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The Molecular Mechanisms of T Cell Clonal Anergy: A Dissertation

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THE MOLECULAR MECHANISMS OF T CELL CLONAL ANERGY

A Dissertation Presented

By

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Submitted to the Faculty of the

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Program in Molecular Medicine

Copyright Information

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Program in Molecular Medicine

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Abstract

A side effect of generating an immune system for defense against invading pathogens is the potential to develop destructive cells that recognize self-tissues. Typically, through the "education" of developing immune cells, the organism inactivates potentially self-destructive cells, resulting in what is called self-tolerance. I proposed to explore the molecular mechanisms responsible for the induction and maintenance of tolerance. Our lab has developed a model of induced immune tolerance to skin and islet allografts utilizing a donor-specific transfusion of spleen cells and a brief course of anti-CD40L antibody. Because the difficulty in isolation of tolerant T cells from this system is prohibitive to performing large screens on these cells directly, I have chosen to study an *in vitro* CD4+ Th1 cell line, A.E7, which can be made anergic via stimulation through the T cell receptor in the absence of costimulation. I hypothesized that anergized T cells upregulate genes that are responsible for the induction and maintenance of anergy and therefore exhibit a unique RNA expression profile. I have screened anergic cells using Affymetrix GeneChips and identified a small number of genes that are differentially expressed long-term in the anergic population compared to mock-stimulated and productively activated controls. The results have been confirmed by quantitative RT-PCR for each of the candidates. One of the most promising, the zinc-finger transcription factor Egr-2, was verified to be expressed long-term by western blotting, demonstrating perfect correlation between Egr-2 protein expression and the anergic phenotype. Silencing Egr-2 gene expression by siRNA in A.E7 T cells prior to anergy induction rescues the cells

from the inability to phosphorylate ERK-1 and ERK-2 and also results in increased proliferation in response to antigen rechallenge. In this study I report that Egr-2 is specifically expressed long-term in anergic cells, protein expression correlates inversely with responsiveness to antigen rechallenge, and that Egr-2 is required for the full induction of anergy in T cell clones.

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Introduction

A fully developed immune system is essential for an organism's defense against invading pathogens. The most comprehensive method to construct a system with sufficient diversity to combat pathogens to which the host has never been exposed is through a random assembly of T and B cell receptors. A side effect of using such a powerful development tool is the inevitable generation of potentially destructive cells that recognize self-tissues. Typically, through the "education" of developing immune cells, the host inactivates potentially self-destructive cells, resulting in what is called "self-tolerance". Occasionally, self-tolerance is disrupted and the host experiences autoimmune destruction of self-tissues, as is seen in diabetes, multiple sclerosis, lupus, and rheumatoid arthritis. Through an understanding of tolerance induction and maintenance, methods are currently being developed to "re-educate" the immune system. Treatments developed to *suppress* the immune response will be useful to reverse and prevent autoimmune disease; additionally, these treatments could be modified to control alloimmune responses to tissue and organ transplants. Conversely, treatments to *enhance* immune responsiveness by breaking tolerance will be useful in vaccination and induction of tumor immunity.

Both T and B cells are important in the host immune response and both have been implicated in autoimmune destruction. In many cases, B cells require T cell help or "permission" in order to become effectors; accordingly, many feel that a thorough knowledge of T cell tolerance mechanisms will allow interventions that will also curb B

cell-mediated destruction. Aside from minor points, only tolerance associated with T cells will be discussed.

Two general methods of mediating self-tolerance have been described. Both methods, *central* and *peripheral* tolerance, appear to be necessary to control the immune system to maintain tolerance (1). Likewise, both methods are being studied and manipulated to induce tolerance at times when it has failed (such as in autoimmunity) or when it would be medically advantageous to the individual (during allogenic transplantation). *Central tolerance* occurs in the thymus as cells enter the organ and then percolate throughout, becoming exposed to a variety of self-antigens presented by resident antigen presenting cells. Potentially autoreactive T cells with high affinity for self-antigen are selectively deleted following engagement with the presenting cells; this process is termed *negative selection* (2). It has been demonstrated that a number of autoimmune mouse models (*e.g.* the NOD mouse) demonstrate an inability to delete certain autoreactive T cells and this results in tissue destruction in the periphery (3), suggesting that defective central tolerance through negative selection is responsible for autoimmune disease. However, it has also been reported that normal healthy individuals also host T cells in the periphery that recognize self-antigens; these autoreactive cells are evidently unable or unwilling to effect an immune response (4;5).

The ability to control autoreactive T cells once they escape central tolerance and enter the periphery proposes a requirement for a second mechanism of tolerance, *peripheral tolerance*. In this case, T cells that escape deletion in the thymus, typically low affinity autoreactive T cells (6), are made unresponsive in the periphery. Peripheral

tolerance has been reported to be mediated in a number of ways: 1) *Ignorance*; when potentially autoreactive T cells released into the periphery never encounter antigen because the antigen is expressed in an immune-privileged site or it never reaches the threshold required to trigger a response. 2) *Phenotype skewing*; T cells are activated but progress to a non-pathogenic phenotype, such as cytokine deviation; 3) *Peripheral deletion*; T cells undergo apoptosis in response to exogenous tolerizing signals. 4) *T cell suppression*; the recruitment of antigen-specific T cells trained during thymic development to prevent the activation and proliferation of other potentially autoreactive cells. And finally, 5) Unresponsiveness or *anergy;* cells are partially stimulated in such a way that they remain unresponsive to full stimulation at a later time, when they have the potential to destroy self tissues (7).

When these mechanisms fail to control autoreactive T cells and autoimmunity ensues, one option to cure the disease is transplantation of the destroyed tissue (*i.e.* β-islets in the case of type I diabetes mellitus). However, a method must be utilized to control immune alloreactivity to the foreign transplanted tissue as well as the recurrence of autoimmunity. Our lab has developed a model of induced immune tolerance to islet allografts utilizing a donor-specific transfusion of spleen cells and a brief course of anti-CD40L antibody into a chemically-induced diabetic host (8). The result is permanent survival of the graft and normalization of blood glucose, effectively curing the host of diabetes in the absence of general immunosuppression. We believe that this protocol involves one or more of the mechanisms of *peripheral tolerance* described above, specifically T cell deletion (9), suppression, and anergy.

I have chosen to study the state of long-term unresponsiveness in peripheral T cells termed anergy. The anergic state has been demonstrated in a number of models of T cell stimulation, both in vitro and in vivo. While these models utilize different methods of induction and vary somewhat in their characteristics, it is widely accepted that they represent actual events that occur in vivo during the induction of tolerance to peripheral tissues.

History of anergy

T cell clones, established from mouse or human peripheral T cells and maintained in vitro, can be stimulated by antigen presenting cells (APC's) pulsed with T cell-specific antigen; this in vitro stimulation is believed to mimick T cell activation in vivo. APC's— which consist of B cells, macrophages, or dendritic cells—stimulate CD4+ T cells via peptide presented on the major histocompatibility complex II (MHC II) which crosslinks the T cell receptor (TCR); additionally, cells receive costimulation through the T cell coreceptor CD28 by the APC ligands B7-1/2. This stimulation plus costimulation results in IL-2 production and proliferation in the T cells (Figure 1).

MHC II

peptide

TCR

CD28 B7.1/2

IL-2

T cell

APC
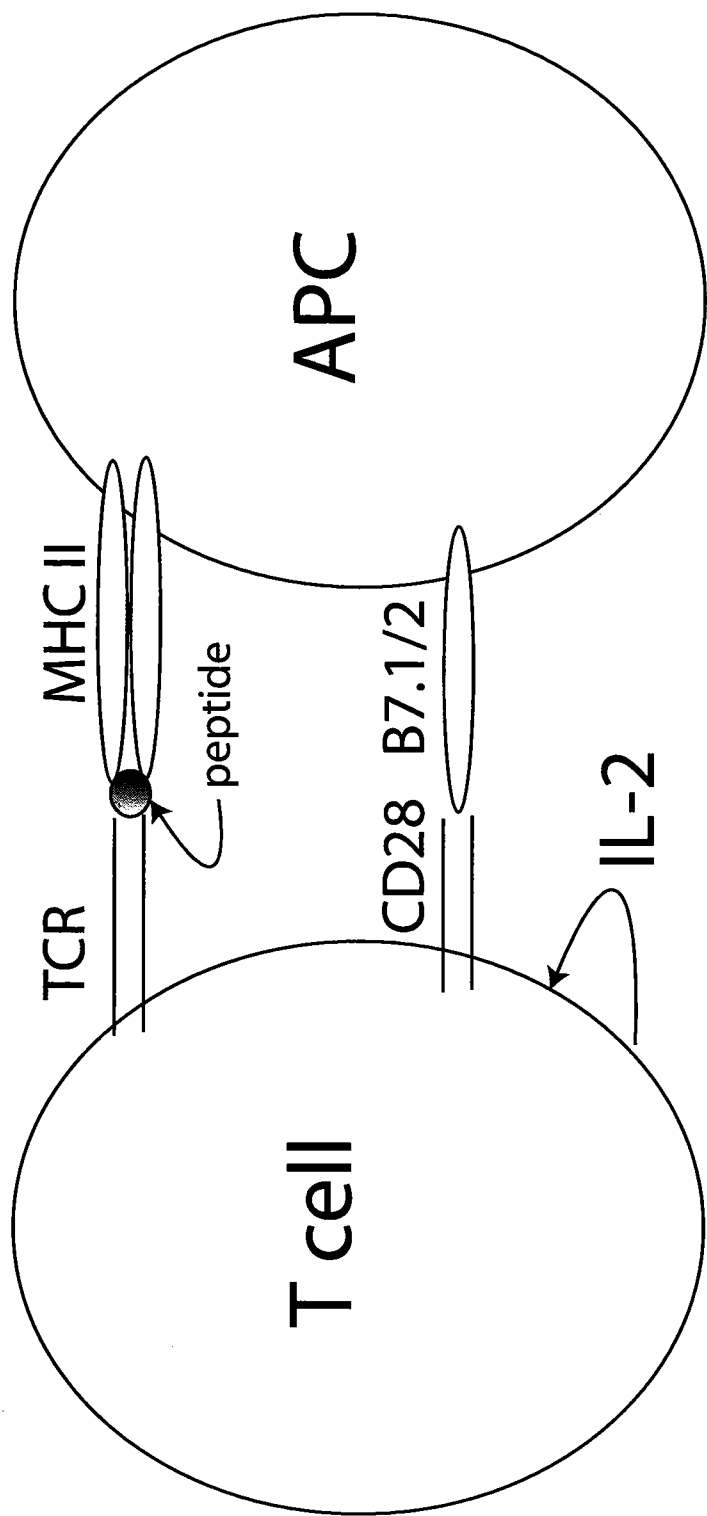
Figure 1

Legend to Figure 1: T cell stimulation through APC peptide presentation. A stimulatory signal is generated through the TCR of a CD4+ T cell via specific peptide presented on the MHC II of the APC. In addition, a costimulatory signal is provided through ligation of CD28 on the T cell by B7.1/2 on the APC. The result is IL-2 production and proliferation in the T cell.

Antigen-induced T cell hyporesponsiveness was first observed by Feldmann and colleagues in 1983 when human CD4+ Th1 clones reactive against the influenza HA epitope were pulsed in vitro with moderate amounts of peptide in the absence of syngeneic APC's; they were later hyporesponsive to rechallenge with peptide presented on APC's (10). Human T cells are unique in that they are able to process soluble antigen and present to other T cells via MHC class II molecules; mouse cells cannot. These makeshift antigen-presenting cells lack B7.1/2 costimulation.

This phenomenon was then further defined and characterized as T cell anergy by Schwartz and colleagues in 1987. They observed that mouse T cell clones stimulated in vitro did not respond optimally when the APC's were chemically modified with protein crosslinker 1-ethyl- 3-(3-dimethylaminopropyl)-carbodiimide (ECDI) (11). In addition, when these T cell clones were rested following the initial stimulation and later exposed to fully functional APC's plus antigen, they were surprisingly unresponsive—producing very low levels of IL-2 and failing to proliferate normally (11). A similar result was observed when T cell clones were stimulated with purified MHC molecules incorporated into planar lipid membranes plus antigen (12), with Conconavalin A (Con A) in the absence of APC's (13), or with calcium ionophore treatment (14;15). This hyporesponsive phenotype was defined as T cell clonal anergy and the common event in the various anergizing stimuli was proposed to be a lack of costimulatory signal through CD28 (16). This hypothesis was confirmed when the anergizing stimulus could be mimicked by treatment of the T cells with platebound anti-TCR mAb (anti-CD3) in the absence of APC's (17) and anergy induction could be avoided if anti-CD28 mAb was

included in the stimulation, resulting in increased IL-2 production and proliferation (18;19).

Additional studies, however, have demonstrated that stimulation of T cell clones by fully competent APC's (with normal costimulatory ability) but presenting altered peptide ligands could also induce the anergic state. In this form of stimulation, the known peptide recognized by the T cell clone was mutated at a single amino acid until an altered peptide was found that failed to induce proliferation. A number of these altered ligands induced anergy in the T cells (20). These anergizing peptides were termed "partial agonists" as opposed to "agonists" which activated the T cells or "antagonists" which failed to induce either proliferation or anergy. These methods of anergy induction are summarized in Figure 2.

A Activated
TCR
TCR agonist +
CD28 agonist

CD28
normal APC+peptide
CD3+CD28 Ab

IL-2

B Anergic
TCR
TCR agonist
alone

CD28
ECDI-fixed APC+peptide
planar lipid mem+peptide
CD3 Ab alone
ConA alone

C Anergic
TCR
TCR partial
agonist +
CD28 agonist

CD28
normal APC+alt. peptide
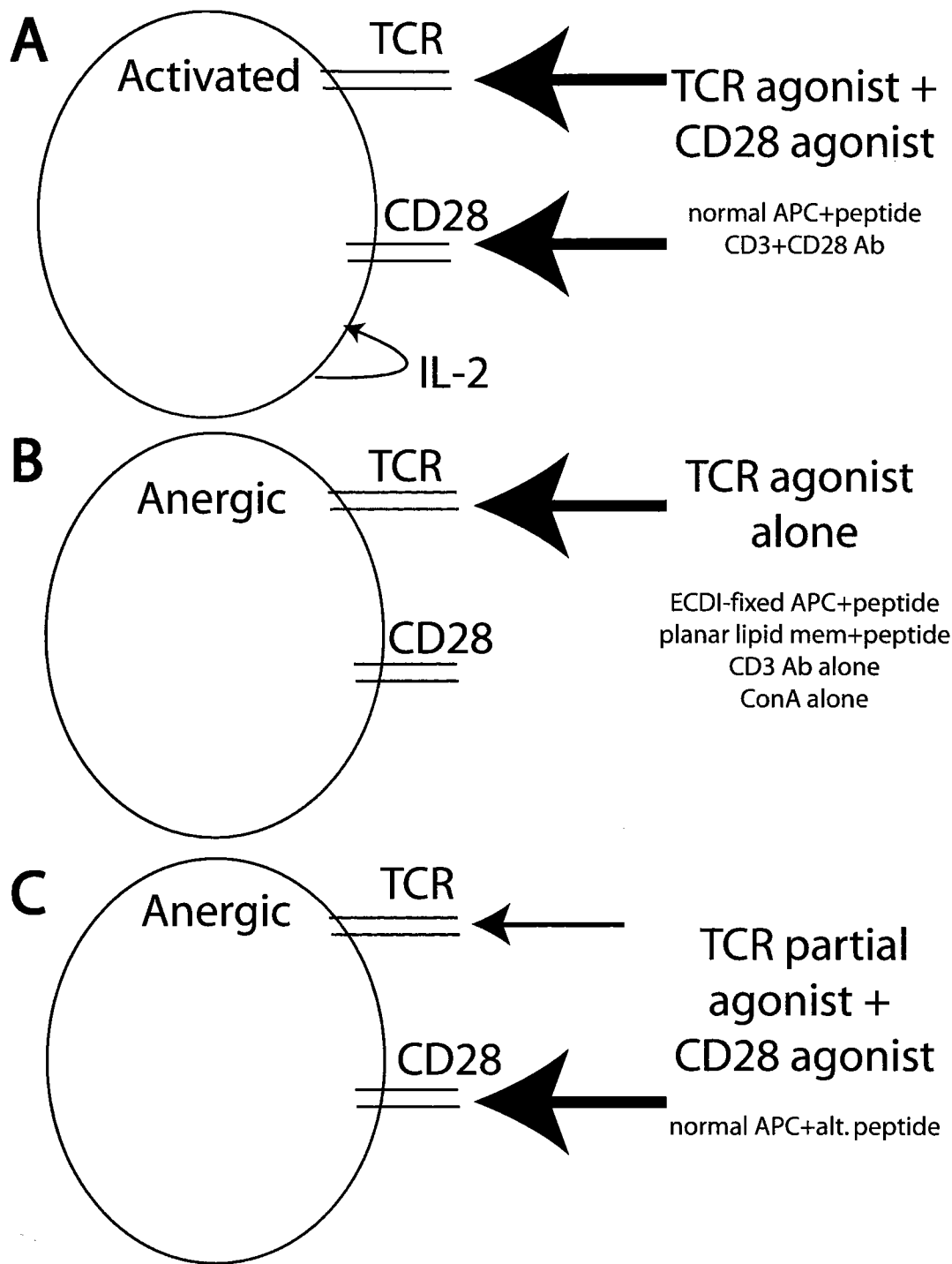
Figure 2

Legend to Figure 2: Methods of anergy induction in CD4+ T cell clones in vitro. **A)** T cells are activated and anergy is avoided following stimulation through the TCR and costimulation through CD28. **B)** Anergy results following stimulation through the TCR alone. **C)** Anergy is induced following TCR stimulation with a "partial agonist" even in the presence of costimulation through CD28.

In all of the anergy models described above, it was observed that the T cell clones failed to produce IL-2 following the anergizing stimulus and that anergic T cells could recover antigen responsiveness following treatment with exogenous IL-2 (21). Later work showed that even full stimulation of T cell clones in vitro could result in anergy induction if IL-2 signaling was interrupted using anti-IL-2 or anti-IL-2-receptor blocking antibodies during stimulation (22). If T cells were fully stimulated but the IL-2 was washed out 12h after stimulation to maintain a low concentration, the T cells did not proliferate and became anergic (23). In parallel, anergy induction could be prevented by signaling through the γ c chain of the IL-2 receptor via cross-linking with αIL-2R antibody (24). All of these reports demonstrated that IL-2 was necessary to prevent anergy induction, but it was unclear what molecular events following IL-2 receptor signaling could both prevent anergy induction and abrogate established anergy in unresponsive clones.

More recent work has demonstrated that preventing proliferation with rapamycin, a drug that blocks G1 to S phase cell cycle transition, induced anergy in fully stimulated T cells. Hydroxyurea, a drug that arrests cell growth in the S phase, did not (25). It was apparent from these experiments that normal T cell stimulation results in the upregulation of "anergic factors" and that these factors are then downregulated with proliferation or, more specifically, G1 to S phase transition; only a stimulated cell that has proliferated avoids anergy. In summary, T cell anergy results from the upregulation of "anergic factors" following TCR engagement and failure to progress from G1 to S phase transition (Figure 3).
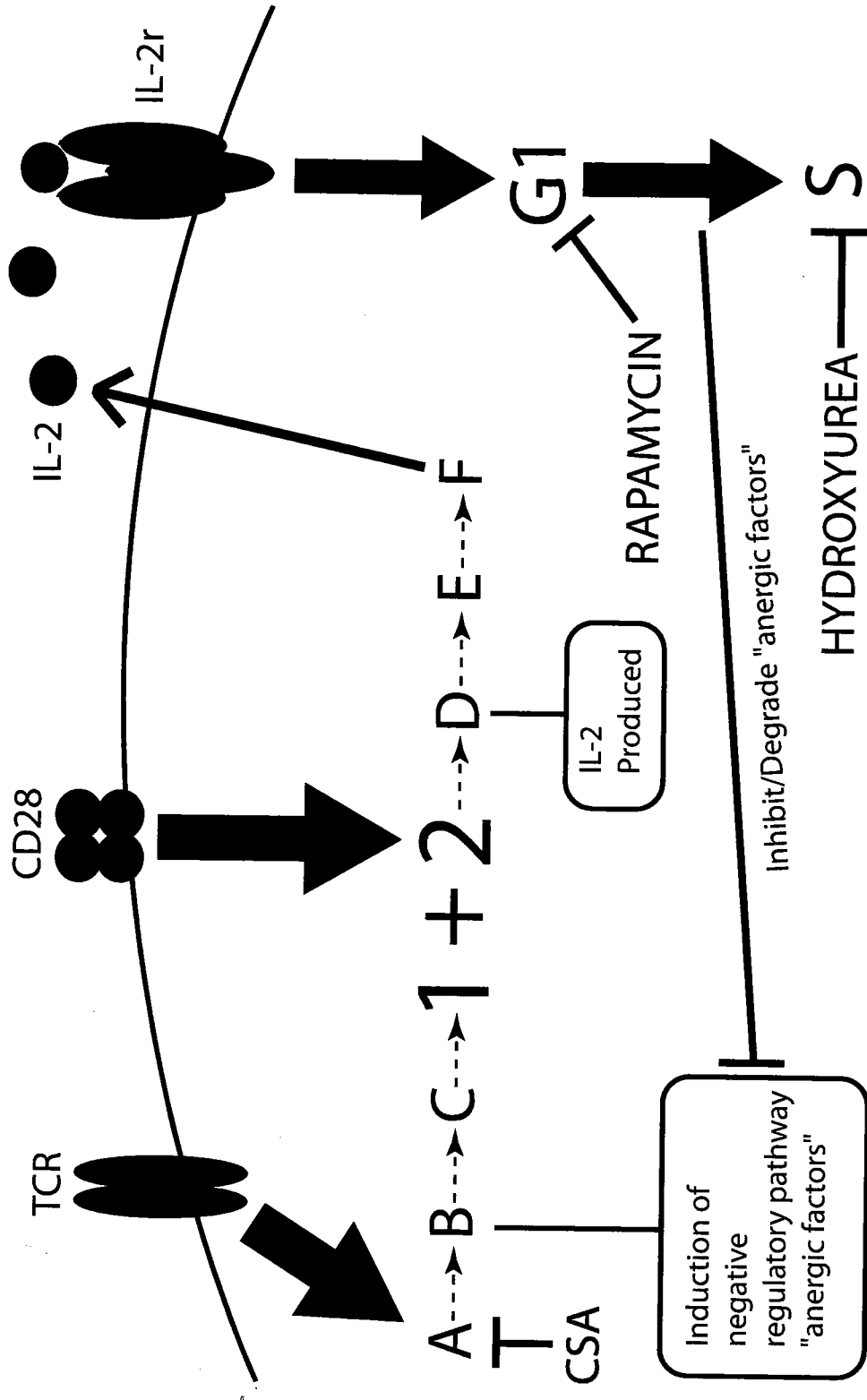
Figure 3 Adapted from Powell et al., JI, 162; 2775

Legend to Figure 3: Model of T cell anergy and the induction of "anergic factors" proposed by Powell et al. (25). TCR stimulation results in multiple signaling processes that accumulate in signal 1. One of the effects of TCR signaling is the generation of "anergic factors" that mediate anergy. CD28 signaling provides an additional signal (signal 2) that is additive to the first, resulting in IL-2 transcription, production and release. Following autocrine signaling of IL-2, cells undergo cell cycle $G1 \rightarrow S$ phase transition and proliferation. IL-2 production is severely impaired following TCR stimulation in the absence of CD28 signaling (lack of signal 2) and results in anergy. In the presence of full costimulation, peptide partial agonists do not produce sufficient signaling to result in IL-2 production, but do induce anergy and, therefore, "anergic factors" (signaling progresses only to "B"). The presence of rapamycin, which blocks cell cycle in G1, induces anergy even in productively stimulated cells while hydroxyurea, which blocks cell cycle in S, does not. Cyclosporine A (CSA), which blocks early TCR signaling, prevents both activation and anergy. The authors conclude that "anergic factors" are induced at signal step "B" and that $G1 \rightarrow S$ phase transition (resulting from productive stimulation or exogenous IL-2 treatment, blocked by rapamycin but not hydroxyurea) causes inhibition or degradation of these factors.

Primer on the biochemical events following T cell activation

In order to more easily describe the biochemical events necessary for anergy induction and maintenance, I will provide a brief description of the biochemical events that occur upon T cell activation, followed by a figure in summary. Following TCR engagement, the subunits of the TCR become phosphorylated by the Src family kinase Lck. These phosphorylated motifs then recruit the tyrosine kinase Zap-70 which then phosphorylates the adaptor molecules LAT and SLP-76. These adaptors then recruit other proteins including phospholipase C (PLC) and son of sevenless (SOS), forming a large complex at the cell surface that results in the activation of three signaling pathways that converge on the IL-2 promoter, activating transcription. A brief description of the three pathways is as follows: **1)** Ras is activated by its exchange factors SOS and RasGRP. RasGRP is dependent on PKC which in turn is activated by diacylglycerol (DAG), a cleavage product of the membrane lipid PIP_2 generated by PLC. Activated Ras recruits Raf and these proteins lead to the activation of a number of kinases that activate the mitogen-activated protein (MAP) kinases ERK1/2, JNK, and p38. These MAP kinases activate transcription of, and directly phosphorylate, the transcription factors fos and jun which dimerize to form **AP-1**, a trascription complex required for IL-2 activation. **2)** MAP kinase kinase kinase (MAPKKK) is also activated by PKC and leads to the degradation of IκB, an inhibitor of NFκB, which leads to the migration of **NFκB** into the nucleus for activation of IL-2 transcription. **3)** In addition to DAG, PLC generates another cleavage product IP_3, which induces the release of intracellular Ca^{++} stores. This

calcium in turn activates cell surface Ca^{++} channels and results in a large flux of calcium

into the cell. The increased Ca^{++} concentration activates the phosphatase calcineurin,

which dephosphorylates **NFAT**, allowing it to enter the nucleus and activate IL-2

transcription (26).

