Immunity, Vol. 6, 419–428, April, 1997, Copyright ©1997 by Cell Press

Different Nuclear Signals Are Activated by the B Cell Receptor during Positive Versus Negative Signaling