The previous discussion of anergy induction methods suggested an important role

for CD28 costimulation in IL-2 production and anergy avoidance. Evidence suggests that

the biochemical effects of CD28 ligation on IL-2 transcription are three-fold: 1) PI3

kinase (PI3K) is recruited to CD28 resulting in activation of Akt and increased

MAPKKK activity in pathway 2, resulting in **NFκB** activation (26). 2) The Ca^{++} flux

following TCR ligation is prolonged by CD28 (however CD28 stimulation alone has no

effect on Ca^{++} flux) with a net increase in pathway 3 accumulating in **NFAT** activation

(27). 3) IL-2 mRNA is stabilized, allowing message to accumulate to high levels and

results in increased IL-2 protein production (28). These concepts of T cell activation

resulting in IL-2 production are summarized in Figure 4.
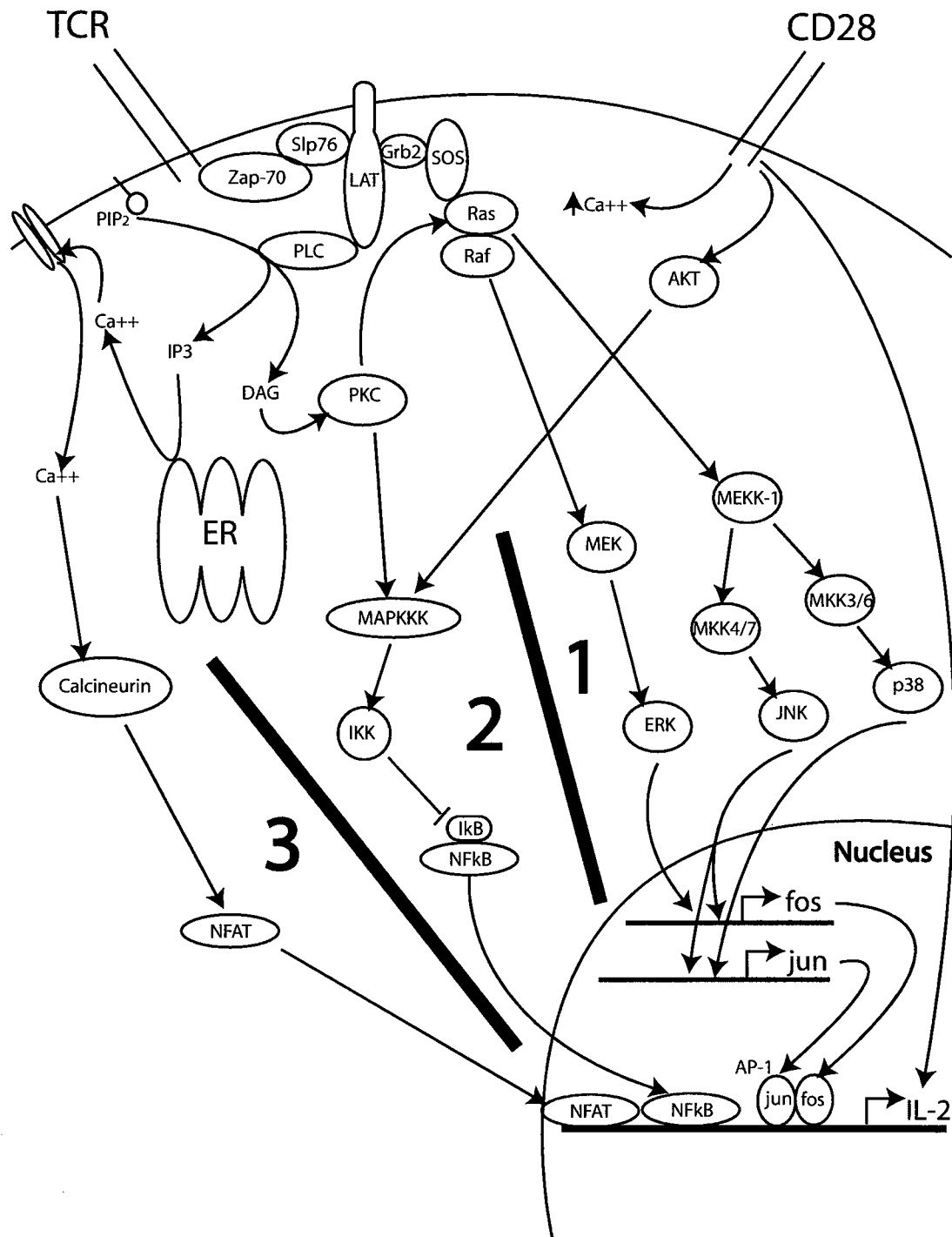
Figure 4

Legend to Figure 4: Signaling events following T cell activation.

Signaling events required for anergy induction

As described above, Powell et al. demonstrated that anergy results from TCR engagement in the absence of proliferation (25). Since TCR signaling results in the induction of three separate pathways, these pathways have been examined to determine which are required for the induction of anergy. Jenkins et al. showed that calcium signaling (pathway 3 in Figure 4) is essential for the induction of the anergic state. Cyclosporine A (CSA), a drug that blocks early T cell signaling at calcineurin and prevents T cell activation, prevents T cell anergy induction as well (17). Anergy induction is also blocked by EGTA, a calcium chelator (29). Calcium ionophore treatment, resulting in increased intracellular calcium levels, can induce anergy (15). One of the major consequences of calcium flux following stimulation in T cells is the activation of the transcription factor NFAT. It was suggested that, downstream of calcium flux, NFAT was responsible for the induction of the anergic factors described in the model. In support of this hypothesis, Macian et al. reported that T cells from NFAT-1-deficient mice (the predominant NFAT in resting T cells) are resistant to anergy induction and that cells transfected with a constitutively active NFAT1 show reduced IL-2 production following anti-CD3 and anti-CD28 stimulation. This occurs even when NFAT1 is modified to prevent interaction with AP-1, suggesting that, independent of AP-1, NFAT is capable of inducing anergic factors (15).

The MAP kinase cascade (pathway 1 in Figure 4) is made up of three parallel pathways—ERK, JNK, and p38. Chemical inhibitors of two of the three pathways (SB203580 blocks p38 and PD90859 blocks ERK-1 and ERK-2) do not prevent anergy

induction. Also, a T cell line derived from the JNK 2 -/-, JNK1 dominant negative mouse could be anergized with anti-TCR antibody (L. Luu, J. Powell & R.H. Schwartz, unpublished data, described in (30)). While calcium mobilization resulting in NFAT activation is required for the induction of anergy, signaling through the MAP kinase cascade and the transcription factor AP-1 is not. It is currently unclear what role, if any, NFκB (pathway 2 in Figure 3) plays in the induction of anergy factors. Since CD28 plays an important role in augmenting NFκB activation and CD28 signaling is typically absent during anergy induction, it is possible that NFκB is also not required for anergy induction. Supporting this is the fact that calcium ionophore alone can induce anergy. Figure 5 incorporates the hypothesis that NFAT activation alone is required for anergy induction.
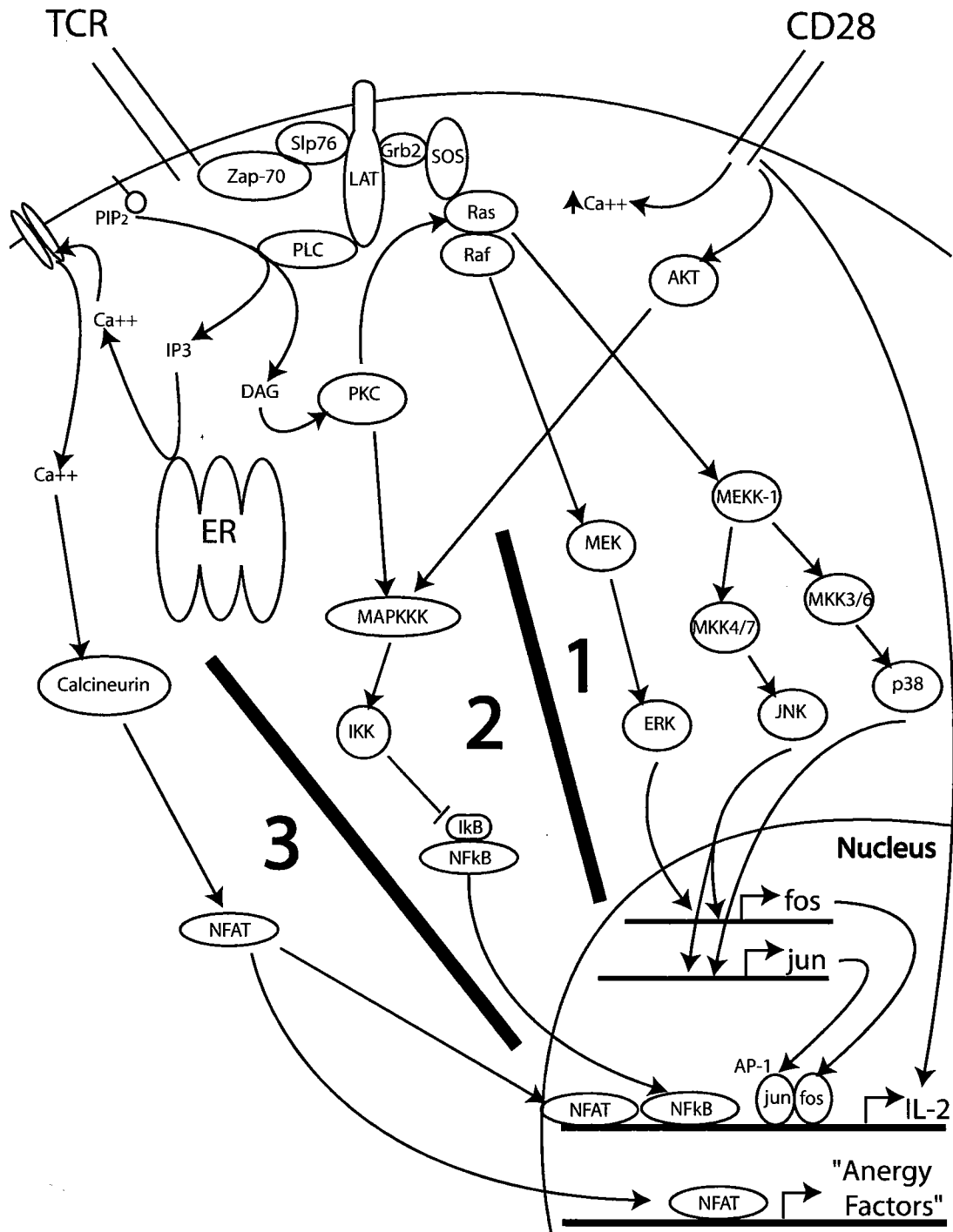
Figure 5

Legend to Figure 5: Anergy factors are upregulated by NFAT following T cell

activation.

Molecular and biochemical observations in anergy maintenance

Since anergy was first described, many groups have been working to elucidate the molecular and biochemical events that are required for the maintenance of the anergic state. One of the first observations made in stimulated anergic cells is that they fail to make IL-2 (17). Since IL-2 is an important cytokine for T cell proliferation, this could explain the lack of proliferation in fully stimulated anergic T cells. However, it was unclear what events between T cell stimulation and IL-2 production were altered.

First, the expression of the TCR was examined. Although most T cells downregulate their TCR in response to stimulation, they restore normal expression after 2-5 days; anergic cells are no different (31). Following stimulation, responsive T cells experience a rapid influx of calcium followed by activation of calcineurin, dephosphorylation and mobilization of NFAT into the nucleus, and activation of IL-2 transcription. While one group reported that anergic cells have constitutively elevated calcium levels (32), Mondino and colleagues have since shown convincingly that anergic cells have normal calcium flux as well as NFAT mobilization and binding following stimulation (31).

In parallel to the calcium-NFAT pathway, the Ras/MAP kinase pathway becomes activated in responsive T cells. This consists of activation of SOS and RasGRP, activation of Ras, and the phosphorylation and consequent activation of the MAP kinases ERK, JNK, and p38. These kinases phosphorylate fos and jun which dimerize to form AP-1 and activate transcription of IL-2. Stimulated anergic T cell clones are defective in

AP-1-mediated transcription (23) and, more specifically, in entry of the AP-1 subunits c-fos, fosB and junB into the nucleus (31). It is unclear if the defective nuclear entry is due to transcriptional repression, translational repression, or a trafficking defect.

Upstream of the activation of the AP-1 transcription factors, each of the three parallel pathways in the MAP kinase cascade—ERK, JNK and p38—is altered. While they are normally activated in response to TCR signaling, the kinases remain inactive in stimulated anergic T cell clones (33;34). In addition Ras, upstream effector of the MAP kinase cascade, was shown to remain in its inactive, GDP-bound form following stimulation. However, the upstream effector son of sevenless (SOS), which facilitates GTP loading on Ras, was activated normally (35). This led to speculation that anergic cells contain a Ras GTPase Activating Protein (GAP) that is responsible for converting Ras-GTP to Ras-GDP, preventing its kinase activity.

Opposing this idea, Gajewski and colleagues demonstrated that anergic cells contain increased activity of the signaling kinase Fyn (32). Its association with Cbl and the CrkL-C3G complex results in constitutive activation of Rap-1, which was demonstrated to inhibit the ability of Jurkat T cells to upregulate IL-2 in response to stimulation. Because activated Rap-1 can associate with Raf-1 protein kinase and prevent its interaction with Ras, the authors argued that activated Rap-1 was responsible for preventing Ras activation in anergic cells (36). However, Fyn deficient mice can be anergized [N. Nabavi and R.H. Schwartz unpublished—described in (30)], lending credibility to the speculation that Ras hypoactivity in anergy is due to an as yet unidentified Ras GAP.

These observations suggest that the defect in producing IL-2 in anergic T cell clones is simply due to a defect in Ras activation, resulting in a hypoactive MAP kinase cascade, and ultimately in defective mobilization of the AP-1 transcription complex. However, in addition to the biochemical signaling block focused around Ras and the MAP kinase cascade, Powell and colleagues argued that the IL-2 promoter contains a site (-180) that is susceptible to active repression represented by a shift in binding from AP-1 transcription factors (activating) to cAMP Response Element-Binding protein/cAMP Response Element Modulator (CREB/CREM) factors (repressing) (37). The CREB/CREM repressor complex is predominant in both resting cells and stimulated anergic cells. It is possible that a competition of transcription factors occurs at this site, with negative regulators (CREB/CREM) predominating in resting cells (to prevent IL-2 transcription in the absence of signaling) and in stimulated anergic cells. Positive regulator AP-1 binding is predominant in stimulated responsive cells. It is unclear whether the imbalance observed in anergic cells is due to a paucity of AP-1 factors, an excess of CREB/CREM factors, or an active regulator that is responsible for shifting the balance (through phosphorylation of CREB/CREM, for example). A summary of the various signaling and transcriptional blocks identified in fully stimulated anergic cells is described in Figure 6.
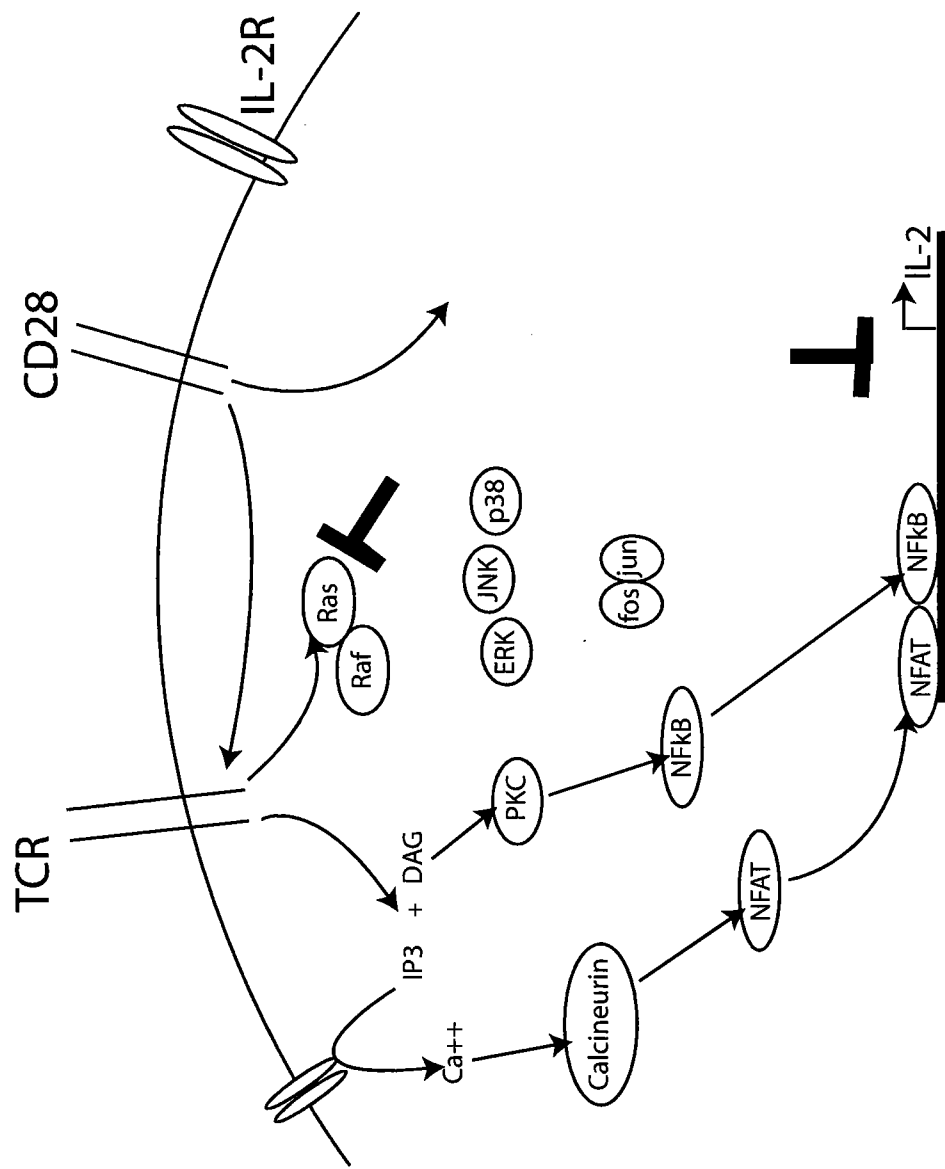
Figure 6

Legend to Figure 6: Signaling and transcription blocks in anergic cells exposed to full stimulation.

Evidently, mechanisms other than the block in Ras activation contribute to hyporesponsiveness in anergic cells. Another study supporting this hypothesis is a recent one by Crespi and colleagues who transfected T cells with a Ras mutant that is constitutively active [Ras (Leu61)]. While transfected T cells elicited TCR-independent activation of the Ras-Raf-ERK pathway and conferred T cells with the ability to secrete IL-2 in response to stimulation with Ca^{++} ionophore alone, it was insufficient to confer resistance to anergy induction via chronic TCR engagement (38).

In order to identify genes responsible for anergy, Korthauer et al. performed differential display analysis on anergic cells using four different methods of anergy induction (39). One of the candidates reported by the group was General Receptor of Phosphoinositides 1 (Grp1), a cell membrane protein first discovered in the lab of Michael Czech at the University of Massachusetts Medical School. Grp1 is a member of the cytohesin family that selectively binds the membrane signaling intermediate PIP_3 and regulates cellular adhesion and membrane trafficking (40). When Grp1 was retrovirally transduced into primary T cells they exhibited normal proliferation and cytokine production. However, transduced cells rapidly lost expression of the protein, suggesting that Grp1 plays a role in limiting T cell expansion or survival. Anergic cells cannot home to the marginal zone of germinal centers in vivo (41); it is possible that the adhesion characteristics of Grp1 mediate this function.

Another group identified a novel gene that was induced in an anergic CD4+ Th1 cell line, named Gene Related to Anergy In Lymphocytes (GRAIL). It was induced after 4 hours of stimulation with engineered fibroblasts that expressed MHC class II without

costimulatory molecules. GRAIL was not expressed in unstimulated cells or in fully stimulated cells and its expression was inhibited by CSA. However, GRAIL was not expressed after 18 hours of anergizing stimulation and so is only transiently expressed in anergic cells. GRAIL is an E3 ubiquitin ligase which, when overexpressed in T cells, inhibited IL-2 production in response to anti-CD3 plus anti-CD28 antibody activation. This inhibition of IL-2 production was dependent on an intact endocytic pathway (42).

Boussiotis et al. reported that, secondary to an increase in intracellular cAMP, the cell cycle regulator $p27^{kip1}$ was upregulated in anergic human T cell clones as well as alloreactive mouse T cells tolerized in vivo. Overexpression of $p27^{kip1}$ in T cells inhibited IL-2 production. They observed that $p27^{kip1}$ interacted with JAB1 in anergic cells but not unstimulated controls and proposed that $p27^{kip1}$ maintained T cells in the anergic state by sequestering JAB1 in the cytoplasm, preventing its interaction with c-Jun and JunD. JAB1 stabilizes the interaction of Jun activators with AP-1 sites, allowing transcription of IL-2 (43). It was reported by others that, in mouse T cell clones, increased $p27^{kip1}$ levels did not correlate with the anergic phenotype. Although treatment with exogenous IL-2 downregulated expression of $p27^{kip1}$, the amount of IL-2 necessary to downregulate this protein was much lower than that required to prevent the induction of anergy. They also showed that while overexpression of $p27^{kip1}$ was able to decrease IL-2 promoter-induced expression, physiological levels of this protein had no effect on the IL-2 promoter. Lastly, they demonstrated that T cell lines from $p27^{kip1}$ knockout mice were as susceptible to anergy induction as those from mice heterozygous for the gene (44).

None of the proteins in the studies described above fits the profile of an "anergic factor" predicted by Powell et al. (25) and none has been demonstrated to be required for anergy induction or maintenance.

Anergy in primary T cells

Anergy is not limited to T cell clones in vitro. In fact, the characteristic unresponsiveness and lack of IL-2 production of anergy has also been observed in primary T cells from the mouse and human, both in vitro and in vivo; demonstrating the far-reaching implications of understanding anergic mechanisms to the management of immune tolerance in the organism. The following section first describes experiments that anergized primary T cells ex vivo and then studies that reported the induction of T cell anergy in vivo.

Mouse and human primary T cells ex vivo

Chai and Lechler induced anergy in highly purified mouse primary CD4+ T cells in vitro via stimulation with platebound anti-CD3 mAb. While a large number of these cells died by apoptosis, the remaining viable cells did not proliferate and failed to make IL-2 when rechallenged with Con A but were hyperresponsive to exogenous IL-2. Anergy induction was seen in both naïve and memory T cells in this study and was inhibited by cyclosporine A (45). Anergy-like unresponsiveness has been demonstrated in human peripheral blood mononuclear cells (PBMC's) in an MLR in the presence of

inhibitors of costimulation such as CTLA4-Ig (46) and in freshly isolated PBMC's

stimulated with anti-TCR antibody in the presence of IL-10 (47).

Wells and colleagues observed an interesting relationship between anergy and cell

division history as well as anergy and CTLA-4 signaling in primary mouse T cells

stimulated in vitro. Pooled spleen and lymph node cells were labeled with CFSE and

stimulated in vitro with soluble anti-CD3 mAb alone or in combination with anti-CD28

mAb (to enhance costimulation) or CTLA4-Ig (to block costimulation through APC B7

interaction with T cell CD28). The cells were stimulated for 4 days, rested for 48 hours,

and then sorted based on CFSE fluorescence. Sorted T cells were then rechallenged with

irradiated syngeneic APC's plus soluble anti-CD3 mAb with or without IL-2. They

observed that when cells were stimulated with anti-CD3 mAb in the presence of CTLA4-

Ig (blocking costimulation through B7 molecules), they divided much less than those

stimulated with anti-CD3 mAb alone. In addition, these cells were hyporesponsive to

rechallenge with anti-CD3 mAb plus fresh irradiated syngeneic APC's (now with

available B7), as assessed by CFSE dilution and by IL-2 production. This

hyporesponsiveness to secondary stimulation was observed regardless of their

proliferation history. However, when provided with exogenous IL-2 in addition to anti-

CD3 mAb, the cells proliferated normally (48). These observations support those seen in

T cell clonal anergy but challenge the notion that cell division in the absence of

costimulation can result in the abrogation of the anergic phenotype. However, as R.

Schwartz notes in a review of anergy, this group only isolated cells that had proliferated 0

times or 2 times to assess for responsiveness based on cell division during the secondary

stimulation. He suggests that maybe cells stimulated under these conditions require more total time spent in G1 to S phase transition in order to circumvent the anergic state, and that the authors could also look at cells that had divided 3 or 4 times (30). Secondly, the authors sorted T cells according to cell division history following stimulation with anti-CD3 alone (with B7 available for costimulation) and rechallenged with anti-CD3 plus irradiated syngeneic APCs with or without IL-2. Under these circumstances, a cell that failed to divide, regardless of the presence of costimulation, was hyporesponsive to rechallenge. This supports the conclusions of Powell et al. (25) that costimulation alone is not sufficient for anergy avoidance, but that cell division is also required to avoid anergy. These cells, however, were also refractory to proliferation even when exogenous IL-2 was included in the secondary stimulation. This appears to be a deeper level of anergy, and the authors suggest that it is a result of B7 stimulation of CTLA-4 in the primary stimulation of the cells that fail to divide (48). These observations led the authors to conclude that anergy avoidance requires a combination of both costimulation and cell division.

Mouse primary transgenic (Tg) T cells in vivo—soluble peptide

Marc Jenkins and his group developed a transgenic model to study T cells tolerized in vivo, their persistence in the host, and trafficking of these cells compared to stimulated cells. Naïve mouse DO11 transgenic T cells were adoptively transferred into syngeneic wild-type recipients; the recipients were then treated with soluble intravenous OVA$_{323-339}$ peptide (tolerizing stimulus), left untreated (unstimulated) or treated with

subcutaneous peptide plus adjuvant (stimulated). The tolerized T cells rapidly expanded and then contracted in vivo; the surviving cells were hypoproliferative and defective in IL-2 production upon rechallenge with peptide in vitro and in vivo. These tolerant cells persisted for at least 3 weeks and for as long as several months in vivo, regaining functional capability over this long rest period if not rechallenged with peptide. Further, in contrast to cells activated with peptide in complete adjuvant, tolerized cells did not efficiently accumulate in the lymph nodes, suggesting that tolerant cells localize differently than responsive cells in vivo (49).

Mouse primary Tg memory T cells in vivo—soluble peptide

Mirshahidi et al. showed that primed (memory) mouse T cells could be made anergic. They isolated 6.5 TCR transgenic CD4+ T cells that recognize the $HA_{110-120}$ peptide presented on $I-E^d$, adoptively transferred the cells into B10.D2 recipient mice, and primed the mice with the HA peptide in complete adjuvant. Five weeks later they treated the mice with low-dose soluble peptide in incomplete adjuvant to induce tolerance. With this treatment, the memory transgenic T cells were hypoproliferative and defective in IL-2 production when rechallenged with peptide in vitro. The tolerant cells expanded, however, when cultured with recombinant IL-2 (50).

Mouse primary Tg T cells in vivo—tissue whole protein expression

Tanchot et al. adoptively transferred transgenic T cells that recognize PCC presented in the context of $I-E^k$ into a transgenic host that expressed the whole PCC

protein under control of an MHC class I promoter, conferring constitutive expression of the antigen in the host. The transferred transgenic T cells experienced an initial expansion and then deletion phase; the remaining cells manifested an 85-95% reduction in all cytokine production and decreased proliferative response to antigen in vitro. If the cells were transferred into a second host without antigen, they partially regained responsiveness. If transferred into a second host that expressed the antigen, the cells initially regained responsiveness, but then slowly entered into an even deeper state of tolerance, demonstrated by further reduced cytokine production and proliferation with rechallenge in vitro (51).

Mouse primary non-Tg T cells in vivo—superantigen

In a non-transgenic model of in vivo tolerance, mice were injected with staphylococcal enterotoxin B (SEB) to stimulate T cells in vivo in a nonspecific manner, resulting in anergy. SEB is a superantigen that binds the TCR and MHC proteins laterally, inducing potent activation of a large number of T cells in the host. When T cells were stimulated with SEB in vivo, they expanded and then contracted, presumably by apoptosis. The remaining T cells were unresponsive in vitro to TCR ligation with either the superantigen or anti-TCR antibodies, failing to proliferate and make IL-2. Interestingly, these cells were also unable to respond to exogenous IL-2 (52;53).

Human primary T cells in vivo (in SCID mouse)

In an effort to reconstitute a SCID mouse with a human immune system, human peripheral blood leukocytes (PBLs) were injected and analyzed over four weeks. The cell population initially expanded but later contracted, leaving a population of human T cells that remained long-term in the host. The model was not particularly useful, however, because the remaining cells could not mount a response when challenged with foreign tissue. When the surviving cells were rechallenged in vitro with anti-CD3 and anti-CD28 mAb, they could not proliferate or make IL-2. The cells did proliferate, however, when treated with exogenous IL-2, suggesting that they were anergic. After sufficient IL-2 stimulation in vitro, the T cells reacquired the ability to respond to stimulation with mitogens or mouse proteins presented on syngeneic APCs (54).

These reports demonstrate that anergy is not just an in vitro phenomenon of T cell clones; there is a role for T cell anergy in primary cells in vitro and in vivo.

T cell suppression—is anergy involved?

T cell suppressor function has recently been demonstrated in a number of T cell subtypes in vitro and in vivo. One of the most potent of the T cell suppressors exhibits constitutive CD25 expression. These cells have been shown to suppress CD25- T cells to a high degree when both are present and stimulated in vitro by APC's or by antibody-coated beads. This suppression requires cell-cell contact. Once the suppressor cells have been activated, suppression is mediated in an antigen-independent manner. CD25+ suppressor cells have been described as "anergic" as they do not proliferate when fully

stimulated in vitro (anti-CD3+anti-CD28 mAb or mature allogeneic dendritic cells) but will proliferate modestly when stimulated in the presence of IL-2 (55). However, they do not exhibit "classical" anergy as they return to an anergic state even after IL-2-induced proliferation.

Ermann and colleagues have proposed that CD25+ suppressor cells mediate suppression by inducing anergy in the CD25- target cells. They demonstrated that when CD25+ suppressors and CD25- targets were stimulated in co-culture, CD25- cells upregulated transcription of GRAIL, a gene specifically induced in anergized T cells that inhibits IL-2 production (described above). IL-2 treatment of the suppressed cells abrogated suppression and permitted proliferation, also implicating the anergic state in the failed proliferation of the CD25- targets (56).

In some cases, originally nonsuppressive T cells that were anergized in vitro gain suppressive ability, both in vitro and in vivo. The 1F8 T cell line, derived from NOD mice and alloreactive for the amino acid substituted H2-A^{g7} molecule (NOD^{asp}), was rendered anergic in vitro with platebound anti-CD3 mAb. Subsequently, they were unresponsive to stimulation in vitro with NOD^{asp} spleen cells but remained responsive to exogenous IL-2. When anergic 1F8 cells were mixed with responsive (unstimulated) 1F8 cells and then exposed to NOD^{asp} splenocytes in vitro, the proliferation of the responsive cells was suppressed. This suppression required cell-cell contact; when anergic cells were separated from the responsive cells in a transwell, no suppression was observed. When NOD recipients are first primed with NOD^{asp} spleen cells and then transplanted with NOD^{asp} tail skin, rejection is typically seen within 10-15 days. However, when recipients

received anergic 1F8 cells on days 1 and 5 post-grafting, a prolongation of graft survival to 25-30 days was observed, demonstrating that anergic 1F8 T cells had some suppressive activity in vivo as well (57).

These studies implicate anergy in T cell suppression. It is unclear whether this role is in the suppressor T cell itself, in the target of suppression, or both.

Evidence for anergy in tolerance to alloantigen

Protocols developed to induce tolerance to alloantigens will be useful in transplantation of allografts. Our lab has developed a protocol utilizing anti-CD40L mAb and a donor-specific transfusion of splenocytes to induce long-term donor-specific tolerance to alloantigen (8). Another group cultured a mixed lymphocyte response (MLR) with purified B6 CD4+ T cell responders and irradiated bm12 stimulators (which differ from B6 at three amino acids in the IA region of the MHC class II); anti-CD40L mAb was added to induce tolerance in the B6 responder T cells. While including anti-CD40L in the MLR prevented proliferation of the responders in the primary MLR, the cells were also hyporesponsive after being washed, rested, and stimulated in a secondary MLR in the absence of the antibody. When IL-2 was included in the primary MLR, it prevented induction of tolerance. In addition, exogenous IL-2 restored the proliferative response of tolerized responders in the secondary MLR. The response of tolerized cells to third party stimulators was much less affected, demonstrating specificity for the tolerizing stimulus. This result suggests that the use of anti-CD40L to tolerize T cells induces anergy in potentially alloreactive T cells (58).

Lechler and colleagues have proposed that anergy is induced in vivo in human peripheral tissues by antigen presentation on MHC class II positive cells that lack costimulatory ability. They have observed that cardiac and renal transplant recipients harbor alloreactive T cells in their periphery that remain unresponsive when stimulated in vitro with donor alloantigen but can recover responsiveness when treated with exogenous IL-2. They exhibit no change in response to third party stimulators. Their model is that inflamed parenchymal tissues upregulate MHC class II molecules in response to IFN-γ secretion but lack costimulatory molecules, inducing a hyporesponsive or "anergic" state in potentially alloreactive T cells in the classical "stimulation without costimulation" sense (59).

Hypothesis/Approach

I hypothesized that anergic T cells exhibit a unique RNA expression profile. Previous data from others (25) indicates that upregulated proteins or "anergy factors" are induced following T cell activation, are sensitive to CSA, and are downregulated with proliferation following either full activation or exposure to exogenous IL-2. I determined to screen for anergic factors with these characteristics by analyzing the RNA expression profile of anergic cells compared to mock-stimulated and fully activated controls at three times: before, during and after proliferation in the activated population. RNA analysis was performed using Affymetrix GeneChips, a method that allows the simultaneous rapid screening of approximately 36,000 expressed sequences. Factors differentially expressed in anergic cells and possessing the expected characteristics were further examined for

functionality in the anergic phenotype.

Materials and Methods

Cells

A.E7 (a generous gift from R. Schwartz, NIH, Bethesda, MD) is a CD4+ Th1

clone specific for the Pigeon Cytochrome c (PCC) peptide 81-104. Cells were maintained

by stimulating 4×10^6 cells for 48 hours with 8 μM whole PCC and 40×10^6 irradiated

(3000 rad) syngeneic (B10.BR) splenocytes in 8 ml complete media [50% RPMI/50%

EHAA cell culture media, 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100

μg/ml streptomycin, 2 mM L-glutamine, 55 μM β-mercaptoethanol] in a 6-well plate.

The cells were then expanded 1:20 into 152 ml fresh media with 15 U/ml rmIL-2. After a

minimum of 12 days, when the IL-2 was consumed and the cells were rested, live cells

were isolated on a ficoll gradient (Lympholyte M, Accurate Chemical & Scientific Corp.,

Westbury, NY) and utilized for anergy induction.

EL-4 is a mouse thymoma T cell line maintained between $0.05\text{-}2 \times 10^6$ cells/ml in

complete media (RPMI cell culture media, 10% Fetal Clone, 100 units/ml penicillin, 100

μg/ml streptomycin, 2 mM L-glutamine, 55 μM β-mercaptoethanol).

JTAg cells are Jurkat human leukemic T cells transfected with the large T cell

antigen (TAg), resulting in the maintenance of transiently transfected plasmids containing

the SV40 origin of replication in high copy number. This cell line (a gift from L. Berg,

UMASS, Worcester, MA) was maintained between $0.05\text{-}2 \times 10^6$ cells/ml in complete

media (RPMI cell culture media, 10% Fetal Clone, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 55 μM β-mercaptoethanol).

Induction of anergy

Live A.E7 T cells were isolated over a ficoll gradient (Lympholyte M, Accurate Chemical & Scientific Corp., Westbury, NY) by centrifuging 100-250x10^6 cells in 12.5 ml of 1% FBS culture media over 7 ml ficoll at 1300xg, 22^{0}C, 20 min. The cells were washed 3 times, resuspended in 10% FBS culture media, and counted by trypan blue exclusion. Either 20-40x10^6 cells or 100x10^6 cells at 1x10^6/ml were stimulated in a T75 or T175 tissue culture flask, respectively (BD Falcon, Bedford, MA) previously coated with anti-CD3 monoclonal antibody (mAb) (clone 145-2C11) (BD Biosciences, Pharmingen, San Diego, CA) at a concentration of 1 μg/ml in PBS at 37^{0}C for 1-2 hours. After incubation with antibody, the flasks were washed 3 times with PBS before cells were added. Activated control cells were incubated under the same conditions with the addition of soluble anti-CD28 mAb (clone 37.51) (BD Biosciences, Pharmingen, San Diego, CA) at 1 μg/ml. Mock-stimulated control cells were incubated in a flask coated with isotype control IgG antibody (BD Biosciences, Pharmingen, San Diego, CA) under the same conditions in the absence of soluble antibody. After overnight incubation, flasks were placed on ice, the cells were scraped, washed 3 times with 1% FBS culture media, and processed for RNA, protein, or rested for a variable amount of time in 10% FBS culture media.

Proliferation assay

Anergized or control cells that had been resting for at least 5 days (unless otherwise specified) were counted and resuspended in 10% FBS culture media. Twenty thousand cells were coincubated with 0.5×10^6 irradiated (3000 rad) syngeneic spleen cells and increasing doses of whole PCC in a total of 200 μl in a 96-well plate in triplicate. As a control, cells were incubated with IL-2 at 10-15 U/ml instead of antigen. Cells were stimulated for 64-70 hours and were pulsed with 1 μCi [^{3}H]-thymidine for the last 16 hours of culture.

IL-2 ELISA

Cells were stimulated in a 96-well plate as above for 14 hours. Instead of IL-2 as a control, cells were incubated in the presence of PMA (75 ng/ml) and Ionomycin (1 μg/ml) (Sigma-Aldrich, St. Louis, MO). The supernatant was then removed and stored at -20^0C until analyzed. Ninety-six-well plates were coated overnight with 100 μl anti-IL-2 capture antibody (clone JES6-1A12) (BD Biosciences, Pharmingen, San Diego, CA) at a dilution of 1:500 in coating buffer (0.1M Carbonate Buffer, pH 9.5) and then washed with PBS-Tween-20 (0.05%). One hundred-fifty microliters recombinant mouse IL-2 (R&D Systems, Minneapolis, MN) standards (8-500 pg/ml) or supernatants were added to the coated plates and incubated for 1 hour at room temperature. Plates were washed with PBS-Tween-20 and then 100 μl biotinylated anti-IL-2 detection antibody (clone JES6-5H4)(BD Biosciences, Pharmingen, San Diego, CA)(1:500) was added for 1 hour

at RT. The plates were washed with PBS-Tween-20 followed by incubation with 100 μl

horseradish peroxidase-avidin D (Vector Laboratories, Inc., Burlingame, CA) (1:2500) in

PBS-Tween-20 at RT for 20 minutes. The plates were washed in PBS-Tween-20,

followed by PBS, and incubated with   100 μl OPD buffer (50mM Na_2HPO_4, 25mM

NaCitrate, pH 5.0) with peroxide added until color developed. The reaction was stopped

by adding 25 μl 3M HCl. The plates were then read at a wavelength of 490 nm in a

Maxline microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Microarray procedures

A.E7 T cells that had been pretreated (mock-stimulated, anergized, or activated)

as described and rested for 0, 2 or 5 days were processed for total RNA (RNeasy, Qiagen,

Valencia, CA) according to the manufacturer's instructions. Fifteen micrograms of total

RNA per sample was reverse-transcribed into double stranded-cDNA with the T7-$(dT)_{24}$

primer   [5'-

GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3'] using the

SuperScript Choice system (Gibco BRL Life Technologies, Rockville, MD) according to

manufacturer's instructions. cDNA was cleaned up by extraction with (25:24:1)

phenol:choloroform:isoamyl alcohol (Ambion, Austin, TX) followed by centrifugation

over a Phase Lock Gel (Eppendorf-5 Prime, Inc., Boulder, CO). cDNA was then

precipitated with 0.5 volumes of 7.5M NH_4Ac and 2.5 volumes of cold absolute ethanol,

washed twice with 80% ethanol, and resuspended in 12 μl of RNase-free water. Five

microliters of cDNA was then used as a template for the in vitro transcription of biotinylated cRNA using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences Inc., Farmingdale, NY) according to manufacturer's instructions. Biotinylated cRNA was isolated using an RNeasy Mini column (Qiagen, Valencia, CA), eluted with water, and quantified by spectrophotometer at OD_{260}. Sixty micrograms of biotinylated cRNA was fragmented in fragmentation buffer (200mM Tris-acetate, pH 8.1, 500mM KOAc, 150mM MgOAc) at 94^0C for 35 minutes at a final concentration of 0.8 $\mu g/\mu l$. Fifty-five micrograms of fragmented cRNA was added to the hybridization cocktail [0.05 ug/ul fragmented cRNA, 50 pM Control Oligonucleotide B2 (Affymetrix, Santa Clara, CA), Eukaryotic Hybridization Controls *BioB, BioC, BioD, cre* (Affymetrix, Santa Clara, CA), 1.5, 5, 25, and 100 pM, respectively, 0.1 mg/mL Herring sperm DNA (Promega/Fisher Scientific, Madison, WI), 0.5 mg/mL acetylated BSA (Gibco BRL Life Technologies, Rockville, MD), 1X Hybridization Cocktail (100mM MES, 1M [Na^+], 20mM EDTA, 0.01% Tween 20)]. Fresh cocktail was prepared and hybridized to each of the mgU74v2 A, B, and C chips (Affymetrix, Santa Clara, CA) in the microarray core facility at the University of Massachusetts according to standard protocol.

Quantitative RT/PCR

Total RNA was prepared using an RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. cDNA was synthesized using oligo-dT primers and reverse transcriptase AMV (Roche, Indianapolis, IN) according to manufacturer's instructions. Quantitative real-time PCR was performed in a Light Cycler (Roche,

Indianapolis, IN) using a SYBR Green I PCR kit (Roche, Indianapolis, IN) and specific primers to amplify 200-1000 bp fragments from the different genes analyzed. A standard curve was generated for each run with total RNA from an unstimulated or a 24-hour ConA-stimulated mouse spleen cell preparation and used as a reference to calculate relative units of expression for each gene in each sample. Melting curves and gel electrophoresis confirmed the purity of the amplified band. Normalization was achieved by including samples with primers for HPRT.

Primer sequences for the genes analyzed are as follows: HPRT (f) 5'-ttagcgatgatgaaccaggttat-3' (r) 5'-tggcctgtatccaacacttc-3'; Pac-1 (f) 5'-ggaaatcttgccctacctgtacttg-3' (r) 5'-accatatcagagcagccgtcat-3'; Bace-2 (f) 5'-ggaagctgtggcacgaacatct-3' (r) 5'-catcacggtcgcaccaatcac-3'; AK011178 (f) 5'-ccggccactgaacgagatg-3' (r) 5'-ggaaggcccaaacataagga-3'; Osteopontin (f) 5'-ccgaggtgatagcttggcttatg (r) 5'-ctgccctttccgttgttgt-3'; Egr-2 (f) 5'-agtgcacgaaaggccctatc-3'; IL-2 (f) 5'-caacagcgcacccacttc-3' (r) 5'-cgaattggcactcaaatgtgt-3'.

Western blotting

At indicated times, cells were washed once in PBS, resuspended in SDS gel loading buffer without bromophenol blue or β-mercaptoethanol, and boiled for 5-10 minutes. Protein concentration was then determined using the BCA protein quantification assay (Pierce, Rockford, IL). Bromophenol blue and β-mercaptoethanol were added to the samples and equal amounts of protein were loaded on a 10% SDS protein gel. Samples were run at 150 volts for 30-60 minutes and then transferred to PVDF

membrane at 100 volts for 1 hour. For phospho-ERK and total ERK blots, the membrane

was blocked in blocking buffer [TBS-tween-20 (0.05%) with 5% BSA for total ERK, 5%

Blotto (Sigma-Aldrich, St. Louis, MO) for all others] for 1 hour at RT. The blots were

then probed with anti-phospho-ERK (Cell Signaling, Beverly, MA) (1:1000) or anti-ERK

(Cell Signaling, Beverly, MA) (1:1000) in blocking buffer at 4^0C overnight. For all

others, blots were blocked overnight (5% Blotto) at 4^0C and then probed with anti-Egr-2

(Covance, Richmond, CA) (1:200), anti-Egr-1 (Santa Cruz Biotechnology, Santa Cruz,

CA) (1:1000) or anti-β-actin (Sigma-Aldrich, St. Louis, MO)(1:50,000) in blocking

buffer (5% Blotto) at RT for 1 hour. After incubation with primary antibody, the

membrane was washed with TBS-Tween-20 and probed with secondary goat anti-rabbit

antibody conjugated to horseradish peroxidase (Chemicon Int., Temecula, CA)

(1:10,000) or goat-anti mouse (Chemicon Int., Temecula, CA.) (1:10,000) in blocking

buffer (5% Blotto) for 45 minutes. The membrane was washed with TBS-Tween-20

followed by a wash in TBS, developed in chemiluminescence (Perkin Elmer, Boston,

MA) (most blots) or enhanced chemiluminescence (Pierce, Rockford, IL) (phospho-

ERK) and exposed to Kodak X-OMAT AR film.

Flow cytometry

For extracellular staining, cells were washed in FACS buffer and incubated with

10 µl anti-CD4 mAb-APC (1:25), anti-CD4 mAb-PerCP-Cy5.5 (1:20), anti-CD25 mAb-

APC (1:40), and/or anti-CD8 mAb-PerCP-Cy5.5 (1:10) (BD Biosciences, Pharmingen,

San Diego, CA) diluted in FACS buffer (0.1% NaAzide, 0.1% BSA in PBS) on ice for 30

minutes. For intracellular staining, cells were washed once in PBS, stained with Live/Dead Blue viability marker (Molecular Probes Inc., Eugene, OR) according to manufacturer's instructions, washed in PBS, and fixed on ice in 200µl 3% formaldehyde for 30 min. Cells were then washed in Staining Buffer (SB) (3% Fetal Clone, 0.1% NaAzide in PBS) and permeablized at room temperature in 100 ul 0.5% Triton X-100 in PBS for 10 min. Next, cells were washed with SB and blocked at room temperature in 45 µl Blocking Buffer (BB) (5% Fetal Clone, 0.1M Tris pH 7.2, 0.01% Triton X-100) for 10 minutes. Five microliters of anti Egr-2 (Covance, Richmond, CA)(1:20 in BB) or anti Egr-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:40 in BB) was added to the block (1:200 or 1:400 final dilution, respectively) and incubated at room temperature for 30 minutes. Cells were then washed in SB and incubated with 50 µl anti-rabbit secondary F(ab')2 antibody fragment conjugated to FITC (1:200) or PE (1:50) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in BB at room temperature for 30 minutes. Cells were washed in FACS buffer, resuspended in 1% paraformaldehyde in FACS buffer, and kept at 4^0C until data was collected on an LSRII flow cytometer (BD Biosciences, San Jose, CA) using DigiFACS software (BD Biosciences, San Jose, CA). Post-acquisition analysis was performed with FlowJo software (Treestar, San Carlos, CA).

CFSE labeling

Cells were washed and approximately 3×10^6 were resuspended in 0.5 ml PBS. An

equal volume of 1 μM CFDA SE (carboxyfluorescein diacetate succinimidyl ester,

Molecular Probes Inc., Eugene, OR) in PBS was added (final concentration of 0.5 μM).

Cells were mixed and incubated at RT for 3 minutes. Labeling was quenched by addition

of an equal volume of FBS, cells were washed twice in PBS, resuspended in complete

medium and total number of cells determined by trypan blue exclusion. One-half million

cells were added to 2.5×10^6 syngeneic (B10.BR) splenocytes with or without 10 μM PCC

in a total volume of 1 ml complete media in a 48-well plate and incubated for 4 days.

Cells were then stained with Live/Dead Blue fluorescence (Molecular Probes Inc.,

Eugene, OR) according to manufacturer's instructions and at least 10,000 live events

were collected on an LSRII flow cytometer (BD Biosciences, San Jose, CA) using

DigiFACS software (BD Biosciences). Post-acquisition analysis was performed with

FlowJo software (Treestar, San Carlos, CA).

Cloning of pfosB-Luc reporter plasmid

Approximately 1.5 kb of sequence upstream of the fosB transcription start site

(portion of the promoter containing Egr-2 binding sites) was PCR-amplified using the

following primers: forward) 5'-actgtgctagcccccggaaaagccacctatcct-3' (reverse) 5'-

actgtctcgagttcagaccgccgctgtacct-3'. Each primer contains an enzyme restriction site 5' to

the annealing portion. The PCR product was digested with NheI and XhoI and cloned

into the same sites in the vector pGL3, a promoterless Luciferase expression plasmid (Promega, Madison, WI). A successful construct was identified by enzyme restriction, verified by sequencing, and tested for inducibility by PMA and Ionomycin in a transient transfection luciferase assay.

Transient transfection

EL-4 thymoma or JTAg cells were electroporated with 15 µg pIL2-Luc (a generous gift from L. Berg, University of Massachusetts Medical School, Massachusetts) or 5 µg pfosB-Luc (cloned as described) or pjunB-Luc (JB2, B. Eggen, University of Groningen, Netherlands), 2 µg pRL-TK (Promega, Madison, WI), and increasing amounts of CMV-HA-Egr-2 expression plasmid (pJDM1118, a kind gift from J. Milbrandt, WUMS, St. Louis, MO) or control pCMV5 plasmid in complete media at 20×10^6/ml in 0.5 ml in a 0.4 cm cuvette (BioRad) at 320 volts, 950 uF. The cells were incubated on ice 10 minutes and then added to 5 ml complete media in a 6 well plate to recover for at least 6 hours.

Stable transfection of EL-4 thymoma cell line

Forty million EL-4 thymoma cells were electroporated as above with 20 µg of CMV-HA-Egr2 expression plasmid pJDM1118 (above) or control plasmid pJDM1118(-), in which the coding sequence of Egr-2 was removed by restriction digestion with BamHI and BglII and the vector re-ligated. Ten million electroporated cells were plated per 24

well plate at 1 ml/well. Electroporated cells were allowed to recover for 24 hours (approximately 2 doublings) after which G418 (Geneticin, GibcoBRL/Invitrogen Life Sciences, Carlsbad, CA)(400 μg/ml) was added to the cultures. Cells growing out 3-6 weeks later were expanded, frozen, and tested for Egr-2 expression by western blotting with anti-Egr2 antibody.

Luciferase assay

Transiently transfected cells were stimulated with PMA (75 ng/ml) and Ionomycin (1 μg/ml) (Sigma-Aldrich, St. Louis, MO) for 12 hours (IL-2 promoter) or 3 hours (fosB and junB promoters). Cells were then harvested, washed once in PBS, lysed with 50 μl 1X PLB (Promega, Madison, WI) at room temperature 15 minutes, spun at max speed in a microcentrifuge at 4^0C for 2 minutes, and supernatants transferred to another tube. Twenty microliters of each lysate was analyzed using the Dual Luciferase Assay Kit (Promega, Madison, WI) according to manufacturer's instructions.

Electroporation of A.E7 with siRNA

Two separate, nonoverlapping Egr-2-specific siRNAs (Dharmacon, Lafayette, CO) were determined to effectively silence Egr-2 expression in anergic cells. The sequences are as follows: Egr2-7) gugaccaccuuacuacucadtdt (sense), ugaguaguaagguggucacdtdt (anti-sense) and Egr2-8) guuugccaggagugacgaadtdt (sense), uucgucacuccuggcaaacdtdt (anti-sense). An irrelevant siRNA was synthesized as control

with the following sequence: con) cagucgcguuugcgacuggdtdt, ccagucgcaaacgcgacugdtdt.
Anergic or resting A.E7 T cells were electroporated with 2, 5, or 20 nmoles of siRNA
duplex in 0.5 ml complete growth medium at a density of 60 x 10^6/ml in a 0.4 cm
GenePulser cuvette (BioRad, Hercules, CA). The electroporation was performed with a
GenePulser electroporator II (BioRad, Hercules, CA) at 310 mV, 950 μF. Cells were
allowed to recover for 10 minutes on ice and then added to complete growth medium.
Cells transfected prior to anergy induction were rested for 4-6 hours at 37^0C before being
anergized.

Cell stimulation for ERK phosphorylation

Five million resting or anergic cells, with or without siRNA as indicated, were
stimulated with immobilized anti-CD3 mAb (clone 145-2C11) (BD Biosciences,
Pharmingen, San Diego, CA) precoated at 1 μg/ml and soluble anti-CD28 mAb (clone
37.51) (BD Biosciences, Pharmingen, San Diego, CA) at 1 μg/ml in a 6-well tissue
culture plate (BD Falcon, Bedford, MA) in 1 ml of media. Immediately after addition of
the cells, the plate was centrifuged at 300xg for rapid contact and incubated at 37^0C for
60 minutes. After the incubation, cells were removed from the stimulus and protein was
isolated for western blotting.

Results

Establishment of A.E7 anergy model

I first established the well-studied in vitro model of T cell clonal anergy developed in the laboratory of R. Schwartz (17). A.E7 T cells were anergized by incubating overnight with platebound anti-CD3 monoclonal antibody (mAb). Non-anergized controls included 1) mock-stimulated A.E7s incubated with platebound IgG control antibody and 2) activated A.E7s incubated with platebound anti-CD3 mAb plus soluble anti-CD28 mAb. After the indicated rest period, these populations were rechallenged with irradiated syngeneic antigen presenting cells (APC's) plus pigeon cytochrome c (PCC) antigen to assess their levels of responsiveness. Stimulated cells were either pulsed with tritiated thymidine to measure proliferation (Figure 7a) or the supernatants of the cells were harvested and assayed for IL-2 production (Figure 7b). I determined that 5 days of rest after removal from the stimulus was the earliest time at which cells could be identified as anergic, i.e. the anergic cells were hyporesponsive while the activated control cells were responsive to antigen rechallenge. At earlier periods, activated controls were equally unresponsive to rechallenge as anergic cells. While activated control cells are responsive to antigen at high doses and appear to respond to the same extent as mock-stimulated control cells after a sufficient rest period, at low antigen dose they are hyporesponsive, similar to anergic cells.
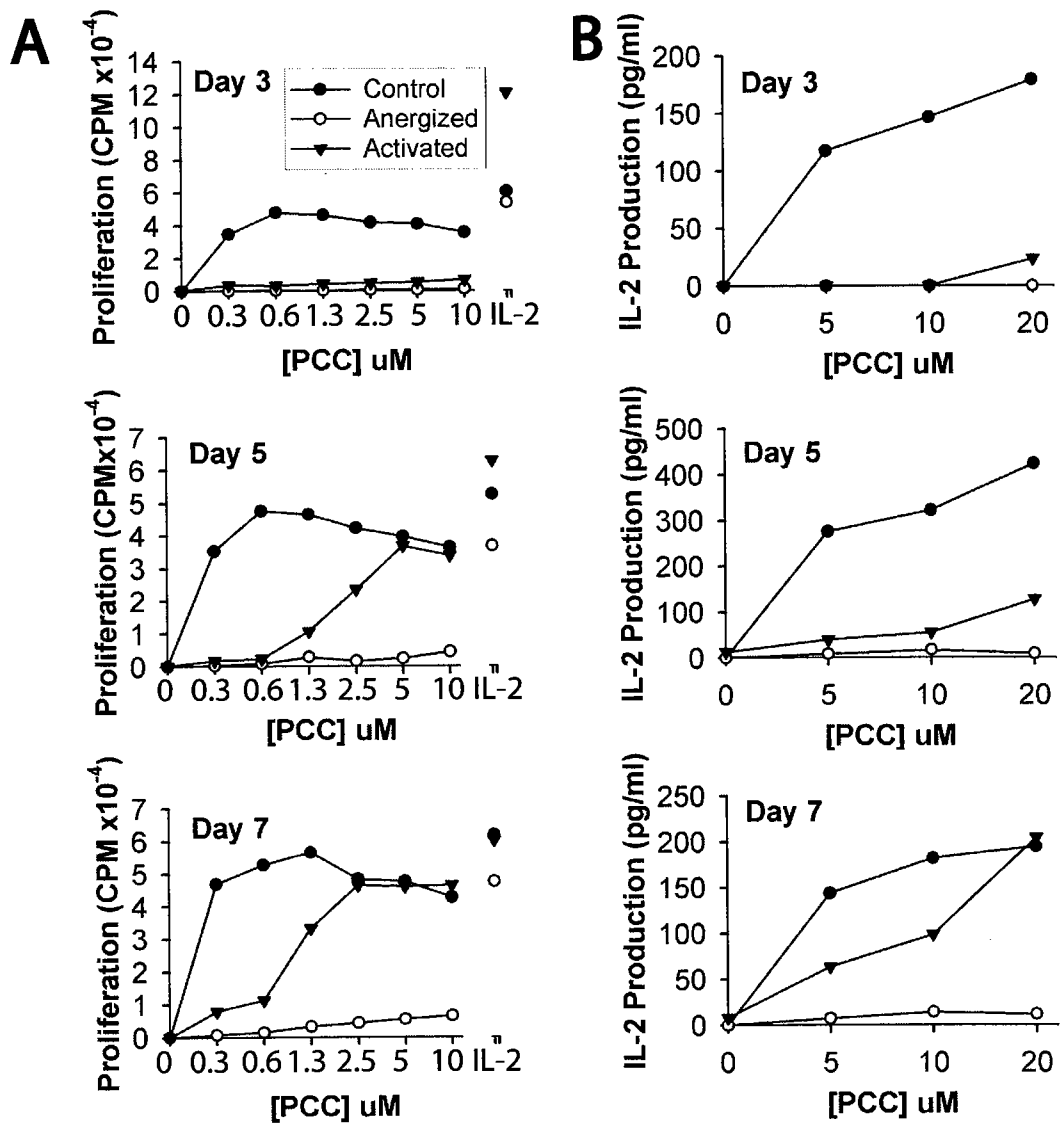
Figure 7

Legend to Figure 7: Following at least 5 days of rest, an anergizing stimulus of A.E7 T cells results in hyporesponsiveness to antigen rechallenge while activated and mock-stimulated cells are responsive. A.E7 T cells were exposed to platebound anti-CD3 mAb (anergized and activated) or platebound IgG antibody (mock-stimulated control) overnight. Activated cells received an additional stimulus of soluble anti-CD28 mAb. The three populations were then submitted to secondary challenge with antigen-pulsed APC's after 3, 5, or 7 days of rest. Level of responsiveness to secondary challenge was assessed by **(A)** tritiated thymidine incorporation into DNA or **(B)** IL-2 secretion.

Microarray analysis

I hypothesized that the hyporesponsive state demonstrated to be a hallmark of T cell clonal anergy was due to expression of key genes in anergic cells. In addition, I felt it probable that the expression profile responsible for anergy involved more than one, and likely many, genes. I felt that the best, unbiased approach to characterizing the anergic expression profile would be a large-scale screen using microarray technology.

In order to identify key players in both the induction and maintenance of anergy, I decided to look at expression profiles at three times—during early, middle and late phases of anergy. The first timepoint was the earliest the cells could be processed for RNA isolation, immediately following 12 hours of stimulation (12 hours). In order to avoid confounding effects of mock-stimulated control cells undergoing apoptosis after prolonged rest, the latest time was the earliest at which the anergic phenotype could be reliably demonstrated, i.e. the activated control cells were responsive while the anergic cells remained unresponsive, which was 5 days following removal from the stimulus (rest day 5). The middle time was 2 days following removal from the stimulus (rest day 2) because it was a time when the activated control cells exhibited a high level of proliferation while anergic cells remained quiescent. These selected timepoints represent a period prior to, during, and after proliferation in the activated control population.

We decided that each timepoint was best assessed in triplicate and so 27 samples (3 populations, 3 timepoints, 3 replicates) were processed for total RNA and prepared for hybridization to the mouse genome U74 version 2 series GeneChips from Affymetrix. The mgU74v2 array series consists of 3 individual chips, each representing ~12,000

expressed sequences, with known genes represented on chip A, and unknown expressed sequence tags (EST's) represented on chips B and C. I performed each replicate and each timepoint as a separate experiment and the samples were prepared and hybridized as soon as they became available. Since the mgU74v2 series consisted of ~36,000 expressed sequences arrayed onto 3 different chips (A, B, and C), I chose to process enough RNA from each experiment to produce a separate hybridization cocktail for each chip, rather than 1 hybridization cocktail per sample and hybridizing it to all 3 chips sequentially as recommended by the manufacturer. This can result in decreased signal after each hybridization, with loss of information for low-expression genes (F. He, Jacobson Lab, UMASS Medical School, unpublished results).

In analyzing the microarray data, I chose to identify genes that were up- or downregulated at least 1.7 fold in anergic cells compared to both mock-stimulated and activated controls. This low-stringency requirement was used by others (60), and was likely to identify a large number of genes that could later be restricted if desired. I also required that candidates appear differentially expressed in each of the 3 replicate experiments, and in the same direction in all of the experiments, i.e. if the gene was upregulated in anergic cells, it must be upregulated in all 3 replicates.

At the earliest timepoint, 12 hours, IL-2 was differentially expressed between anergic and mock-stimulated control cells, as well as between anergic and activated control cells (Figure 8a). These results were confirmed by quantitative RT-PCR (qRT-PCR) using the RNA from one of the microarray experiments (Figure 8b). Aside from IL-2, only 1 other gene fulfilled the criteria; Granzyme B is upregulated to a greater

extent in anergic cells at this early timepoint, but is not expressed on days 2 and 5 (Figure 8c).

At the middle timepoint, rest day 2, a large cohort of genes are differentially expressed in anergic cells compared to controls. Because this is a time when the activated control cells are proliferating and anergized cells are blasting (but remain undivided), it is not surprising that widely varying expression profiles were observed. Because of the large number of differentially expressed genes at this time, it is particularly difficult to identify interesting candidates to pursue. Therefore, candidates that are only differentially expressed at day 2 will not be further discussed.

Because genes continuing to be differentially regulated on rest day 5 are most likely to play a direct role in the anergic phenotype, these candidates were analyzed further, and the expression levels at all times are reported. I gave particular attention to genes that were induced at 12 hours (prior to proliferation) in both anergic and activated cells, downregulated after proliferation in the activated population, and still present in anergic cells on day 5. This is the pattern of expression predicted of an "anergic factor" in the study by Powell et al. (25). On rest day 5, a small number of genes are differentially expressed according to the above requirements. Two ESTs have an interesting expression profile. AI152789 is upregulated early in both anergic and activated cells, becoming differentially expressed in anergic cells primarily on day 2 but also day 5 (Figure 9a). The other, AK011178, is not fully expressed until day 2 and remains at increased levels in anergic cells through day 5 (Figure 9b). Quantitative RT-PCR confirmed the observed expression profile of AK011178 (Figure 9c). Osteopontin, a secreted cytokine first

identified in bone matrix, is expressed early in both anergic and activated cells but is differentially expressed on rest days 2 and 5, downregulated more quickly in the activated population (Figure 10a). Beta-APP cleaving enzyme 2 (Bace-2) is a membrane protein with secretase activity that is expressed only on days 2 and 5 in anergic cells and, to a much lesser extent, activated cells (Figure 10b). Phosphatase of activated cells-1 (Pac-1) is an ERK phosphatase that is differentially expressed according to the requirements in only 2 of the 3 experiments but is worth reporting here. Its expression is increased in anergic cells on day 5 only, narrowly missing the 1.7 fold upregulation requirement in one replicate (Figure 10c). The expression profiles of these genes were confirmed by qRT-PCR (Figure 10d-f).

Egr-2, a zinc-finger transcription factor, is upregulated at 12hr in both anergic and activated cells and is not expressed in mock-stimulated controls. It is present at high levels only in anergic cells on rest days 2 and 5 (Figure 11a). It should be noted that, although expression levels of Egr-2 reported as "average difference" do not appear to show differential expression in the 3rd day 5 replicate experiment, the analysis software reports a 3-fold upregulation in anergic cells compared to activated cells and a 6-fold upregulation over mock-stimulated cells. This discrepancy is due to an error in the algorithm utilized by the analysis software. The observed pattern of expression on the GeneChip was confirmed by qRT-PCR analysis of the transcript (Figure 11b).

A IL-2

Average Difference

B IL-2

IL-2/HPRT

C Granzyme B

Average Difference
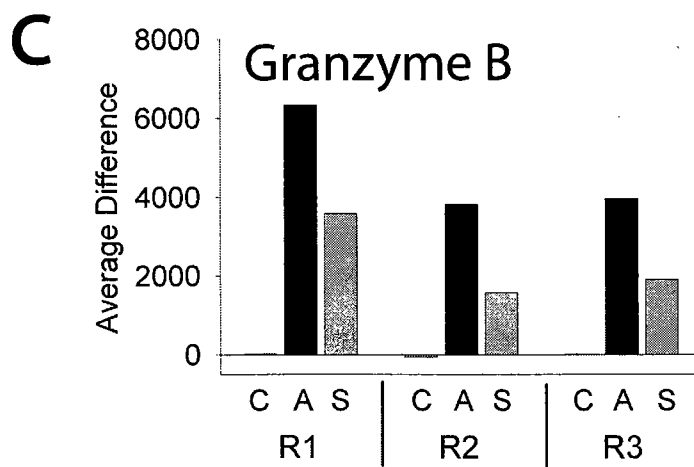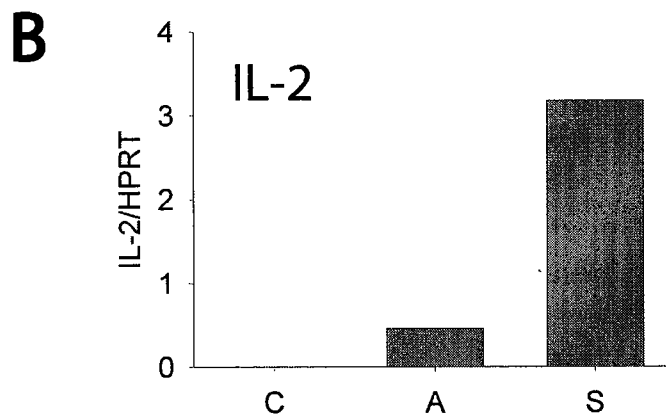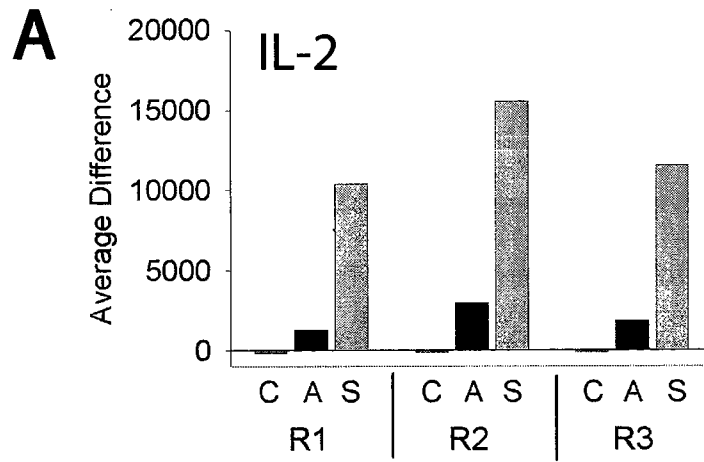
Figure 8

Legend to Figure 8: Analysis of mRNA profiles immediately following 12 hours of

mock-, anergizing, or activating stimuli. A.E7 T cells were exposed to platebound IgG, a

control mock-stimulus (C), to platebound anti-CD3 mAb, an anergizing stimulus (A), or

platebound anti-CD3 plus soluble anti-CD28, an activating stimulus (S) for 12 hours.

Total RNA was isolated, processed into cRNA, and hybridized to Affymetrix GeneChips

or used directly for qRT-PCR. GeneChip signal is reported as the average signal

difference between the gene probe and a nonspecific control. All 3 replicates are shown

(R1-3). The qRT-PCR signal is reported relative to HPRT. **(A)** GeneChip analysis shows

that IL-2 is not present in mock-stimulated control cells and is upregulated slightly

following an anergizing stimulus, but to a much greater extent following a full, activating

stimulus (6-fold higher). **(B)** qRT-PCR confirms this observation. **(C)** Granzyme B is not

present in mock-stimulated cells but is greatly induced in anergic cells. It is induced to a

smaller extent in activated cells.

A AI152789

B AK011178

C AK011178

Figure 9

Legend to Figure 9: Identification of expressed sequence tags (ESTs) differentially

expressed in anergic cells after 5 days of rest. A.E7 T cells were treated as in Figure 8

[mock-stimulated (C), anergized (A), activated (S)] and processed immediately for RNA

(12 hr) or rested 2 days (Day 2) or 5 days (Day 5) before RNA isolation. Total RNA was

processed and analyzed by GeneChip or qRT-PCR as in Figure 8. The criteria required

for genes to be reported include differential expression on day 5. Expression profiles are

reported for all 3 replicates (R1-3) at all 3 times. **(A)** The EST AI152789 is induced by 12

hours in both anergized and activated cells but remains elevated in only anergic cells at

day 2, and to a lesser extent at day 5. **(B)** The EST AK011178 is not significantly

expressed until rest day 2, at which point it is expressed at highest levels in anergic cells.

This differential expression is maintained on rest day 5, but the expression is lower than

on rest day 2. **(C)** qRT-PCR confirms the GeneChip results for AK011178.
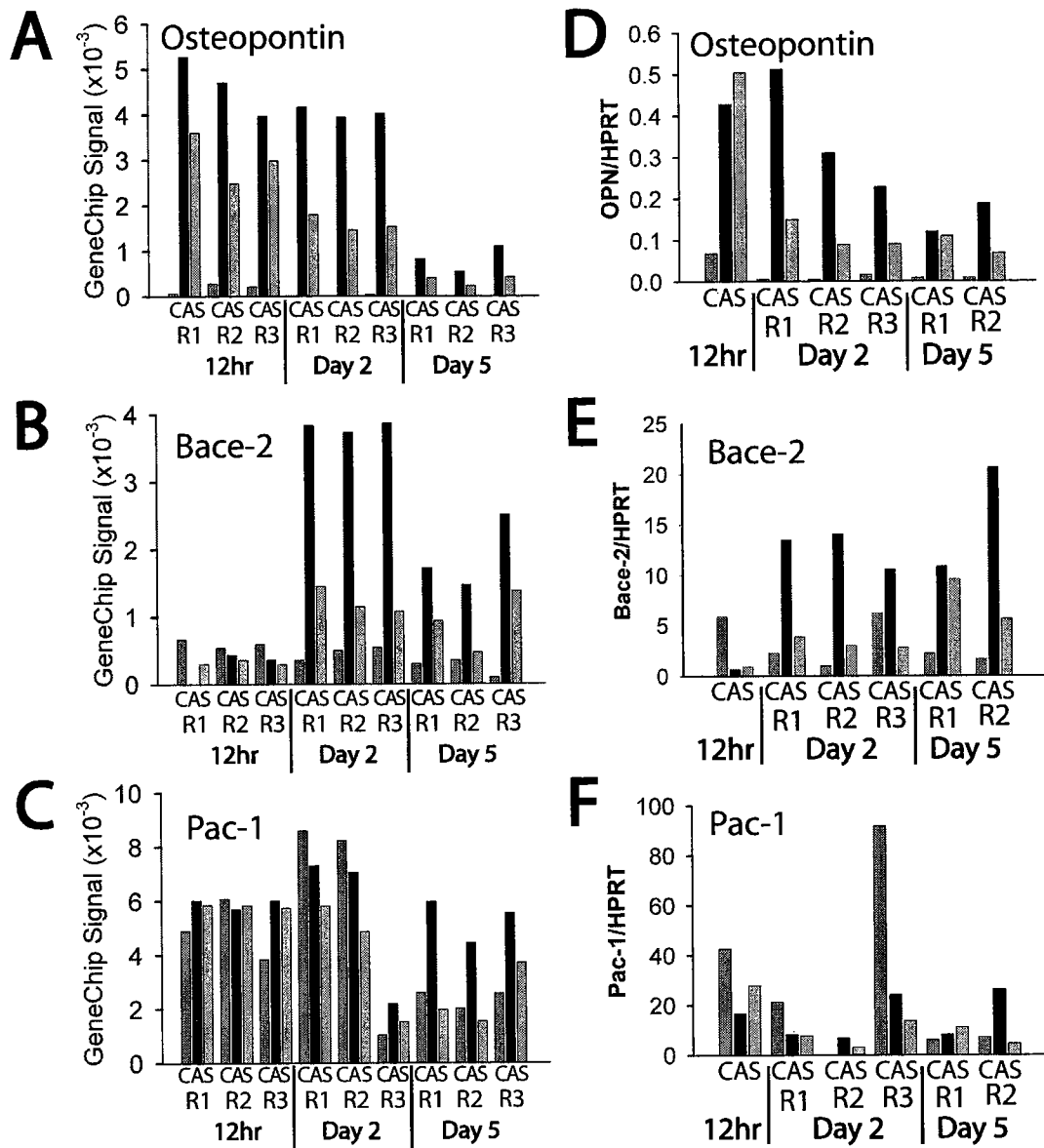
Figure 10

Legend to Figure 10: Identification of known genes differentially expressed in anergic cells after 5 days of rest. A.E7 T cells were treated as in Figure 8 [mock-stimulated (C), anergized (A), activat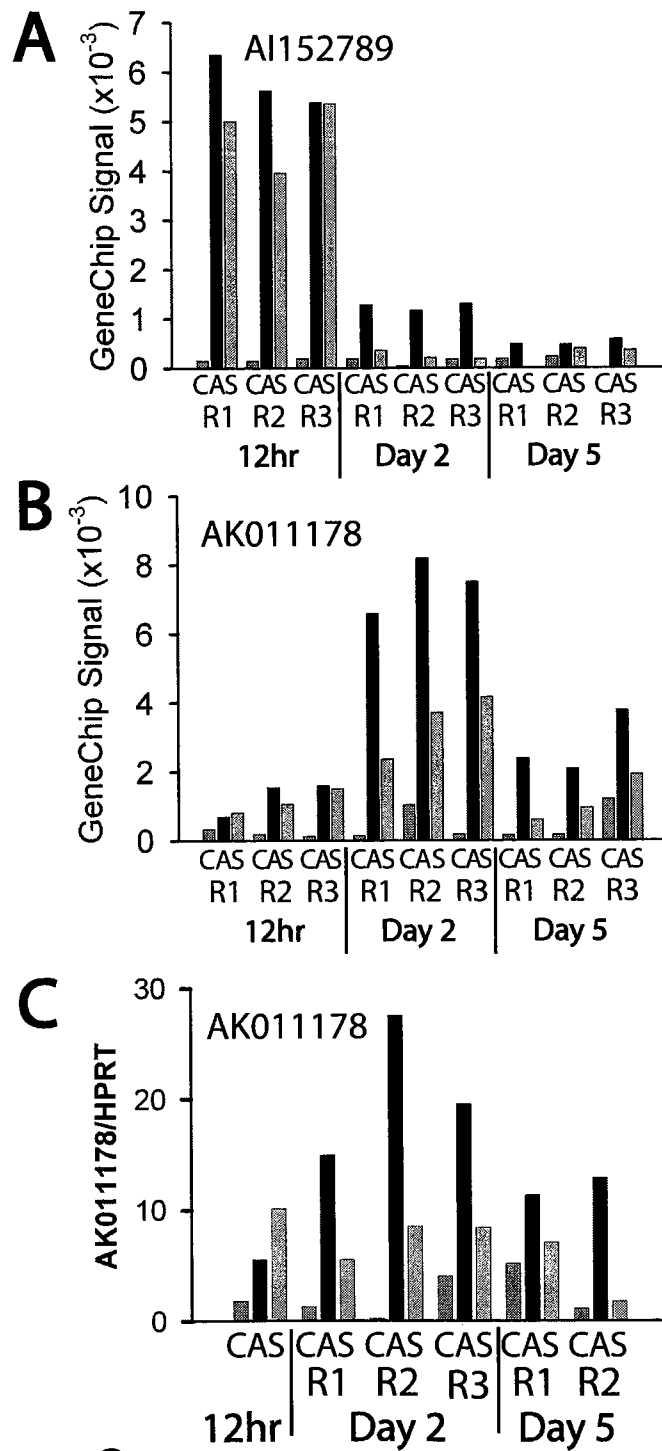ed (S)] and processed immediately for RNA (12 hr) or rested 2 days (Day 2) or 5 days (Day 5) before RNA isolation. Total RNA was processed and analyzed by GeneChip or qRT-PCR as in Figure 8. The criteria required for genes to be reported include differential expression on day 5. Expression profiles are reported for all 3 replicates (R1-3) at all 3 times. **(A)** Osteopontin is induced to similar levels following a 12-hour anergizing or activating stimulus. Differential expression is more evident in anergic cells after 2 days of rest. It is still expressed in anergic cells at low levels after 5 days and to a lesser extent in activated cells. **(B)** Bace-2 is not expressed after 12 hours of anergizing or activating stimulus. It is highly expressed after 2 days in anergic cells and, at this time, is upregulated in anergic compared to activated cells. Differential expression is maintained on day 5. **(C)** Pac-1 expression, while highly variable in the samples, is only differentially expressed on day 5. **(D,E)** qRT-PCR confirms the GeneChip data for Osteopontin and Bace-2 expression. **(F)** qRT-PCR analysis of Pac-1 expression is as variable as the GeneChip data, and only one of two replicates on day 5 shows increased expression in anergic cells.
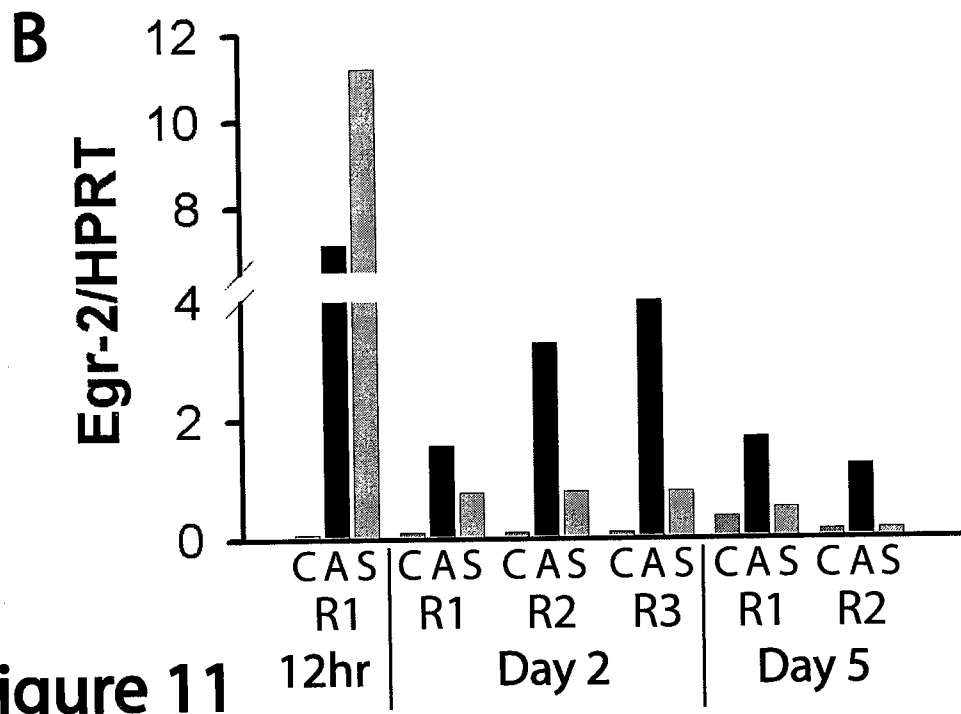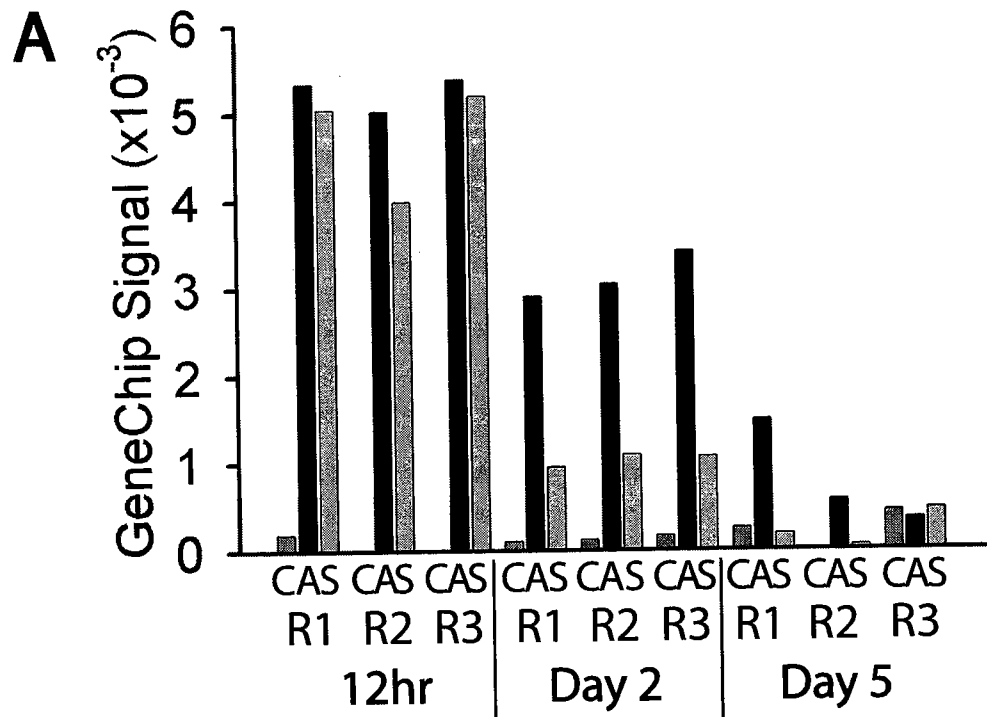
Figure 11

Legend to Figure 11: Egr-2 expression in mock-stimulated, anergic, and activated cells. A.E7 T cells were treated as in Figure 8 [mock-stimulated (C), anergized (A), activated (S)] and processed immediately for RNA (12 hr) or rested 2 days (Day 2) or 5 days (Day 5) before RNA isolation. Total RNA was processed and analyzed by GeneChip or qRT-PCR as in Figure 8. Expression profiles are reported for all 3 replicates (R1-3) at all 3 times. **(A)** Egr-2 expression is increased in both anergic and activated cells following 12 hours of stimulation but remains at an elevated level only in anergic cells on days 2 and 5. The third replicate on day 5 appears not to show differential expression; however, this is due to an error in the algorithm used by the GeneChip analysis software. **(B)** qRT-PCR confirms the GeneChip results.

Egr-2 protein levels correlate with anergy

I analyzed Egr-2 protein levels in anergic and control A.E7 T cells over 9 days by western blot (Figure 12a). Egr-2, while not expressed in mock-stimulated T cells, is upregulated equally in anergic and activated control cells after 12 hours of stimulation. Over the following days, Egr-2 is diminished in activated cells compared to anergic cells. By day 5, coinciding with the earliest day when activated control cells are responsive to antigen rechallenge, protein levels are essentially at resting levels in this population. Interestingly, the rapid downregulation of Egr-2 protein level in activated cells between rest days 3-5 coincides with the greatest increase in cell number at that time, reflecting proliferation of these cells (Figure 12b). The cells were stimulated on rest day 9 to assess responsiveness to antigen rechallenge (Figure 12c).

Because the addition of exogenous IL-2 induces proliferation in anergic cells and abrogates the anergic phenotype, I assessed protein levels of Egr-2 in anergic cells 1, 3, 5, 7, and 10 days following exposure to IL-2 (Figure 12d). The cells were stimulated on rest day 10 to assess responsiveness to antigen rechallenge (Figure 12e). Similar to proliferating cells following activation, proliferating anergic cells exposed to IL-2 downregulate Egr-2 protein and are more responsive to stimulation than anergic cells not exposed to IL-2.
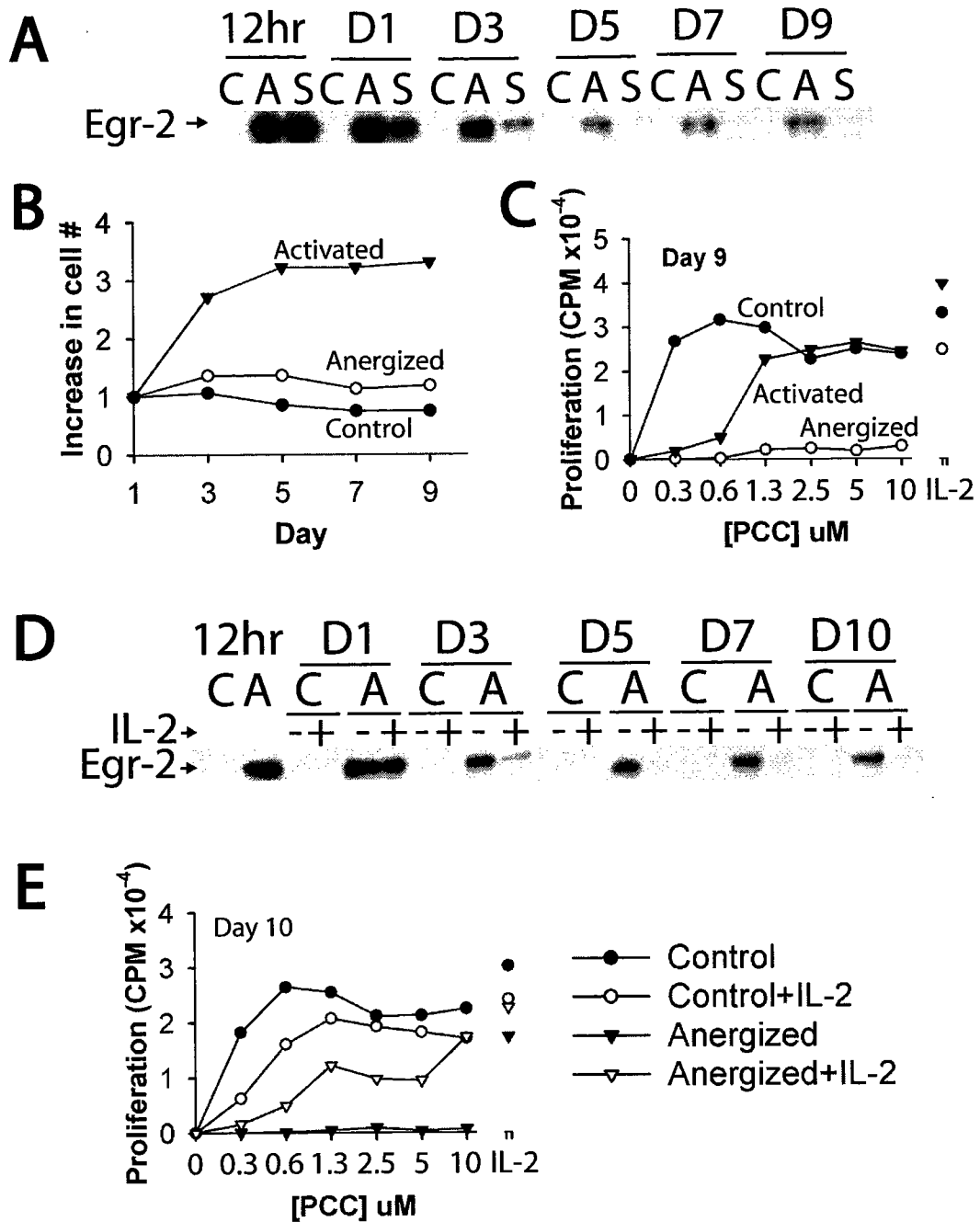
Figure 12

Legend to Figure 12: Egr-2 protein levels correlate negatively with proliferation and with responsiveness to antigen rechallenge. **A)** Egr-2 protein levels were assessed by western blot in cells that were mock-stimulated (C), anergized (A), or activated (S) as in Figure 8. Cells were processed immediately after removal from stimulus (12 hr), and after 1, 3, 5, 7, and 9 days of rest (D1-9) in media. **B)** Live cells were counted by trypan blue exclusion after 1, 3, 5, 7, and 9 days of rest for comparison of Egr-2 protein levels to proliferation (increase in cell number). Proliferation is reported as a ratio of the cell number on the indicated day to the cell number on day 1. **C)** After 9 days of rest, the cells were submitted to antigen rechallenge to assess responsiveness by [^{3}H]-thymidine incorporation into DNA. **D)** Egr-2 protein is downregulated in anergic cells treated with exogenous IL-2. Egr-2 protein levels were assessed by western blot in cells that were mock stimulated (C) or anergized (A) as above. Cells were processed immediately after removal from stimulus (12 hr), and after 1, 3, 5, 7, and 10 days of rest (D1-10) in either media alone or with exogenous IL-2 (10 units/ml) to induce proliferation and break anergy. **E)** After 10 days of rest, the populations were submitted to antigen rechallenge to assess level of responsiveness by tritiated thymidine incorporation.

Kinetics of Egr-2 and Egr-1 upregulation following stimulation

Following 6 days of rest, I submitted mock-stimulated, anergic, and activated populations to secondary stimulation with platebound anti-CD3 mAb and soluble anti-CD28 mAb for 0, 4, 8, and 12 hours and analyzed the Egr-1 and Egr-2 protein level by western blot. While Egr-2 is only expressed in anergic cells prior to stimulation, it is quickly induced in all cell populations following stimulation (Figure 13a). Egr-1 is not expressed in any of the populations prior to stimulation but is upregulated quickly following stimulation. However, in anergic cells, Egr-1 is not upregulated to the same extent as in previously mock-stimulated cells. This "blunted" upregulation in anergic cells is also observed to some extent in the previously activated, or "partially anergic", population (Figure 13b).

In order to determine the kinetics of Egr-1 and Egr-2 protein expression in A.E7s stimulated in a more physiological way, I mixed previously mock-stimulated, anergic, and activated cells with T cell-depleted APC's that had been loaded with a high (10uM) or low (0.3uM) concentration of PCC for 0, 3, or 6 hours. Cells were then harvested, permeablized and stained for Egr-1 or Egr-2 for analysis by flow cytometry. As was determined by western blot, before stimulation (0hr) Egr-2 is most highly expressed in anergic cells (Figure 14a) while Egr-1 is not expressed by any of the three populations (Figure 14b).

Egr-2 is upregulated to maximal levels in previously mock-stimulated and activated cells by 3 hours and remains at high levels for at least 6 hours. In anergic cells,

Egr-2 begins at increased levels but is further induced to maximal levels with stimulation after 3 hours (Figure 14a). Egr-1 is also upregulated to maximal levels in previously mock-stimulated cells by 3 hours but begins to return to low levels after 6 hours. A high antigen dose results in a more rapid upregulation of Egr-1 than a low dose. In agreement with the western blot, anergic cells fail to upregulate Egr-1 to levels of mock-stimulated cells when stimulated with both low and high antigen concentrations. Also, in contrast to previously mock-stimulated cells, expressed levels of Egr-1 in anergic and, to some extent, previously activated cells quickly return to prestimulated levels after peak expression (Figure 14b).

A

	0hr			4hr			8hr			12hr		
	C	A	S	C	A	S	C	A	S	C	A	S

Egr-2 →

actin →

B

	0hr			4hr			8hr			12hr		
	C	A	S	C	A	S	C	A	S	C	A	S

Egr-1 →

actin →
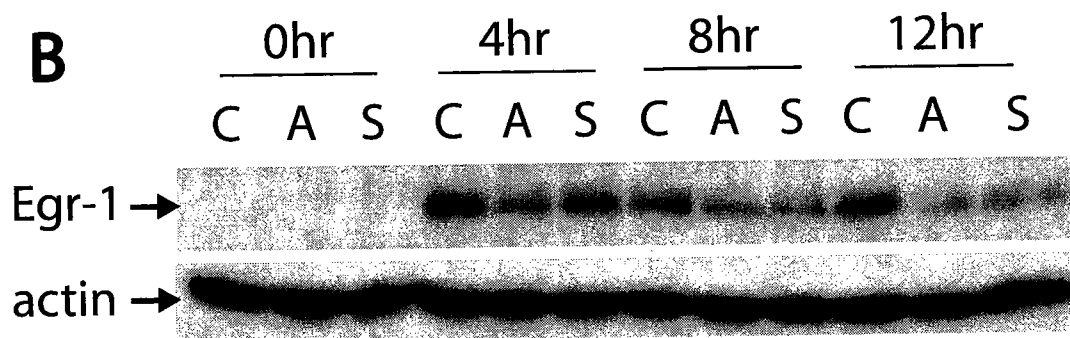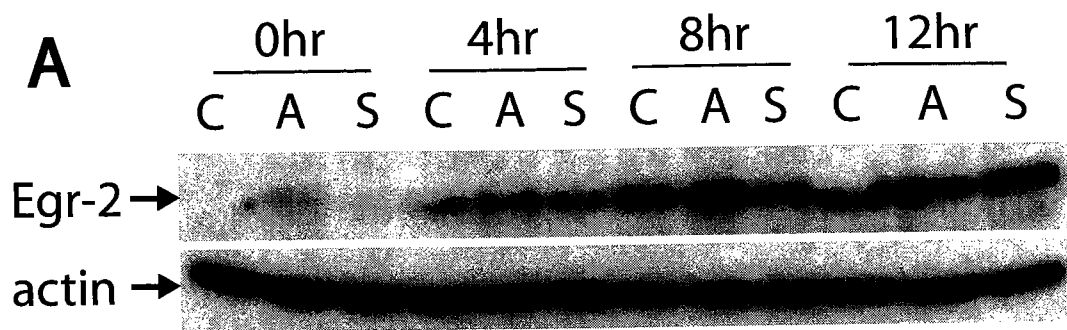
Figure 13

Legend to Figure 13: Western blotting of Egr-1 and Egr-2 protein levels in mock-stimulated (C), anergized (A), and activated (S) cells following secondary antibody stimulation. Cells were treated as in Figure 8 [mock-stimulated (C), anergized (A), activated (S)], rested for 6 days, and submitted to secondary stimulation for 0, 4, 8, or 12 hours with platebound anti-CD3 and soluble anti-CD28 mAb. Protein levels of **(A)** Egr-2 and **(B)** Egr-1 were then analyzed by western blot. Blots were stripped and reprobed with an antibody for β-actin to control for protein loading.

A PCC: 0.3 uM   10 uM
0hr ——
3hr ——
6hr - - -

Mock

Anergized

Activated

Egr-2 →

B PCC: 0.3 uM   10 uM
0hr ——
3hr ——
6hr - - -

Mock

Anergized
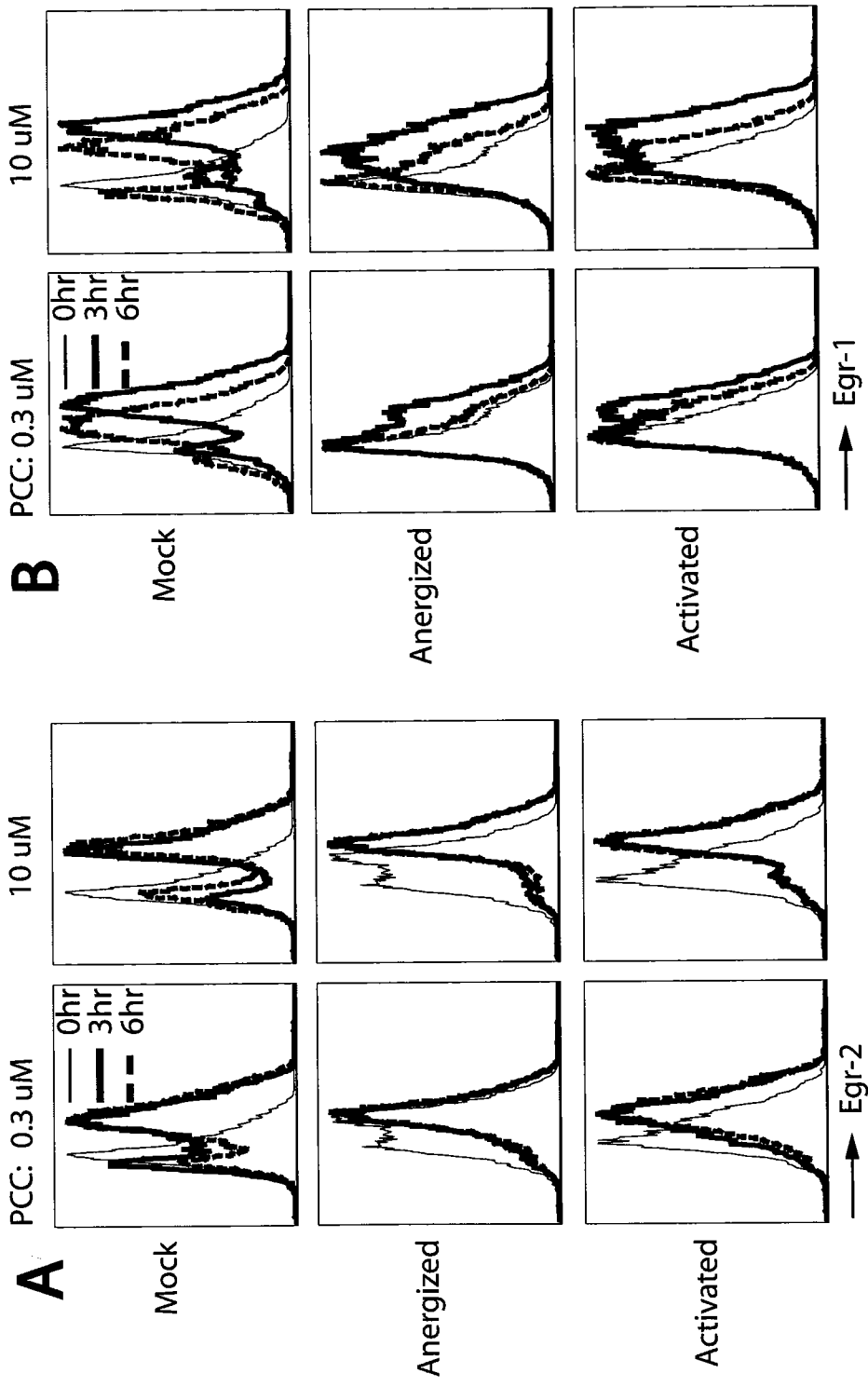
Activated

Egr-1 →

Figure 14

Legend to Figure 14: Flow cytometry analysis of Egr-1 and Egr-2 protein levels in mock-stimulated, anergized, and activated cells following antigen rechallenge. Cells were treated as in Figure 8, rested for 6 days, and submitted to antigen rechallenge for 0, 3 or 6 hours with 0.3 or 10 μM PCC antigen and T cell-depleted syngeneic APC's. Cells were then intracellular stained for **(A)** Egr-2 and **(B)** Egr-1 and analyzed by flow cytometry.

Effect of transient Egr-2 overexpression on IL-2, fosB, and junB promoters

Because one of the hallmarks of the anergic phenotype is defective expression of IL-2, I searched the IL-2 promoter as well as the promoters of known activators of IL-2 transcription for potential Egr-2 binding sites. When promoters were unpublished, I did this by searching the Celera genome database for each of the genes, identified the transcription start site, and examined the upstream 2 kilobases using TESS transcription search tool on the internet. I found that the promoters of IL-2, fosB, and junB all contained putative Egr-2 binding sites.

I tested these promoters for susceptibility to Egr-2 repression by obtaining vectors with the promoters cloned into firefly luciferase reporter plasmids. The JTAg human T cell line or EL-4 thymoma T cell line was transiently transfected with one of the three reporter plasmids, a CMV-Egr-2 expression plasmid, and a TK-Renilla Luciferase plasmid for transfection control. Transfected cells were allowed to recover for at least 6 hours, stimulated with PMA + Ionomycin overnight (IL-2 promoter) or 3 hours (fos and jun promoters), harvested, and assessed for Luciferase activitiy. None of the promoters appeared to be sensitive to Egr-2 repression (data not shown).

Stable transfection of CMV-Egr2 into EL-4 T cells

EL-4 thymoma T cells were stably transfected with a CMV-HA-Egr-2 expression plasmid and selected via G418 drug selection. A number of stable clones grew out with

resistance to G418. However, only two clones appeared to produce detectable levels of Egr-2 by western blot (Figure 15a). One of the clones, 5C1, produced Egr-2 at the expected size. Another clone, 5D3, produced Egr-2 at a size slightly larger than expected. The clone 5C1 grew at a much slower rate than the others. In order to assess if there were any cell cycle defects in this clone, I analyzed 5C1 by PI staining compared to the faster-growing controls. Strikingly, there was a much higher proportion of dead cells in the 5C1 compared to controls—11.5% versus 2.0-2.2% (Figure 15b).

The clones were then tested for IL-2 production in response to antibody stimulation. They were exposed to platebound anti-CD3 mAb and soluble anti-CD28 mAb for 20-24 hours when the supernatant was harvested for analysis by ELISA. There was wide variability of IL-2 secretion among the clones. Interestingly, the clone 5D3 produced little to no IL-2 in response to antibody stimulation (Figure 15c), while production in response to PMA and Ionomycin stimulation was copious (data not shown).

A

5C1 5D3 con Egr-2

Egr-2 →

B

1C2 2B2

5C1 5D3

→ PI

C

IL-2 Production (ng/ml)

0.8

0.6

0.4

0.2

0.0

1C2

5C1

2B2

5D3

0/0 1/1 2.5/1 5/1 10/1

Antibody Stimulation
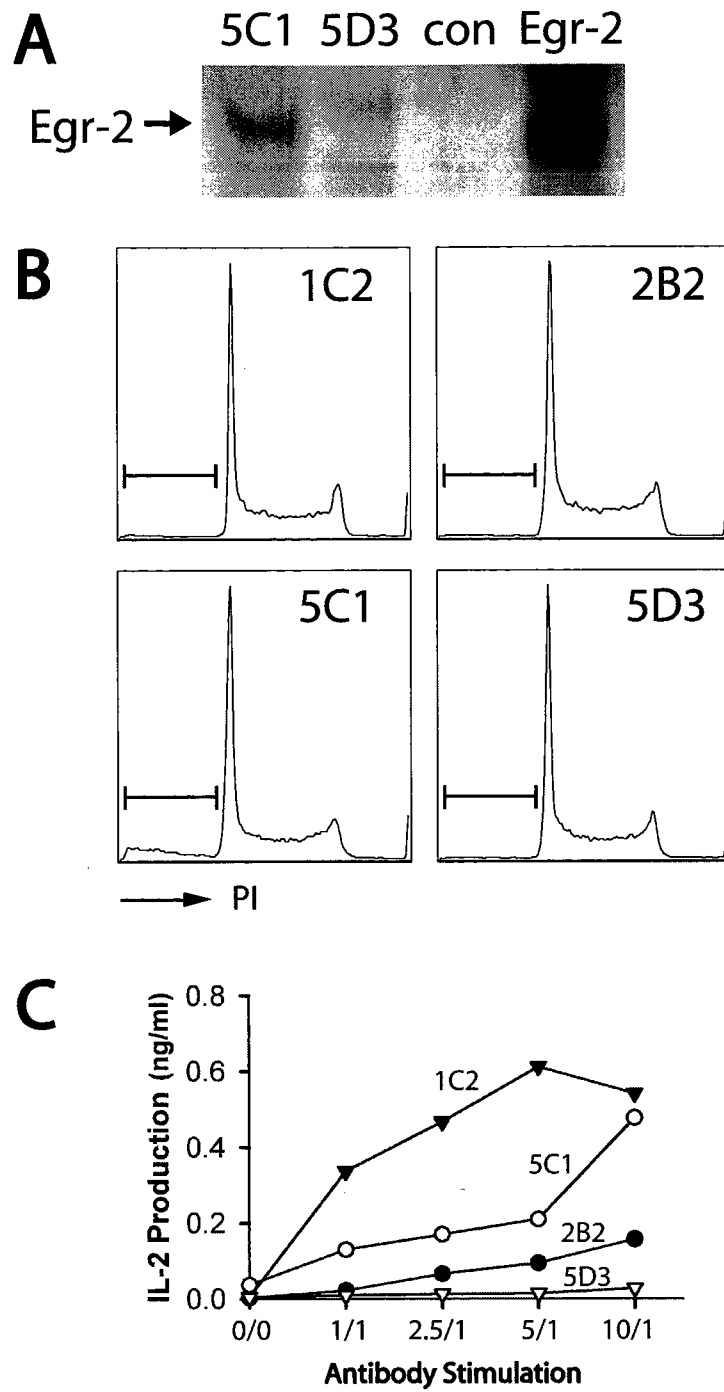
Figure 15

Legend to Figure 15: Transfection of an Egr-2 expression vector in EL-4 thymoma cells followed by G418 drug selection results in 2 stable clones expressing Egr-2; one with a growth defect, one with an IL-2 production defect. **(A)** Western blot of stable clones overexpressing Egr-2. Clone 5C1 expresses Egr-2 at the expected size while 5D3 makes a product that is larger than expected compared to Cos cells transiently transfected with the vector (Egr-2). Control transfected cells (con) make no Egr-2. **(B)** PI staining of EL-4 stable clones shows cells in different stages of the cell cycle. The sharp peak represents cells in G1 phase and cells with less staining (gate shown) represent dead cells. The slow-growing 5C1 clone has 11.5% cells within this gate compared to the other clones (2.0-2.2%). **(C)** IL-2 production in stimulated EL-4 stable clones. Clones were stimulated with increasing amounts of platebound anti-CD3 (0-10 μg/ml) and soluble anti-CD28 mAb (1 μg/ml). Antibody labeling on the x-axis is represented as anti-CD3/anti-CD28 in μg/ml. Supernatants were harvested after 20-24 hours and analyzed for IL-2 by ELISA.

Analysis of allotolerant mice bearing long-term skin allografts

In order to correlate the anergy phenotype with tolerance in vivo, long-term skin allograft recipients were sacrificed and analyzed for the presence of Egr-2-expressing CD4+ T cells. Skin graft recipients that had maintained skin allografts for at least 70 days, cohorts that were actively rejecting their grafts, or recipients of intact isografts were sacrificed and either lymph nodes draining the graft or nodes irrelevant to the graft site were collected for analysis by flow cytometry. Cells were extracted from the nodes, counted, and subsequently stained for flow cytometry for CD4, CD8, CD25, and intracellular stained for Egr-2 or Egr-1 (a control protein associated with activation but not anergy). In general, there was little evidence for correlation of Egr-2 expression with long-term tolerance of skin allografts when compared to rejecting or isograft controls. In one experiment, however, 2 out of 3 mice with intact allografts had an increased percentage of Egr-2 positive, CD4+ T cells in their draining lymph nodes when compared to irrelevant nodes in the same mouse and to nodes draining isograft recipients (Figure 16). There was no correlation between Egr-2 and CD25 expression on CD4+ T cells in any of the mice examined and, as expected, there was no increased expression of Egr-1 in tolerant mice (data not shown).
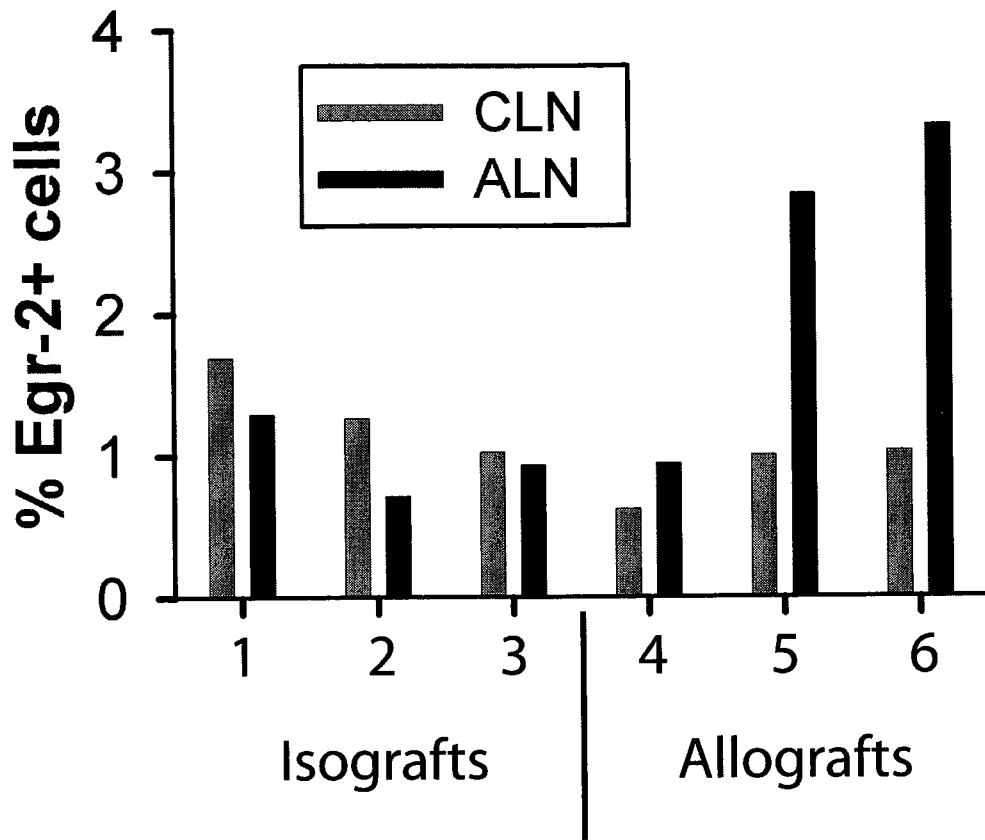
Figure 16

Legend to Figure 16: Egr-2 expression by CD4+ T cells in lymph nodes draining a skin

allograft in mice tolerized by DST plus anti-CD40L. CD4+ T cells from the axillary

lymph nodes (ALN) draining a long-term (70-80 days) graft of tolerized mice were

examined for Egr-2-expression by flow cytometry. Controls were pooled cervical nodes

(CLN) from the tolerant mice or axillary nodes from non-tolerant mice with isografts.

CD4+ cells were gated and analyzed as forward scatter vs. FITC (Egr-2 staining). % Egr-

2 positive cells represent cells with staining increased over isotype control.

Egr-2 gene silencing using siRNA

In order to assess the role Egr-2 plays in the anergic phenotype, eight siRNA oligos were designed from the mRNA sequence of Egr-2. Each of the oligos was tested for its ability to knock down Egr-2 protein levels in anergic A.E7 T cells. The cells were anergized, rested for 5 days, and then electroporated with 20 nanomoles of each siRNA. Protein levels were assessed by intracellular staining and flow cytometry 2 and 3 days after treatment with siRNA (Figure 17). Two of the eight oligos (Egr2-7 and Egr2-8) were very effective in knocking down Egr-2 protein levels and were utilized for further study.

Oligo Egr2-7 was tested for its ability to knock down protein levels when administered prior to anergy induction. A.E7 T cells were electroporated with media alone or 20 nanomoles of control or Egr-2 siRNA. They were allowed to recover for 4-6 hours and then anergized for 12 hours. Some cells were also electroporated with media alone and left unstimulated as a non-anergic control. After 2 and 8 days of rest, cells were stained for Egr-2 protein via intracellular staining and analyzed by flow cytometry (Figure 18). While Egr-2 protein is still induced to significant levels in anergized cells treated with Egr-2 siRNA on rest day 2, protein is reduced in a population of cells to very low levels after 8 days of rest when treated with Egr-2-specific siRNA. It appears that not all cells are affected by siRNA knockdown, as there is a population (higher staining peak) in the Egr-2 siRNA group that still expresses Egr-2 at a high level. Note, in addition, that even cells receiving no siRNA develop two staining populations of cells for Egr-2 (high

and low). This was not observed in cells in which anergy was induced prior to siRNA treatment.

Different quantities of siRNA were tested for the ability to knock down Egr-2 protein levels when administered prior to anergy induction, with the intention of using the lowest possible amount of siRNA. In one experiment, I compared 5 and 20 nanomoles side by side; 5 nanomoles was sufficient for effective knockdown (Figure 19a). In a later experiment, I compared 5 nanomoles to 2 nanomoles and, while I still saw a significant knockdown of protein using the lower amount (Figure 19b), I decided to use 5 nanomoles for future experiments.

In addition to flow cytometry, I chose to analyze Egr-2 protein knockdown by western blot. When cells were removed for intracellular staining by flow cytometry, they were also processed for total cell protein. The mild effect of knockdown observed with flow cytometry is contrasted with an apparently much more significant effect when analyzed by western blot (Figure 19c.).

Egr2-4 Egr2-3 Egr2-2 Egr2-1

— not anergic
▪▪ con siRNA
▬ Egr-2 siRNA

Egr2-8 Egr2-7 Egr2-6 Egr2-5
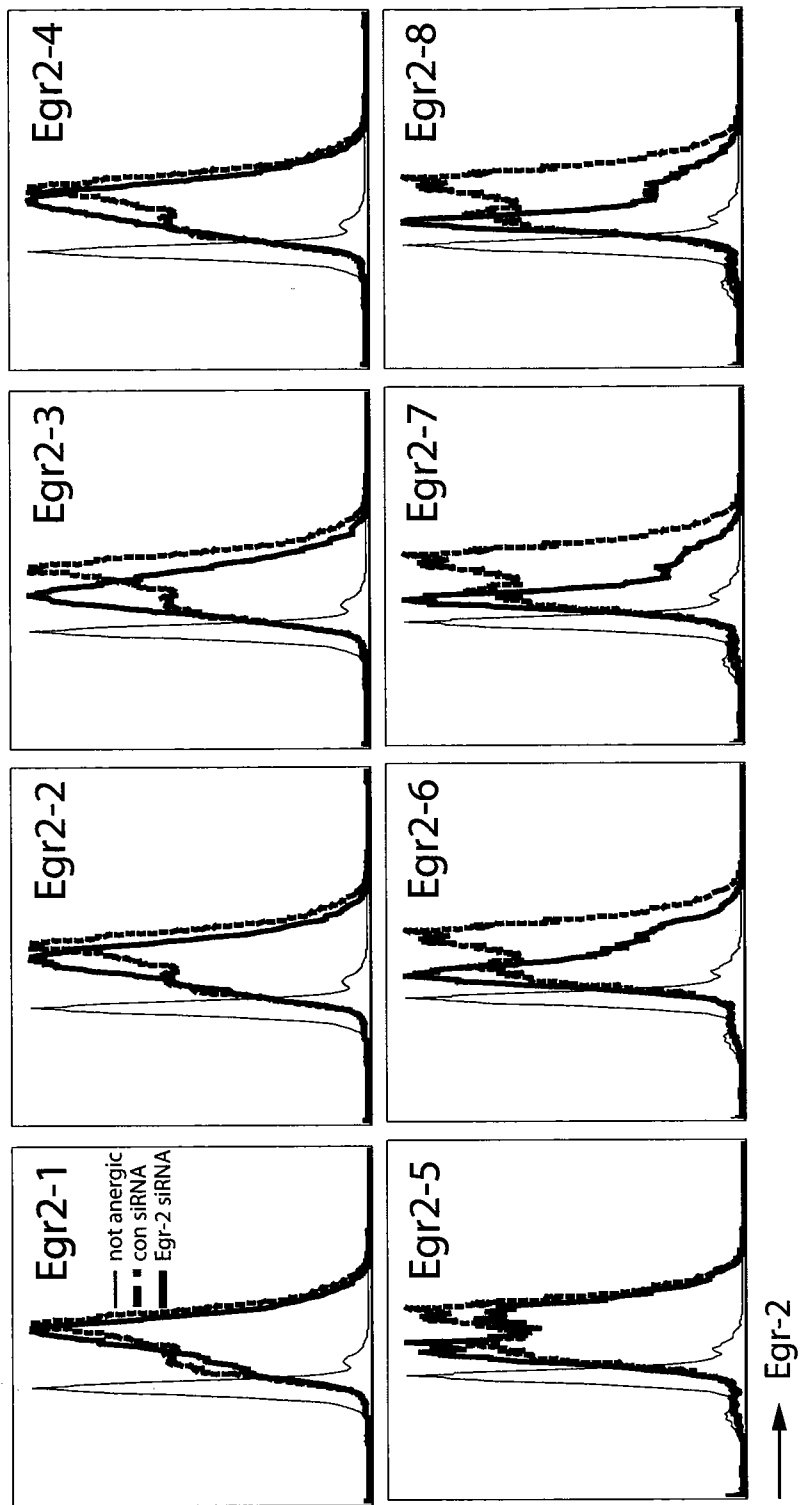
Egr-2

Figure 17

Legend to Figure 17: Eight siRNA duplexes [Egr2-(1-8)] designed from different

regions of Egr-2 mRNA were tested for the ability to silence gene expression in anergic

A.E7 T cells. Twenty nanomoles of each siRNA was electroporated into anergic cells that

had rested for 5 days. Egr-2 protein level was assessed by flow cytometry 2 days after

siRNA treatment (rest day 7). The thin histogram in each box represents non-anergized

control cells (no Egr-2 expression). The dashed line represents cells treated with control

siRNA. The thick solid line represents cells treated with the indicated Egr-2-specific

siRNA. Duplexes Egr2-1 through Egr2-4 were compared in one experiment and Egr2-5

through Egr2-8 were compared in another. Of the first 4 duplexes, Egr2-3 had the

greatest gene silencing effect. Of the last 4 duplexes, Egr2-6, 7, and 8 all had an effect,

and Egr2-7 and 8 were the most effective.

Day 8

Day 2

not anergic
con siRNA
Egr-2 siRNA
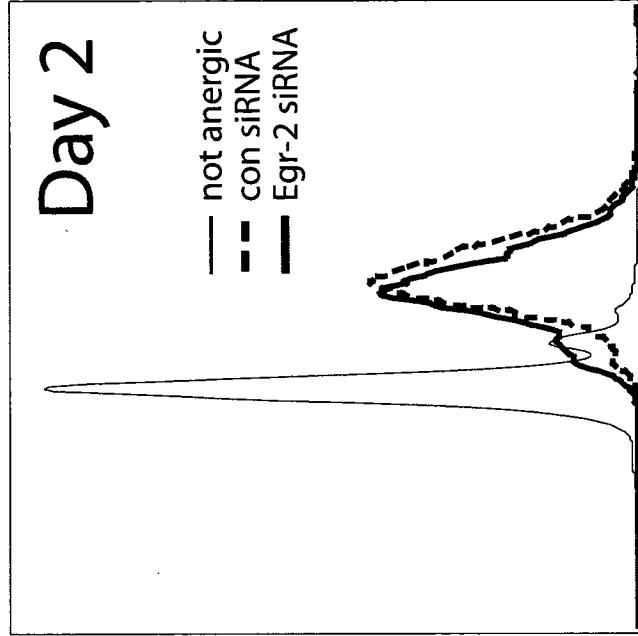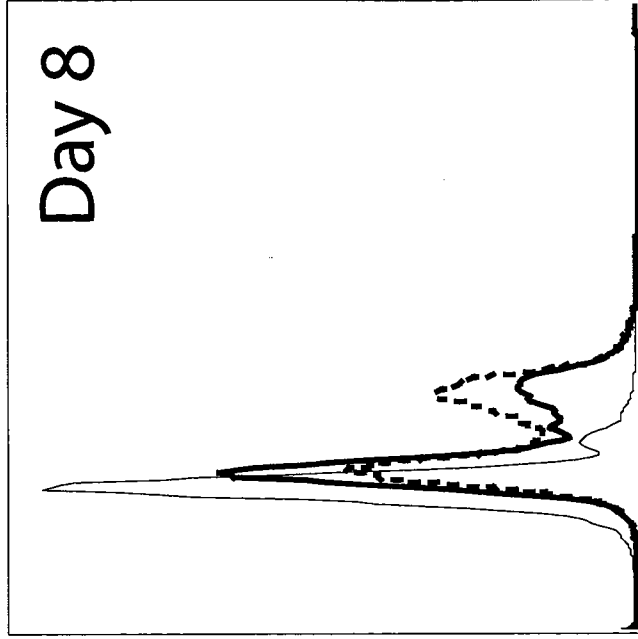
→ Egr-2

Figure 18

Legend to Figure 18: Gene silencing in cells transfected with siRNA before being

anergized. Cells were transfected with 5 nanomoles of siRNA and then anergized. Egr-2

protein was analyzed by flow cytometry following 2 and 8 days of rest. The thin

histogram in each box represents non-anergized control cells (no Egr-2 expression). The

dashed line represents cells treated with control siRNA. The thick solid line represents

cells treated with Egr-2-specific siRNA. Very little Egr-2 gene silencing is evident after

only 2 days of rest following anergy induction. After 8 days, a population of cells

downregulates Egr-2 to low levels.
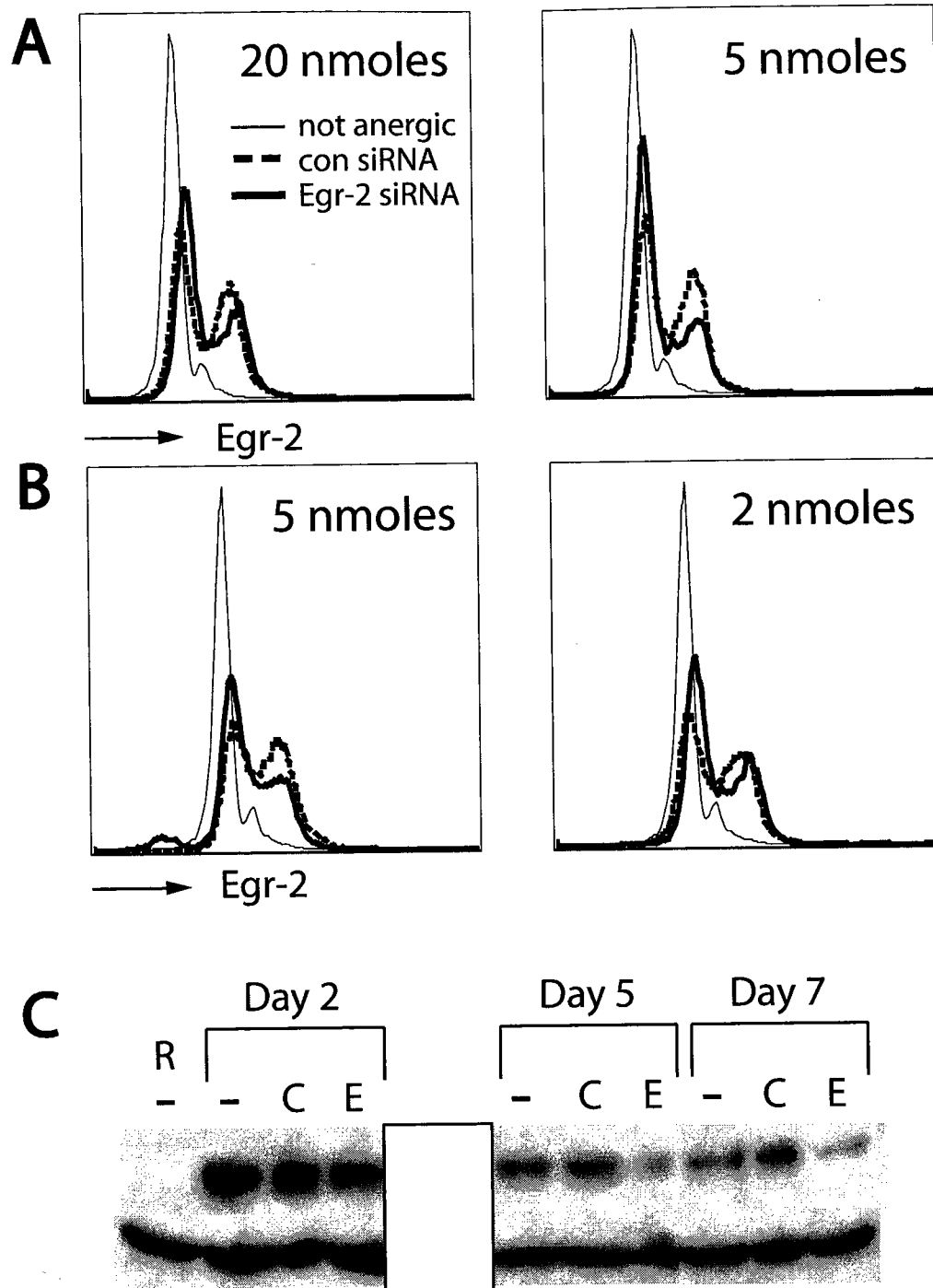
Figure 19

Legend to Figure 19: Egr-2 gene silencing in cells treated with 20, 5, or 2 nanomoles of

siRNA. **(A)** Cells were transfected with 20 or 5 nanomoles of siRNA and then anergized.

Egr-2 protein was analyzed by flow cytometry after 5 days of rest. The thin histogram in

each box represents non-anergized control cells (no Egr-2 expression). The dashed line

represents cells treated with control siRNA. The thick solid line represents cells treated

with Egr-2-specific siRNA. Five nanomoles of siRNA is more effective at gene silencing

than 20 nanomoles. **(B)** Cells were transfected with 5 or 2 nanomoles of siRNA and then

anergized. Egr-2 protein was analyzed by flow cytometry after 5 days of rest. Five

nanomoles of siRNA demonstrates similar gene silencing to 2 nanomoles. **(C)** Cells

treated with 5 nanomoles of siRNA described in (B) were also analyzed for protein

expression by western blot after 2, 5, and 7 days of rest. The first lane, labeled "R" is

protein from resting cells that do not express Egr-2; all other lanes contain protein from

anergic cells electroporated with no siRNA (-), control siRNA (C) or Egr-2 siRNA (E).

The blot was then stripped and reprobed with antibody for β-actin to control for protein

loading. After 5 and 7 days of rest, 5 nanomoles of siRNA was very effective at silencing

Egr-2 gene expression.

Egr-2 is required for anergy induction

To determine the role of Egr-2 in anergy maintenance, anergic cells were subjected to electroporation with Egr-2 siRNA after 5 days of rest. The cells were analyzed for decreased expression 48 hours later (rest day 7) by flow cytometry and then rechallenged to determine if Egr-2 played a direct role in maintaining hyporesponsiveness in anergic cells. In a preliminary experiment, knockdown of Egr-2 was significant but had no effect on the responsiveness to antigen rechallenge (rest day 12) of anergic cells when compared to cells transfected with a control duplex (Figure 20).

To determine the role of Egr-2 in anergy induction, A.E7 T cells were electroporated with Egr-2-specific siRNA, rested for 4-6 hours, and then anergized overnight. A population of cells was electroporated and left unstimulated to serve as a non-anergized control. Egr-2 protein levels were followed by flow cytometry (Figure 21a) and by western blot (Figure 21b). As described above, Egr-2 protein was decreased significantly on rest days 5 and 7 in anergic cells receiving Egr-2-specific siRNA compared to control. Cells were rechallenged on rest day 5, 6, or 7 and those with Egr-2 knockdown consistently demonstrated a significant increase in responsiveness to antigen rechallenge assessed by both tritiated thymidine incorporation (Figure 21c) and by analysis of CFSE, a fluorescent marker that binds to cellular proteins and is diluted in half with each cellular division (Figure 21d).
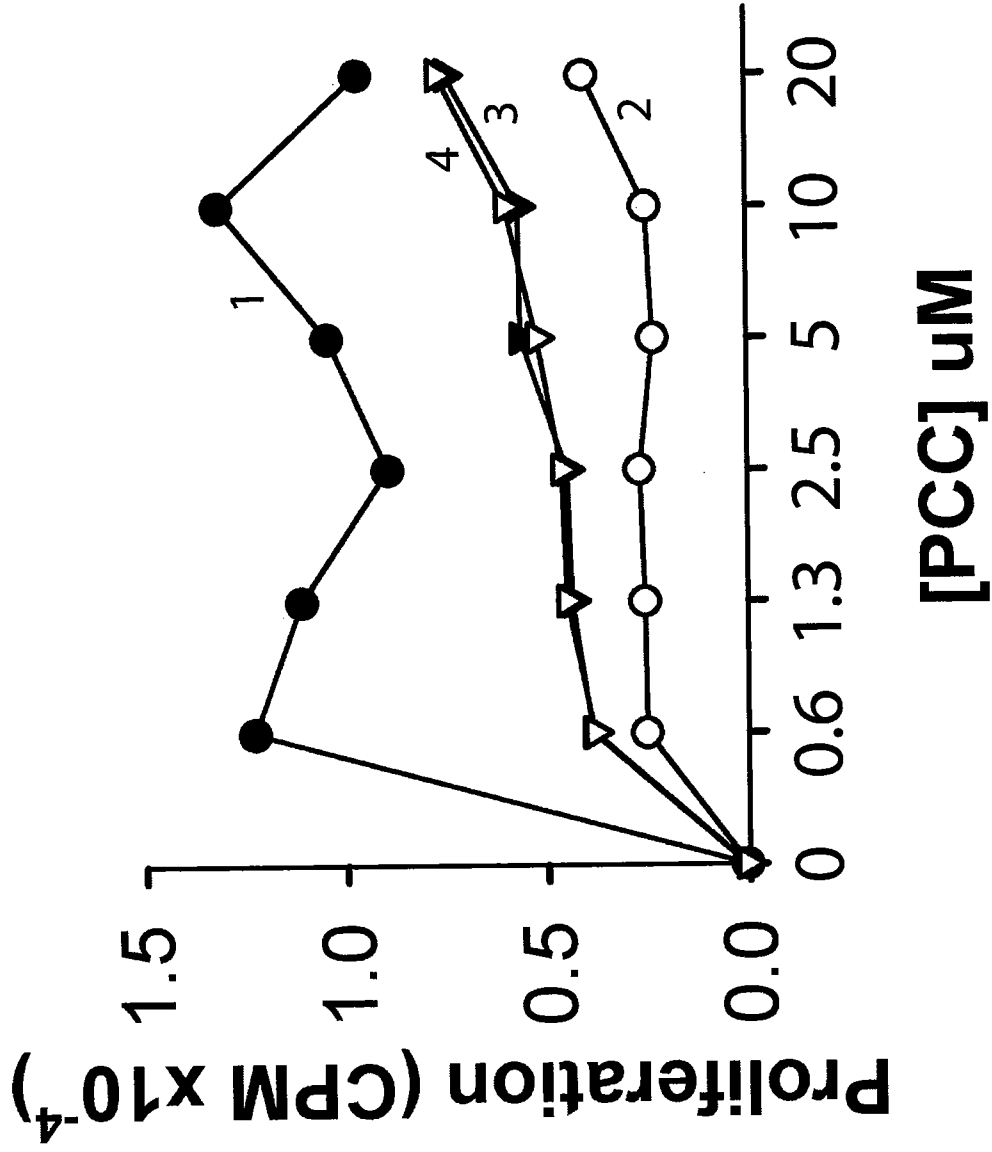
Figure 20

Legend to Figure 20: Egr-2 gene silencing after anergy induction has no effect on responsiveness to antigen stimulation. In a preliminary experiment, A.E7 T cells were anergized with platebound anti-CD3. Some cells were left unstimulated as a non-anergized control (1). Following 5 days of rest, anergic cells were electroporated with media alone (2) or 20 nanomoles of control siRNA (3) or Egr-2-specific siRNA (4). Knockdown of Egr-2 protein was confirmed 2 days later (7 days of rest—data not shown) and the cells were then submitted to antigen rechallenge (12 days of rest). There was no effect on the ability to proliferate in response to antigen rechallenge in anergic cells with Egr-2 knockdown compared to those electroporated with control siRNA. In this experiment there does appear to be a nonspecific effect of siRNA on anergic unresponsiveness.

A

Egr2-7 | Egr2-8

not anergic
con siRNA
Egr-2 siRNA

Egr-2

B

Resting | Anergized

1 | 2 3 4 5

Egr-2 →

actin →

siRNA none none con Egr2-7 Egr2-8

C

Proliferation (CPMx10⁻³)

1
5
4
2 3

[PCC] uM

D

Resting | Anergized

siRNA: none | none | control | Egr2-7

1
97%

2
52%

3
49%

4
73%

CFSE
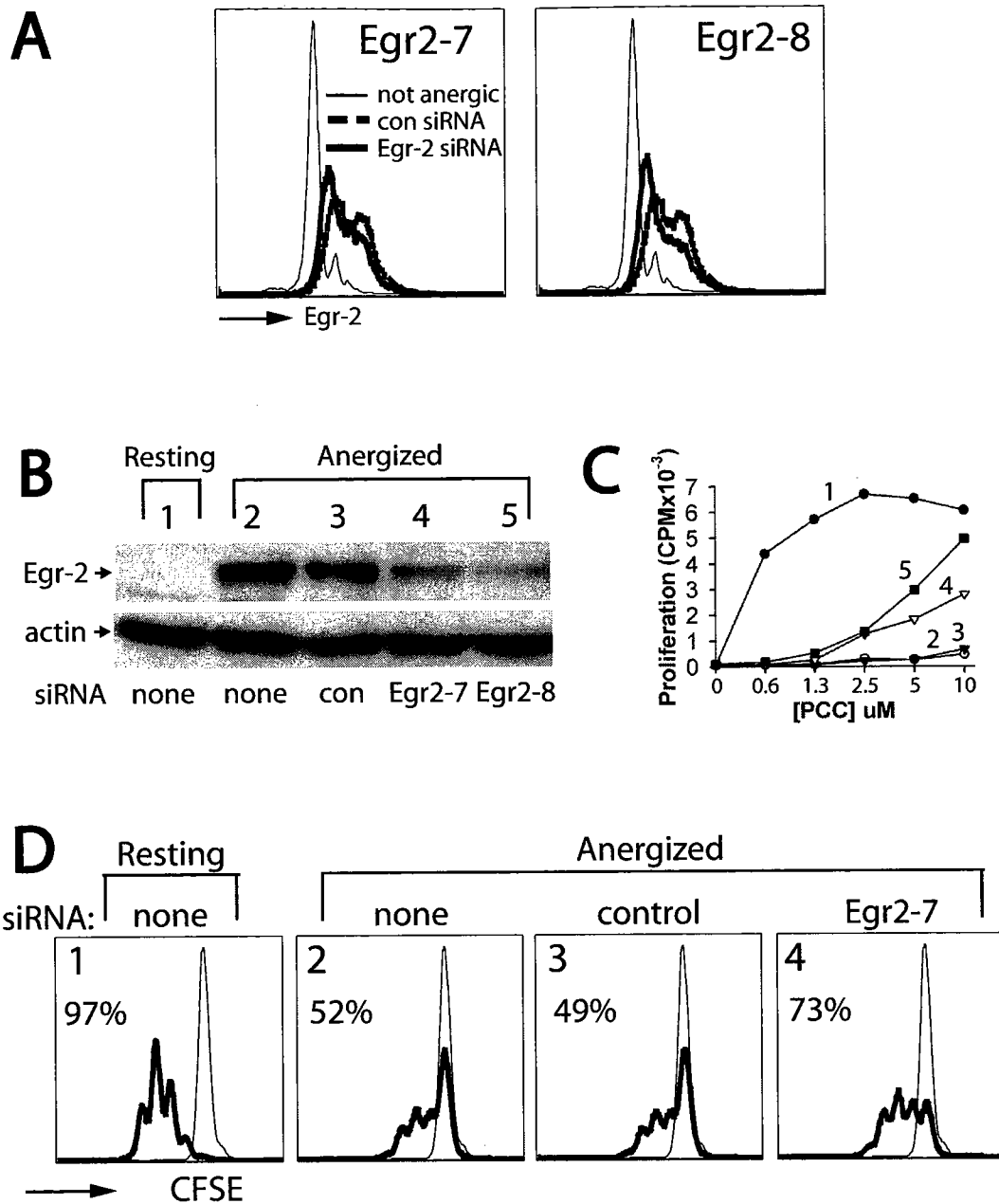
Figure 21

Legend to Figure 21: Treatment with two independent Egr-2-specific siRNAs prior to

anergy induction results in effective knockdown of Egr-2 and rescues anergic cell

unresponsiveness to antigen rechallenge. A.E7 T cells were electroporated with the

following: media alone (2-none), control irrelevant siRNA (3-con), or one of two distinct,

nonoverlapping siRNAs specific for Egr-2 (4-Egr2-7 or 5-Egr2-8). Electroporated cells

were rested for 4-6 hours before being anergized. A population of cells was

electroporated with media alone and left unstimulated (resting) as a responsive control (1-

none). **(A)** Cells were analyzed for Egr-2 gene silencing by flow cytometry 5 days after

removal from the anergizing stimulus. **(B)** Also at day 5, protein was extracted from the

cells for analysis by western blot. The membrane was then stripped and reprobed with

antibody against β-actin to verify equal loading. **(C)** Proliferative responsiveness to

antigen rechallenge was assessed by [^{3}H]-thymidine incorporation into DNA and by **(D)**

CFSE cell division history with flow cytometry. Cells were labeled with CFSE and then

stimulated with syngeneic splenocytes plus or minus PCC. The light histogram in each

graph represents CFSE fluorescence in cells not exposed to antigen (0 divisions) while

the heavy histogram represents fluorescence in cells stimulated with 10μM PCC. CFSE

fluorescence decreases two-fold with each division. While 97% of resting cells have

proliferated at least once when rechallenged with antigen, only 52% and 49% have

proliferated in anergic cells with no siRNA or control siRNA, respectively. Cells treated

with Egr-2-specific RNA have 73% proliferating cells.

Egr-2 is required to establish the block in ERK phosphorylation

In collaboration with Ken Bishop, ERK phosphorylation was analyzed in A.E7 T cells in response to platebound anti-CD3 mAb plus soluble anti-CD28 mAb. Ken optimized the time required for stimulation of resting A.E7s to observe ERK phosphorylation by western blot with an antibody against phospho-ERK and determined that 45-90 minutes of stimulation resulted in peak phosphorylation of ERK-1 and ERK-2 while levels of total ERK-1 and ERK-2 protein were unchanged.

We then used these conditions to analyze ERK phosphorylation in anergic cells that had been treated with media alone, control siRNA, or Egr-2-specific siRNA. A population of cells was electroporated with media alone and left unstimulated as a non-anergic control. Cells were treated as indicated, rested for 4-6 hours, and anergized for 12 hours. The cells were then removed from the stimulus and after 5 days Egr-2 protein levels were analyzed by flow cytometry and western blot. On rest day 6, the cells were stimulated with platebound anti-CD3 mAb plus soluble anti-CD28 mAb for 60 minutes, removed from the stimulus and processed for protein isolation. Thirty micrograms of protein was run on an SDS protein gel, transferred to PVDF membrane, and probed with anti-phospho-ERK antibody. The blot was then stripped and reprobed with anti-ERK antibody (Figure 22). It is apparent that, while anergic cells are defective in their ability to phosphorylate ERK-1 and ERK-2 in response to stimulation compared to resting control cells, Egr-2 gene silencing in anergic T cells restores this ability.

No Stimulation   anti-CD3+anti-CD28

1   2   3   4   5   1   2   3   4   5

ERK-p

ERK
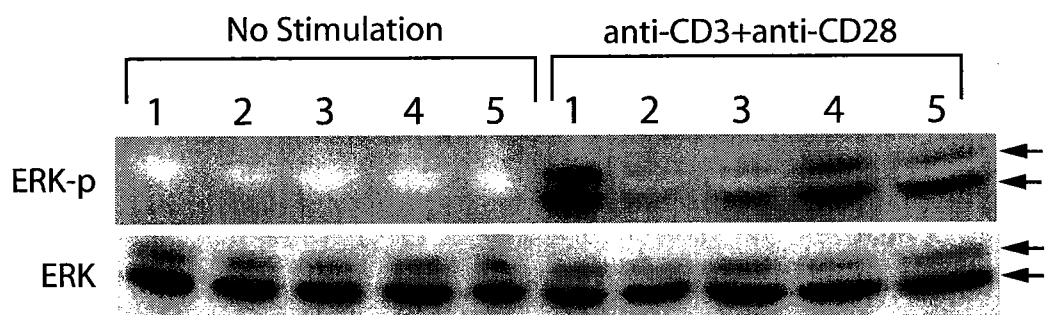
Figure 22

Legend to Figure 22: siRNA-mediated gene silencing of Egr-2 in anergic cells results in increased ERK phosphorylation in response to stimulation. A.E7 T cells were electroporated with the following: media alone (2), control irrelevant siRNA (3), or one of two distinct, nonoverlapping siRNAs specific for Egr-2 (4-Egr2-7 or 5-Egr2-8). Electroporated cells were rested for 4-6 hours before being anergized. A population of cells was electroporated with media alone and left unstimulated as a responsive control (1). Following 6 days of rest, cells were stimulated with immobilized anti-CD3 mAb and soluble anti-CD28 for 60 minutes, removed from the stimulus, and protein was isolated for western blotting with anti-phospho-ERK. The blot was then stripped and reprobed with anti-ERK antibody to verify equal protein loading. Arrowheads mark ERK-1 (top) and ERK-2 (bottom).

Discussion

It has been well documented that stimulation of T cells through the TCR in the absence of costimulation can result in long-term hyporesponsiveness to rechallenge, termed anergy. Although the anergic phenotype has been well studied in vitro and in vivo, the molecular events responsible for the induction and maintenance of anergy are still largely a mystery. Anergic cells appear to have at least two defects when stimulated; 1) a block in T cell signaling, evidenced by ERK hypophosphorylation, Ras hypoactivation and Rap1 hyperactivation and 2) repression of the IL-2 promoter and/or activators of IL-2 transcription. Some candidates have been published that may play a peripheral role in the anergic phenotype, but none has been demonstrated to be required for anergy. Anergy is most likely a complex process involving a number of genes that serve to mediate the blocks to rechallenge mentioned above.

I have demonstrated that the A.E7 T cell line can be anergized in vitro by stimulating the cells with platebound anti-CD3 monoclonal antibody (mAb) in the absence of costimulation, an observation that confirms the findings of other groups (17;23;33-35;39). The induction of the anergic phenotype can be avoided by costimulating the cells with soluble anti-CD28 mAb which results in much greater IL-2 production, proliferation and, after a sufficient rest period, a greater responsiveness to rechallenge with syngeneic APCs plus antigen. It is evident that the minimum period of rest required after an anergizing stimulus before rechallenge is 5 days. At a shorter rest period, although the anergized cells are indeed unresponsive to rechallenge, the activated control cells are equally unresponsive, an observation consistent with published reports

that activated T cells must have a period of rest before regaining responsiveness to stimulation. The activated control is important in the study of anergy because one must be careful identifying T cell unresponsiveness as anergy, when in fact the observed hyporesponsiveness could be simply a result of rechallenging the cells too soon after even productive stimulation. Whether this early unresponsiveness to rechallenge in productively stimulated cells is due to the same factors as long-term anergy has not been resolved. It will be interesting to answer this question when molecular mediators of the anergic phenotype have been identified and tested for association with early post-stimulation unresponsiveness. In the following discussion concerning expression levels of potential anergy mediators at various time points following the initial anergizing stimulus, it should be noted that until activated control cells are responsive to rechallenge (identified in this study to be 5 days), hyporesponsive cells cannot be truly considered anergic. When "anergic cell population" and "activated control population" are used throughout this discussion, these descriptions will refer to the phenotype observed in the populations after at least 5 days of rest, regardless of the time being examined.

In terms of the activated cell population, used as a control for anergy, it is important to note that while these cells are responsive to antigen at high doses and appear to respond to the same extent as mock-stimulated control cells, at low antigen doses they appear to be anergic. Although these cells will be referred to as "activated" and "responsive" controls throughout this discussion, we could more accurately call these cells "partially anergic". This may be useful in the future when correlating molecular events associated with anergy—one would expect that the activated population may

manifest an anergic phenotype at a low antigen dose while overcoming these limitations at higher doses.

I chose the earliest timepoint of 12 hours to examine anergic expression profiles because it allowed time for the upregulation of genes that have a slow induction rate in response to stimulation. However, this approach will not identify genes that are differentially expressed transiently, disappearing before 12 hours. Indeed, one such gene , named GRAIL, has been published that the authors have suggested plays a role in anergy induction, antagonizing IL-2 transcription in cells that do not receive costimulation (42). The authors report that GRAIL is upregulated transiently (during the first few hours of stimulation) only in cells stimulated through the TCR in the absence of costimulation, and that overexpression of GRAIL leads to IL-2 suppression. However, it can be argued that such a gene only plays a peripheral role in anergy induction—that it obligates a T cell to receive costimulation in order to prevent its upregulation and allow IL-2 production; which, incidentally, results in anergy avoidance. Put another way, since anergy can be induced even in fully stimulated cells (such as in the presence of rapamycin), anergy can be induced in the absence of GRAIL. Genes like GRAIL, which will not be identified in this screen, hold a limited interest since they are not present long-term (beyond a few hours) and don't appear to have a direct effect on the upregulation of anergy factors.

The hypothesis offered by Powell et al. (25), that anergy genes will be expressed early in both anergized and activated T cells and then downregulated in the proliferating activated cells, is convincing but incomplete. Indeed, I will refer to factors that follow

this pattern as anergy *induction* genes, in contrast to anergy *maintenance* genes. I make

the distinction because early genes necessary for the anergic phenotype may serve simply

to induce other factors that actually mediate the molecular events responsible for anergy.

If this is true, genes following the hypothesized pattern are important in that if they are

prevented from being expressed long-term, anergy will not follow. However, they may

play little or no role in directly mediating the hyporesponsiveness of a T cell to

rechallenge. If an identified anergy *induction* gene were eliminated after it has

upregulated an effector or *maintenance* gene, there would be no effect on the

rechallenged phenotype. However, if the gene were prevented from being upregulated (as

is thought to be the case with CSA treatment of anergized cells), it would have a

profound effect on the rechallenged phenotype, resulting in a cell that is responsive to

antigen rechallenge. If this assumption concerning both *induction* and *maintenance* genes

is correct, then one might expect to see maintenance genes not being expressed until later

times, after the induction genes have initiated their transcription. These maintenance

genes would be differentially expressed from the start. Such genes need not follow the

pattern in order to be essential for anergy maintenance, because if the induction gene is

not activated, then the maintenance genes would not be upregulated and anergy would

not follow. Again, these genes I would call *maintenance* genes and would expect these

proteins to have direct effector functions at key points in signaling and transcription in

stimulated anergic cells. Of course, it is possible that a single gene could have both

induction and *maintenance* effects, and this scenario will be considered in the discussion

of the candidates themselves.

The analysis of Affymetrix GeneChip data from anergic and control cells at three times following the primary stimulus was revealing about the events occurring in anergy induction when compared to full activation. If the model of anergic factor upregulation described by Powell et al. (25) is accurate, it would follow that the earliest timepoint analyzed in this study, after 12hr of stimulation, would demonstrate no difference in expression of "anergy factors" between the anergized population and the activated control. In fact, that appears to be the case—although many genes are differentially expressed in anergic cells compared to mock-stimulated control cells at this early time, I identified only two that are significantly differentially expressed between anergic and activated control cells in all three replicate experiments. The first is IL-2, which is slightly upregulated in anergic cells over mock-stimulated controls (more than 1.7 fold) but is expressed to a much greater extent in the activated control. This was an expected result as anergized T cells fail to produce IL-2 at the same level as productively stimulated cells (17) but still produce some IL-2 in response to stimulation. It is precisely this difference that appears to mediate the induction of anergy in partially stimulated cells. Therefore, IL-2 served as an excellent internal control to determine the success of the microarray hybridization and analysis. The slight upregulation of IL-2 in anergized T cells at this time point does not appear to be sufficient to induce proliferation and, ultimately, anergy avoidance.

The second gene identified at this time was Granzyme B, a secreted perforin typically produced by cytotoxic T cells and natural killer cells; it has the ability to induce apoptosis in the target cell (61;62). Implications of anergic cells making cytotoxic

substances include a role in T cell suppression—it is possible that an anergic cell could use cytotoxic substances to kill an APC presenting antigen to it, resulting in a decrease in antigen presentation to other potentially reactive T cells. Anergic cells have been reported to suppress T cell activation (57) and others have reported the ability of anergic T cells to kill antigen presenting cells (63), but the A.E7 cell line does not suppress activation in vitro (R. Schwartz, personal communication). The differential expression of Granzyme B narrowly fulfills the requirements set forth for anergy candidates, that the gene be up- or downregulated at least 1.7 fold in anergic cells as compared to both mock-stimulated and activated controls. The question remains whether this is a true anergic factor differentially expressed prior to proliferation in the activated control (negatively regulated by CD28 stimulation) or if it is simply an artifact narrowly passing the criteria out of over 36,000 expressed sequences. The paucity of differentially expressed factors at this early time prior to proliferation supports the model offered by Powell et al. (25).

Notable genes differentially expressed in anergic cells at day 5 of rest include Bace-2, Osteopontin, Neuritin, Pac-1, Egr-2, and two ESTs—AK011178 and AI152781. Beta-APP cleaving enzyme-2 (Bace-2) is a transmembrane protein located in the cell membrane, golgi apparatus, and endosomes (64). Bace-2 message is expressed in a large number of tissues including brain, heart and pancreas. There is moderate expression of Bace-2 in lymph nodes, low expression in spleen, and no detectable expression in thymus or blood leukocytes (65). Bace-2 is homologous to another protein, Bace-1, and both enzymes possess secretase activity, meaning that they can cleave other membrane-bound proteins, releasing them (secreted) from the cell. The most studied target of the Bace

enzymes is β-APP, a protein that, when cleaved, accumulates in senile plaques in Alzheimer's disease (66). While Bace-1 has significant β-secretase activity of β-APP, Bace-2 has much less. Bace-2 also acts as an α-secretase (64) and it is currently unclear what physiological role Bace-2 has in the brain. Bace-2 expression has not yet been reported in T cells. A recent report has shown that, in cultured cells, its homolog, Bace-1, localizes to the lipid raft (67), a very important structure for T cell activation. I have had two different antibodies made to this protein, targeting the extracellular domain. Ken Bishop has made a Bace-2 expression vector and shown that both antibodies recognize protein from Cos cells transfected with this vector. It will be interesting to see if the antibodies confirm differential protein expression of Bace-2 in anergic cells and whether the antibodies will be useful for flow cytometry. Because Bace-2 is a cell surface protein, flow cytometry and cell sorting could be performed to isolate live, Bace-2-expressing cells. In addition, experiments could be performed using these antibodies as blocking agents to assess the role of extracellular Bace-2 enzyme activity in T cell anergy.

Osteopontin is a secreted cytokine well-studied in bone dynamics as well as the immune system. It has been reported to have alternate splice forms and multiple post-translational modifications, resulting in numerous different proteins, each with unique activities. Osteopontin is essential in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), as the osteopontin knockout mouse is more resistant to induction of EAE as well as its progression (68). One of the primary functions of osteopontin is as a chemoattractant for T cells and macrophages; the knockout mouse has limited macrophage accumulation in damaged tissues (69). Other groups have identified a

role for osteopontin in rheumatoid arthritis (70). While all of these reports suggest a proinflammatory role for osteopontin rather than one consistent with decreased T cell responsiveness, there are other possibilities. First, the many different isoforms and modifications of osteopontin could result in one that has a blocking effect, preventing macrophage accumulation. Another possibility is that osteopontin has an as yet unidentified, direct role in preventing T cell activation. Third, and possibly most interesting, stems from the discovery that two independent companies have observed upregulation of osteopontin in CD25+ suppressor cells following activation (conversations with representatives at Keystone conference). It is possible that osteopontin has direct suppressive effects on T cells or macrophages, or that its role is to cause migration of macrophages to areas of tolerance induction. One of the companies that identified osteopontin secretion in CD25+ suppressor T cells displayed data using an ELISA kit. I have obtained this kit and we are prepared to test anergic cells for the secretion of this protein.

Neuritin has recently been discovered, and is upregulated in neurons that were stimulated to promote neurite outgrowth. It is a GPI-anchored protein that localizes to the cell surface. Addition of soluble recombinant neuritin to cultured neurons mimicks stimulation, suggesting that neuritin binds a receptor on the cell surface to induce an activation signal (71). Neuritin's GeneChip expression pattern is strikingly similar to that of Bace-2, and could be examined as a potential target of Bace-2 secretase activity, resulting in a secreted product—possibly with immune-modulating activity.

Phosphatase of Activated Cells-1 (Pac-1) is a well-studied protein that is quickly upregulated in stimulated T cells, migrates to the nucleus, and has MAP kinase phosphatase activity (72). Constitutive expression of Pac-1 inhibits MAP kinase activity normally induced in response to T cell receptor crosslinking, resulting in decreased activation of ERK-1 and ERK-2 and decreased transcriptional activity of c-fos (73). The potential significance of this protein to anergy maintenance is particularly evident given the fact that there has been a well-demonstrated defect in ERK phosphorylation in stimulated anergic cells (25;35). In fact, Li et al. even suggested that this defect may be due to either Pac-1 or another protein phosphatase induced in anergic cells (34). At the same time, another group reported that Ras is defective in stimulated anergic cells, suggesting that this explained the defect in ERK activity (35). However, it is possible that anergic cells utilize two approaches to limit signaling through the MAP kinase cascade.

It is worth noting that upon close examination by Ken Bishop, one of the ESTs differentially expressed in anergic cells, AK011178, shares some identity with RhoGAP8, a GTPase that inactivates Rho by cleaving GTP within the active form. This method of inactivation is also one way Ras is inactivated, so AK011178 could be pursued as a potential RasGAP. Even if this EST does not have RasGAP activity, inhibition of Rho can have major consequences on changes in the cytoskeleton and therefore also on immune synapse formation and T cell activation. This EST is being investigated further to elucidate its potential role in anergic T cells.

Early Growth Response-2 (Egr-2), first identified in a fibroblast cell line to be induced within minutes after serum stimulation (74), is a member of the family of early

growth response zinc-finger transcription factors. This family is a particularly well-studied group, and consensus binding sites (75-79) and target genes (Table 1) (74;75;80-95) have been reported. All four members of the Egr family are expressed in T cells in response to mitogenic stimulation (79;96-102). Transcription of Egr-2 and 3 is blocked when T cells are pretreated with cyclosporine A (CSA) while Egr-1 transcription is reported to be superinduced at the mRNA level (96;103). This suggests that: 1) Egr2 and 3 transcription is NFAT-dependent, 2) Egr-1 transcription is independent of NFAT and 3) possibly Egr-1 is negatively regulated by a CSA-sensitive protein. These are important observations, since CSA has been reported to block the induction of anergy as well (17).

Factor	Target genes	
Egr-1	IL-2	ICAM-1
	TNF-α	VCAM-1
	TGF-β	CD44
	insulin-like growth factor	CD23
	PDGF-α, β chain	Fas
	Chromogranin A	Egr-1
	Luteinizing hormone β	flt-1
	nur77	BTEB2
Egr-2,3	FasL	hox1.4
Egr-4	Egr-4	

Table 1

Legend to Table 1: Genes reported to be targets of Egr transcription factors.

The functional regions of Egr-2 DNA-binding and transcriptional activities have been mapped. It appears that only the zinc fingers (C-terminal) are required for DNA binding while two domains located N-terminal to the zinc fingers are responsible for transactivation. Deletion of these regions completely abolished the activating ability of Egr-2 without affecting DNA binding or nuclear localization (104).

Egr-2 and Egr-3 have so far been implicated only in activating transcription at the Fas ligand promoter. In fact, either factor alone is able to induce transcription of Fas ligand (80;101). This was an obvious first choice to pursue for the function of Egr-2 in anergy, however I found no increase in Fas ligand transcript in anergic cells on the GeneChip or protein expression on anergic cells using flow cytometry (data not shown).

The *lpr* mouse is a natural Fas ligand knockout that has a severe systemic lupus erythematosus (SLE)-like syndrome (105). Since Egr-2 and 3 activate transcription of FasL, peripheral T cells from the *lpr* mouse were examined for Egr-2 and Egr-3 expression and it was discovered that they constitutively express Egr-2 protein at a very high level; comparable in fact, to wild-type T cells stimulated with PMA and Ionomycin. Egr-3 was not constitutively expressed (101). As a natural mouse model overexpressing Egr-2, it is interesting to note that *lpr* T cells are hyporesponsive to in vitro stimulation with concanavalin A (Con A), failing to make IL-2 or proliferate; they also appear to have a decreased responsiveness to ConA stimulation plus exogenous IL-2 (106;107). These cells make normal amounts of IL-2 when PMA is added to the stimulation (108). Despite the defect in IL-2 production, *lpr* T cells produce normal amounts of IFN-γ (109). *lpr* T cells also have elevated Fyn kinase activity due to increased levels of Fyn

protein (110). Apart from the decreased responsiveness to IL-2, this description of *lpr* T cells correlates perfectly with T cell clonal anergy (32;111;112). While I cannot rule out the effects of a disrupted FasL pathway in these cells, it is possible that Egr-2 induces the hyporesponsive phenotype and increased Fyn levels in *lpr* mice. One unresolved issue is that if these cells are hyporesponsive in vivo as well, why do they exhibit severe autoimmune disease? It is possible that a small responsive subpopulation of T cells escapes programmed cell death in vivo, homes to the proper site of autoantigen, and proliferates, causing destruction of tissue. However, this population may not be large enough to proliferate to a large extent in vitro.

Another observation is that Egr-2 is induced in fibroblasts when they are treated with TGF-β (113). Since treatment with TGF-β has been demonstrated to induce anergy in stimulated T cells (114;115), it would be interesting to examine if TGF-β also induces Egr-2 in T cells.

GeneChip analysis revealed that Egr-2 expression followed the pattern of an "anergy factor" according to the model hypothesized by Powell et al. (25). This was confirmed by RT-PCR. While Egr-2 protein was equally upregulated in anergic and activated cells prior to proliferation, it was downregulated in activated controls at approximately rest day 3-5, which coincides with the time of greatest proliferation in this population. This suggests that, indeed, proliferation may be responsible for the downregulation of Egr-2. Protein levels remained detectable in anergic cells by western blot through rest day 9 in this experiment, demonstrating that the factor is present during rechallenge and the observed hyporesponsive phenotype. When cells were anergized and

then rested with or without exogenous IL-2 to break anergy, Egr-2 protein levels dropped quickly in the anergic cells exposed to IL-2, again coinciding with proliferation. This shows excellent correlation of Egr-2 expression with the anergic phenotype, since stimulation of anergic cells exposed to IL-2 for 10 days demonstrated dramatically increased responsiveness of these cells.

Interestingly, Egr-1 is an activator of IL-2 transcription, binding to specific Egr consensus sites in the IL-2 promoter (91;116;117). However, in one study, Egr-2, 3 and 4 did not bind the promoter as assessed by EMSA in Jurkat T cells (91), suggesting that Egr-2 does not directly affect IL-2 transcription. Others reported that Egr-1 acted on its own promoter to repress transcription, in a negative feedback loop (75). This opened the possibility that Egr-2 may repress IL-2 transcription indirectly by repressing Egr-1. For this reason, I decided to study the kinetics of Egr-1 and Egr-2 protein expression in previously mock-stimulated, anergized and activated cells to determine if Egr-1 is indeed repressed in anergic cells during rechallenge. After a sufficient rest period following mock-stimulation, anergy induction or activation, cells were stimulated with platebound anti-CD3 mAb plus soluble anti-CD28 for various times and protein expression was analyzed by western blot. As expected, Egr-2 protein level was high in anergic cells prior to stimulation and increased in all populations shortly after. Egr-1 was not expressed by any of the three populations prior to stimulation and was quickly upregulated in previously mock-stimulated cells. Interestingly, Egr-1 expression was very weak following stimulation in anergic cells.

This pattern was also observed when the same populations were rechallenged with APC's plus antigen (at a high or low dose) and protein levels of both Egr-1 and Egr-2 were analyzed by flow cytometry. Again, Egr-2 protein level was selectively higher in anergic cells prior to stimulation and quickly upregulated in all populations at both antigen doses. The fact that anergic cells are able to increase Egr-2 protein levels is interesting, considering the defect in signaling as well as the lack of AP-1-mediated transcription in these cells. Either the signaling block is incomplete, allowing only factors requiring a low threshold of stimulation to be upregulated, or the block is selective and pathways upstream of Egr-2 are unaffected—either is possible. First, Crespi and colleagues reported that bypass of the MAP kinase block with a transfected Ras mutant that is constitutively active cannot abrogate anergy induction (38), challenging the theory that the MAPK signaling block is necessary for anergy maintenance. Second, Egr-2 is NFAT-dependent (CSA blocks its expression) and anergic T cell clones do not have a calcium mobilization or NFAT transcription defect (31). It is possible that Egr-2 is not dependent on an intact MAPK cascade for upregulation in T cells. Supporting this latter hypothesis is the observation in B cells that a chemical inhibitor of the ERK branch does not affect Egr-2 induction (118).

In contrast to the responsiveness of anergic cells demonstrated by Egr-2 upregulation, anergic cells are severely defective in the ability to express Egr-1 following stimulation. This is evident in both the western blotting and flow cytometry. At both high and low antigen concentrations, Egr-1 expression is blunted in anergic cells at the peak time of expression and rapidly returns to prestimulation levels. This is also the case to a

milder extent in previously stimulated control cells. This observation has not been previously reported, and is a similar result to what has been observed with fos and jun proteins in stimulated anergic cells (31), also activators of IL-2.

Since Egr-1 expression was indeed inhibited in anergic cells, I chose to develop a transient transfection assay to assess if Egr-2 could repress Egr-1 promoter activity. I obtained a CMV-Egr2 expression plasmid (P. Mittelstadt, NIH, Bethesda, MD) and a chloramphenicol acetyltransferase (CAT) reporter vector driven by the Egr-1 promoter (X. Cao, National University of Singapore, Singapore). Jurkat T cells were transfected with both plasmids, stimulated with platebound anti-CD3 mAb, and processed for CAT activity. Although no strong inhibition of the Egr-1 promoter by Egr-2 was evident, the lack of a transfection control plasmid made it difficult to draw conclusions from the data (data not shown). Additionally, it has since come to my attention that the CMV promoter is not a particularly effective promoter in T cells (L. Berg, UMASS, Worcester, MA), and therefore it is possible that significant expression of Egr-2 was not obtained in these cells.

Because the IL-2 promoter contains putative Egr-2 binding sites, I utilized an IL-2 expression assay to assess if Egr-2 has an ability to repress the IL-2 promoter. Oddly, although EL-4 T cells make large amounts of IL-2 with anti-CD3 and anti-CD28 mAb stimulation, no IL-2 promoter-driven luciferase is detectable in these cells when stimulated under the same conditions. Others have also made this observation in other T cell lines (discussions at meetings). Because of this phenomenon, EL-4 cells transiently transfected with an IL-2 promoter-driven luciferase plasmid were stimulated with PMA and Ionomycin, which resulted in large amounts of luciferase being produced. Under

these conditions, CMV promoter-driven Egr-2 had no effect on IL-2 promoter activity.

This result could be explained by the fact that, while Egr-1 binds and activates the human

IL-2 promoter, Egr-2 does not bind (91). I felt that, despite this report, it was important to

test Egr-2 action on IL-2 transcription because it was possible that Egr-2 acted differently

in mouse T cells or that it repressed IL-2 transcription indirectly by either 1) repressing

an activator of IL-2 or 2) activating a repressor of IL-2. I was unsure if such an indirect

action would be detectable using a transient transfection assay, given that the IL-2-

luciferase reporter plasmid is present in many copies in transfected cells and might prove

overwhelming to endogenous transcription mechanisms. Another difficulty with this

approach is that, in order to elicit IL-2 promoter activity, it required stimulation of

transfected cells with PMA and Ionomycin—mitogens that result in maximal T cell

stimulation and bypass upstream signaling pathways. If Egr-2 upregulated a factor that

blocked signaling, PMA and Ionomycin stimulation would bypass the block in this assay.

It is also possible that PMA and Ionomycin stimulation is so powerful that it can

overcome a transcriptional block. In fact, PMA/Ionomycin stimulation abrogates the

anergic phenotype, resulting in IL-2 secretion and proliferation of anergized T cells

(34;35;112) (and unpublished results). In addition to the IL-2 promoter, I also tested the

fosB and junB promoters, which contain Egr-2 binding sites and were reported to be

altered in anergic cells (31). Again, using the same Egr-2 expression plasmid, I found no

effect of Egr-2 on the activity of either promoter in response to PMA and Ionomycin

stimulation.

Because of the limitations of the transient overexpression assays mentioned above, I chose to create stable EL-4 clones expressing Egr-2 and then analyze them for growth impairments and endogenous IL-2 production defects. The stable transfection resulted in two clones that made protein detectable by western blot. One clone, 5D3, expressed protein that was larger than the expected size and one, 5C1, expressed Egr-2 of the appropriate size. Interestingly, clone 5C1 grew much slower than the others. When 5C1 cells were stained with PI and analyzed by flow cytometry, a large percentage (11.5%) fell within the dead gate, while controls had only 2-2.5% fall within this gate. The 5C1 and control clones were subcloned by limiting dilution to obtain cell lines from single cells. These subclones were also analyzed by PI staining and four subclones from the 5C1 stable cell line all grew very slowly and 22-45% of the cells fell in the dead gate, while controls had only 2-5% dead cells (data not shown). The observation that Egr-2 overexpression may induce cell death is consistent with the fact that anergic cells undergo a wave of cell death after rest day 5 (17). Also consistent with these observations, Unoki and colleagues reported that Egr-2 expression is induced after overexpression of PTEN and that expression was decreased in a large number of ovarian tumors compared with corresponding normal tissues. When Egr-2 was overexpressed in tumor cells lines, it suppressed the growth of these cells significantly. Antisense oligonucleotides for Egr-2 effectively inhibited its expression and accelerated cell growth (119).

The stable clones were subjected to antibody stimulation and analyzed for IL-2 production by ELISA. There was variability among the clones in IL-2 production, even

between the two controls tested. The stable clone producing Egr-2 at the correct size,

5C1, produced normal amounts of IL-2 in response to stimulation in three separate

experiments. Interestingly, clone 5D3, which produced a slightly larger Egr-2 protein

product than expected, produced extremely low amounts of IL-2 in response to

stimulation in all three experiments. This clone did, however, produce large amounts of

IL-2 in response to PMA and Ionomycin stimulation. Since I have not pursued this effect

further, I am unable to make any conclusions about the state of IL-2 transcription in these

cells compared to control clones. Because only two stable clones tested positive for Egr-2

by western blot (one at the expected size, one larger than expected), it is difficult to base

any meaningful conclusions on data generated from the clones. Although one clone had a

severe growth defect with increased cell death and one had a defect in IL-2 production,

neither clone manifested both defects. The observed phenotypes of these clones could

simply result from differences in the original cell that established the clone, since

variability is expected within a transformed cell line carried in vitro.

I next chose to examine our in vivo model of tolerance induction, the mouse

tolerized to a skin allograft after treatment with DST and anti-CD40L. Because of the

association of suppressor T cells with tolerance induced by this method (120) as well as

the association of anergy with cells that are suppressed (56), I chose to examine the

lymph nodes draining the graft of long-term allotolerant mice for expression of Egr-2 by

flow cytometry, hypothesizing that suppression would occur in the lymph nodes and

would be present long-term. In a preliminary experiment, 2 of 3 tolerant mice had an

increased percentage of CD4+ cells from the draining nodes that fell within the Egr-2-

high gate compared to control mice and compared to tolerant mice in non-draining nodes. Analyzing mice with long-term grafts is difficult because, since "tolerant" mice eventually do reject their grafts, I can't know whether or not a long-term recipient is in the process of losing tolerance and in the early stages of rejecting the graft. In addition, although it is likely that T cell suppression occurs in the lymph nodes draining the graft, it is possible that suppression occurs in the graft itself, a location difficult to inspect for Egr-2-expressing cells. Further, tolerized cells may not remain in the draining lymph nodes but may leave them to circulate in the blood stream or migrate to extranodal sites, an observation made in the Jenkins soluble antigen model of tolerance (49). Others in the lab have explored the effect of depleting CD25+ cells in grafted mice on tolerance induction. If CD25+ cells are depleted immediately prior to and/or during tolerance induction with DST and anti-CD40L, grafts are rejected more rapidly, implicating T cell suppression in the induction phase of tolerance (S. Banuelos, unpublished results). These observations are important because it is possible that suppressed anergic cells would best be identified early in tolerance induction and may be best observed in the blood of these animals. I have not explored in vivo Egr-2 expression further, but this is an interesting venue to pursue.

With the demonstration of a correlation between Egr-2 protein expression and the anergic phenotype in vitro, I wanted to explore whether Egr-2 had a causative role in anergy. I chose siRNA-mediated gene silencing as the method to test this theory. As described above, if anergic factors that play a role in the *maintenance* of anergy are knocked down in anergic cells late in the process but before antigen rechallenge, I would

expect to observe increased responsiveness to rechallenge. Conversely, if the factor were only an *induction* factor, knockdown at some time after it has induced other effector molecules would have no effect on responsiveness to rechallenge. For this reason, two approaches were designed to assess the effect of siRNA-mediated Egr-2 knockdown. First in a preliminary experiment, A.E7 T cells were anergized, rested for 5 days, and then electroporated with Egr-2 siRNA or control siRNA. Cells were assessed two days later (rest day 7) for the effectiveness of knockdown by flow cytometry. After observing significant knockdown of Egr-2 levels in these anergic cells, I then proceeded to submit the cells to antigen rechallenge (rest day 12). The cells with greatly reduced levels of Egr-2 demonstrated no increased responsiveness to stimulation over controls. This suggested that either Egr-2 was not necessary for direct effector functions in anergic T cells, or that Egr-2 protein was not reduced sufficiently on rest day 12 to observe these effects.

Next, resting A.E7 T cells were electroporated with Egr-2 siRNA, allowed to recover for a few hours, ficolled to remove dead cells, and anergized. These cells were assessed two days after removal from the stimulus (rest day 2) for Egr-2 knockdown by flow cytometry. Analysis demonstrated that Egr-2 levels were marginally affected by siRNA treatment at this time. This was not surprising, considering the massive amounts of Egr-2 produced upon stimulation, as evidenced by mRNA levels on the GeneChip and by RT-PCR and protein levels by western blot and flow cytometry. When cells were analyzed three days later (rest day 5), an increased proportion of cells (76% vs. 55%) treated with Egr-2 siRNA expressed very low levels of Egr-2 compared to controls as assessed by flow cytometry. Knockdown was confirmed by western blot. Evidently Egr-2

siRNA needs time to "catch up" after the initial bolus of Egr-2 production following stimulation.

The mild effect of Egr-2 knockdown as assessed by flow cytometry is contrasted by a much larger apparent effect when analyzed by western blot. This discrepancy may be explained by the very different approach each method takes to quantify protein levels. Flow cytometry was initially my method of choice, because it provides information on protein level in each cell. Western blotting only indicates the total amount of protein expressed in an entire population of cells. For example, if 50% of a population of cells has 100% knockdown of Egr-2 protein, then flow cytometry will show two peaks, each with 50% of the cells in it. One will represent a low-staining population with no Egr-2 and the other a higher staining peak with normal levels of Egr-2. If 100% of the cells only reduce their protein levels 50%, then flow cytometry will show one peak that contains 100% of the cells that stain at 50% of the control anergic population. In both scenarios, analysis by western blot will report the same result—a 50% reduction in protein.

Because of this phenomenon, it appears that flow cytometry provides more information about the entire population of interest. However, it is likely that flow cytometry is limited by a threshold of detection—a lower limit of protein that must be present in a cell in order for staining to be bright enough to exceed background. In this case, cells that contain Egr-2 protein at levels just below this threshold (whatever it may be), will be in a low-staining peak at background level, representing an Egr-2-negative population. These "below threshold" cells will have the same fluorescence as cells that have 100% reduced Egr-2 levels. As observed in the experiments when cells are treated

with siRNA prior to anergy induction, even control anergic cells have a high and low

staining peak. The low peak in these cells may represent cells making Egr-2 below

threshold (which may be a high level) while this same peak in cells with Egr-2

knockdown may have no Egr-2. So, when cells with Egr-2 knockdown are analyzed by

flow cytometry and the result is an increase in the low-staining peak compared to control

anergic cells, there may actually be significantly less Egr-2 in the low staining peak of the

cells with knockdown compared to the same peak in controls. Because protein analysis

by western blot is additive over the entire population and therefore has no lower threshold

of detection on a per-cell basis, the same two populations compared with this method

may show a much more impressive difference in protein level. Adding another level of

complexity, western blotting is dependent on chemiluminescence for detection and

exposure to film. This can result in a narrow linear range that may limit quantification by

this method. In summary, while flow cytometry provides more qualitative information

about the population, it may lose quantitative ability if there is a moderately high

threshold of protein detection per cell. While western blotting provides more quantitative

information about the total population, it fails to give the qualitative information about

expression in the cells of that population.

Anergic cells pretreated with siRNA as indicated were stimulated on rest day 7

and pulsed with tritiated thymidine after 48 hours to assess their responsiveness to

antigen rechallenge. Cells that had been treated with Egr-2 siRNA showed increased

responsiveness to rechallenge compared to untransfected and control siRNA-transfected

cells. In another experiment, these cells were first labeled with CFSE, mixed with

stimulator APC's plus PCC antigen, and assessed for proliferation by flow cytometry 4 days later. As observed with tritiated thymidine incorporation, cells with reduced levels of Egr-2 were more responsive than either untransfected or contol-transfected cells to antigen rechallenge, resulting in increased proliferation assessed by CFSE dilution. This result implicates Egr-2 as an anergy induction gene, since in a preliminary experiment protein knockdown at day 5 with antigen rechallenge at day 12 appears to have no effect whereas early knockdown by transfecting siRNA prior to anergy induction results in rescue of antigen responsiveness in anergic cells. Because Egr-2 is a transcription factor, I am currently looking at the expression of late anergy candidates in siRNA-treated cells (potential maintenance effector genes) to assess if knocking down Egr-2 prior to anergy induction affects their expression.

Because Egr-2 knockdown allowed anergic cells to recover the ability to proliferate when rechallenged, I considered whether other aspects of the anergic phenotype were also restored by Egr-2 knockdown. One of the hallmarks of anergic cells, a defect in ERK-1 and ERK-2 activity in response to stimulation, has been documented (33;34). Defective phophorylation of ERK-1 and ERK-2 has also been reported in anergic cells, correlating with the loss of enzyme activity (25;35). In collaboration with Ken Bishop, I observed that the defect in ERK phosphorylation in stimulated anergic cells was corrected by Egr-2 knockdown.

Crespi et al. recently reported that constitutive Ras activation in primary T cells was insufficient to confer resistance to anergy induction via chronic TCR engagement (38). This data suggests that while the block in the ERK arm of the MAP kinase cascade

is a characteristic of anergy, it is not necessary for anergy maintenance; the

hyporesponsiveness of anergic cells may be maintained by redundant mechanisms. Other

groups have reported that active repression of the IL-2 promoter contributes to the

inability of anergic cells to transcribe IL-2 in response to stimulation (37;121). If, in fact,

there are multiple blocks utilized in anergic cells, it appears that Egr-2 is required for

inducing all of them, since its removal alone relieves anergic hyporesponsiveness. While

I have observed the rescue of ERK hypophosphorylation when Egr-2 is knocked down, it

is difficult to draw conclusions about direct Egr-2 involvement in other possible

pathways, since decreased ERK activity can contribute to downstream defective AP-1

activation and IL-2 production.

Interestingly, high expression of Egr-2 was found in other studies to be associated

with anergy, but the initial findings were not extended. Lechner et al. (60) screened for

genes expressed in primary T cells anergized in vivo, and reported the induction of Egr-2

expression in the anergized T cell population. However, in these studies comparison was

made with purified primary T cells stimulated with a mitogenic dose of anti-CD3 for 16

hours, a time point prior to the downregulation of Egr-2 and other putative anergy-

specific factors. Indeed these stimulated control cells also showed high Egr-2 expression

(60). In a second study of gene expression profiles, Macian et al. (15) also observed

upregulation of Egr-2 following an anergizing stimulus but evaluated only early times

after T cell anergy induction, prior to proliferation in the activated control population.

Another study using microarrays directed to tolerized B lymphocytes found high Egr-2

expression compared to unstimulated control cells (118), suggesting that Egr-2 may be important in B cell tolerance as well.

Because Egr-2 knockdown after anergy had been induced may have little or no effect on the responsiveness of anergic cells, and since Egr-2 is unlikely to directly inhibit signaling components, I believe that the role of Egr-2 in anergy is primarily to induce effector molecules that mediate the hyporesponsive phenotype. In collaboration with Ken, I am searching for Egr-2 targets of transcription that can regulate activation of the upstream components of the MAP kinase cascade. In fact, one of the anergy candidates upregulated in anergic cells at later timepoints is PAC-1—a protein with demonstrated MAP kinase phosphatase activity. Another, AK011178, is an EST that appears to have homology to RhoGAP8, a protein that inactivates Rho by converting its GTP to GDP. This is significant because Ras, the upstream activator of the MAP kinase cascade, is inactivated in a similar way, leaving the possibility that AK011178 could have Ras-GAP activity. As mentioned in the introduction, many believe that anergic cells upregulate a RasGAP, however, none has yet been identified. In addition to these two candidates, there are a number of others that have yet to be characterized for their function in anergy, including Bace-2, neuritin, osteopontin, and other EST's.

The future for the project described in this thesis is very exciting, as the data generated from the GeneChip screen has just begun to be tapped with the characterization of the role for Egr-2 in anergy. It is possible that any of the other genes identified in the screen could also play a significant part in the anergic phenotype. Approaches to further characterizing other anergy candidates may take many different forms. One is to identify

those with the greatest differential expression to pursue first or those with characteristics that make the most sense to anergy. I have begun this way, flagging the AK011178 EST because of its impressive differential expression, Bace-2 because of its expression and cell membrane localization, and Pac-1 because of its MAP kinase phosphatase activity.

A second approach would be to utilize other published microarray studies of anergy, including those by Ali et al., Lechner et al., and Macian et al. (15;60;122) to cross-reference the genes induced in this system, including the many genes at rest day 2, with the large number of genes reported in the other systems to identify those worth pursuing.

A third approach may be to induce anergy in other ways or in other cells, in vitro and in vivo, isolate RNA and perform qRT-PCR to analyze the expression of the candidates and discover which are universally associated with anergy. This "subscreen" would be limited to a small number of candidates (i.e. the day 5 candidates described above for which primers and conditions are already optimized) because qRT-PCR is not a high-throughput method of analysis. A fourth, higher throughput approach would be to design custom cDNA microarrays to screen all of the candidates identified from all of the timepoints (including the many on rest day 2).

Fifth, a functional approach to screening anergy candidates is to create expression vectors for a number of them, transfect them into A.E7s (difficult) or a transformed T cell line like the EL-4 or Jurkat (easier), and screen the cells for disrupted IL-2 production. Cells could be transfected with the candidate tagged with GFP, stimulated to produce IL-2, and analyzed by two-color flow cytometry with intracellular staining for IL-2.

Sixth, an interesting approach that I have instituted, is to take advantage of the

fact that Egr-2 is necessary for anergy induction and that it could be the sole factor to

fulfill the pattern described by Powell and colleagues (25). This implicates Egr-2 as a

possible "master switch" that sets in motion the induction of the anergy effector

molecules, which have yet to be identified. From this hypothesis, one could search for

candidates that are induced by Egr-2, involving the identification of candidates that are

not induced immediately with stimulation (not upregulated at the 12 hr timpoint) but are

induced and differentially expressed on day 2 or day 5. Genes following this pattern

include Bace-2, AK011178, and Pac-1. Then, one can utilize genome databases to pull

out ~2kb upstream of the transcription start site of these genes and scan them with a web-

based transcription factor binding site seach tool (such as TESS). Because Egr-2 binding

sequences have been well studied, it is relatively easy to determine if binding sites exist

in the promoter of a gene, the orientation in which it would bind, and how many sites

exist. After performing this search for Neuritin, Pac-1, and Bace-2, I have discovered an

Egr-2 binding site in each of the promoters, and two of the promoters have a single site

located on the negative strand and beginning between 36 and 47 base pairs upstream of

the transcription start site. In contrast, irrelevant promoters (2 separate 2kb promoters of

housekeeping genes) or random DNA sequence (up to 10kb) did not contain a single site.

This is exciting—not only does it lend additional credibility to these factors in anergy,

but they may be the important effectors directly downstream of Egr-2. In addition, if this

small sequence at this location in a promoter responds to anergy induction (even if Egr-2

alone doesn't induce it), then it could be linked to a reporter gene (GFP) and a transgenic

mouse generated that would, in theory, produce green T cells when anergic. This would allow visualization of the role of anergy in tolerance directly in vivo.

While there is much to be done concerning the other anergy candidates, exciting opportunities also exist for further study of Egr-2. First, because Egr-2 can be detected using flow cytometry, more inspection of cells for Egr-2 expression could be performed in mice tolerized to skin allografts using DST and anti-CD40L, the system instituted in our lab. I have already shown some data on Egr-2 expression in T cells from these mice but have only looked in mice with long-term grafts.

Second, one of the observations described above is that Egr-2 upregulation occurs in stimulated anergic cells despite the block in the MAP kinase cascade and despite the fact that junB (31) and Egr-1 are not induced. This suggests that Egr-2 upregulation in T cells in response to stimulation is independent of signaling through the MAP kinase cascade, since each branch of the cascade (ERK, JNK, p38) as well as Ras activity is defective in anergic cells (33-35). Interestingly, Schwartz reported that blockade of each branch of the MAP kinase cascade through inhibitors (ERK and p38) or knockout mice (JNK) does not inhibit anergy induction [L. Luu, J. Powell & R.H. Schwartz, unpublished data, described in (30)]. This lends additional support for the importance of Egr-2 in anergy induction. It also may explain why anergic cells require exposure to their antigen in order to remain anergic. Tanchot and colleagues describe a system of anergy induction to peptide in vivo in which the T cells survive in vivo and remain tolerant as long as antigen is present. If the anergic cells are transferred to a new host not expressing the peptide, the cells regain responsiveness without proliferation. If the cells are transferred

to a new host expressing the antigen, they are induced into an even deeper level of anergy (51). In the system described by Pape, et al., in which anergy is induced in TCR transgenic cells in a normal host with peptide injection in the absence of adjuvant, anergic cells lose unresponsiveness after a period of time. This loss of responsiveness is preceeded by a clearing of antigen in the host; if the tolerizing antigen is repeatedly introduced into the host the anergic cells remain hyporesponsive longer than with a single injection (49). I have also observed this loss of anergy in the A.E7 cell line over time and it correlates with a loss of Egr-2 expression (data not shown). It is possible that Egr-2 maintains the transcription of anergy effector molecules long-term, and that the loss of Egr-2 expression explains the impermanence of anergy. Since even full stimulation of an anergic cell is able to induce Egr-2 to maximal levels without inducing IL-2 transcription or proliferation, maybe the presence of antigen in the in vivo models discussed above maintains hyporesponsiveness long-term by repeatedly inducing Egr-2. Further work should be done, including directly showing that inhibitors of the MAP kinase cascade do not affect Egr-2 induction. I would be interested to know if Egr-2 expression correlates with anergy and its decline in the in vivo systems. It would also be interesting to know, in the injected peptide system of Tanchot et al., whether periodic exposure of the in vivo anergic cells to peptide in a context of productive activation would also maintain anergy over prolonged periods. I would hypothesize yes, if the rechallenge effect is due to Egr-2 induction, since it is induced without proliferation even with full stimulation.

Third, concerning the role of Egr-2 in anergy in vitro, one could make a stable transfectant of A.E7 T cells with Egr-2 expressed under an inducible promoter. This

would allow observations to be made concerning direct and indirect roles of Egr-2 in IL-2 production, proliferation and the activation or repression of other genes.

Fourth is to stably transfect a vector containing hairpin siRNA under an inducible promoter into A.E7 T cells. This would allow inducible knockdown of Egr-2 without the confounding effects of electroporation and the transience of siRNA. Hairpin siRNA consists of a short transcript of antisense RNA similar to siRNA but also contains the sense sequence in the reverse order separated from the antisense by 5-9 nucleotides. This results in a transcript that acts similar to transiently transfected double stranded siRNA because, with the 5-9 nucleotides as a hairpin loop, the antisense and sense portions anneal together. This has been reported to work well as a stable system to silence genes (123).

Lastly, in order to study the effects of Egr-2 on anergy in vivo, an Egr-2 knockout mouse would be valuable. The knockout mouse has been produced and reported but is embryonic lethal due to defective hindbrain formation (124). The only way to study the effect of eliminating Egr-2 on anergy in vivo would be to create a conditional knockout mouse, lacking Egr-2 in only its T cells so that brain development and the development of all other tissues is normal. However, this is a lengthy enterprise—for a description of the method of making a conditional knockout using the Cre/lox system, see the publication by Brian Sauer (125). Fortunately, another group has already produced and published a mouse with floxed Egr-2 in order to study brain development (126). I have contacted P. Charnay, the corresponding author, and he has agreed to provide us with these mice in a collaboration effort. Mice expressing Cre under a T cell-specific promoter

can be purchased and crossed to these mice, producing the conditional T cell Egr-2

knockout. With this mouse, not only could anergy induced by various methods be studied

in vivo, but more complicated systems of tolerance could be examined for the role that

Egr-2 and anergy play.

Conclusions: Modified model of anergy induction

T cell stimulation resulting in IL-2 production and proliferation requires two

signals—a TCR signal and an additive CD28 signal. TCR crosslinking induces 3

downstream pathways: 1) Ras and Raf activation, activation of the MAP kinases ERK,

JNK, and p38, dimerization of fos and jun transcription factors (AP-1), production of

Egr-1, and their translocation into the nucleus; 2) generation of DAG, activation of

protein kinase C (PKC) leading to the degradation of IκB, and translocation of NFκB into

the nucleus; 3) generation of IP$_3$, release of calcium into the cell, activation of

calcineurin, and dephosphorylation and translocation of NFAT into the nucleus. AP-1,

NFκB, and NFAT coactivate IL-2 transcription while NFAT alone induces transcription

of Egr-2. CD28 signaling strengthens the TCR signal possibly increasing NFκB

activation, prolonging the Ca^{++} influx and, most importantly, stabilizing IL-2 mRNA. IL-

2 production and autocrine signaling induces G1$\rightarrow$S phase transition and proliferation

which diminishes Egr-2 production. When Egr-2 production is shut down between 2 and

5 days following stimulation, anergy effectors are not induced. These cells remain responsive to rechallenge with antigen (Figure 23a).

TCR stimulation without CD28 costimulation results in failure to produce IL-2, probably through suboptimal NFκB activation and IL-2 mRNA instability. Because IL-2 production is not sufficient, proliferation does not occur and the Egr-2 protein level remains elevated. As a result, Egr-2 activates other "anergy factors", effectors that directly mediate anergic hyporesponsiveness. Candidate anergy factors with late induction identified in the Affymetrix GeneChip screen include: 1) the EST AK011178, with potential Rho or Ras inactivating activity; 2) Pac-1, a known MAP kinase phosphatase; 3) Bace-2, a membrane-bound secretase with unknown function in T cells; and 4) Neuritin, a GPI-linked protein with unknown function in T cells (Figure 23b).

When anergic cells are rechallenged with both signals, NFAT is activated normally. However, Ras and the MAP kinase cascade are inhibited, fos, jun and Egr-1 are not induced and translocated into the nucleus, and IL-2 transcription may be actively repressed. These effects are probably due to as yet unidentified anergy effector proteins; any of the candidates described above may contribute to these functions. While activation of NFAT alone (and possibly NFκB) is insufficient for IL-2 production (failing to cause proliferation), it is able to re-induce Egr-2 to maximal levels. This strengthens the anergic state, which otherwise disappears coincident with the loss of Egr-2 (Figure 24).

While anergy is probably mediated by a number of factors that work in concert, Egr-2 may be a primary factor that activates a program of gene transcription inducing effector molecules that mediate the many different aspects of the anergic phenotype

observed, including hyporesponsiveness, differential homing, and cytokine production (41;127). The fact that other studies have observed Egr-2 induction in tolerized lymphocytes both in vitro and in vivo suggests that Egr-2 may function in anergy induced by a variety of mechanisms. Based on the data presented here, identifying the factors controlled by Egr-2 in these models should provide important insights into the mechanisms involved in the maintenance of immune tolerance.
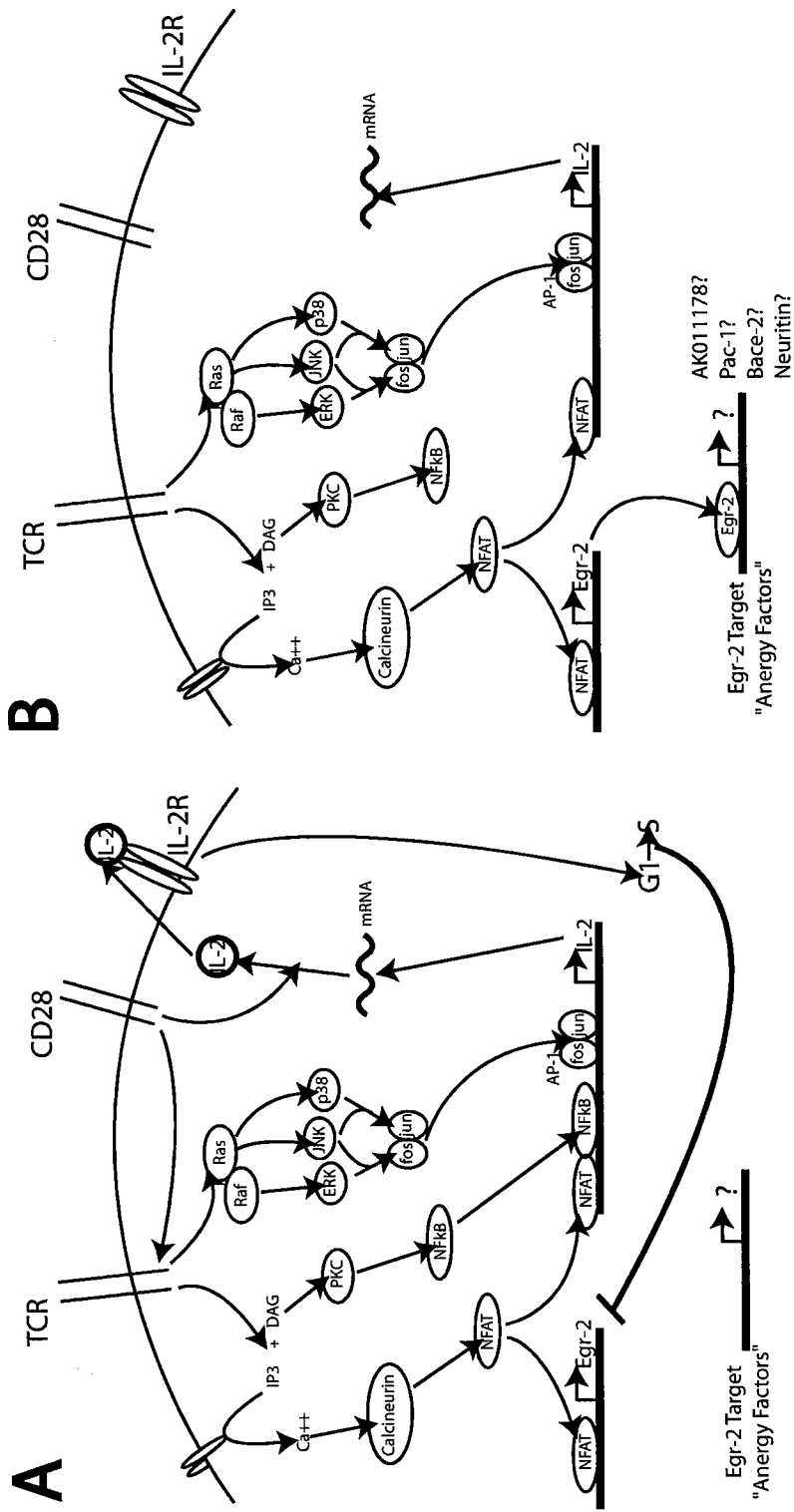
Figure 23

Legend to Figure 23: Model of T cell activation and anergy induction. **(A)** T cell stimulation through the TCR and CD28 results in IL-2 production, proliferation, and anergy avoidance. **(B)** T cell stimulation through the TCR alone does not induce sufficient IL-2 or proliferation and results in anergy induction.

Figure 24

Legend to Figure 24: Model for failed proliferation following antigen rechallenge of anergic cells. Even full stimulation of anergic cells does not induce IL-2 production or proliferation. This is probably due to anergy effector proteins induced by sustained Egr-2 expression. Egr-2 is re-induced in these cells, prolonging anergic hyporesponsiveness.

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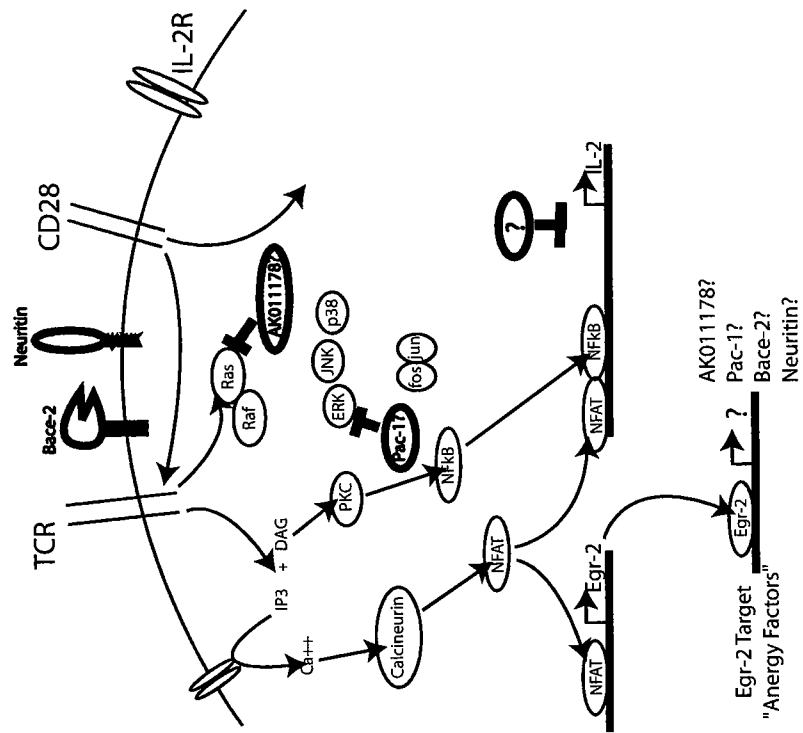
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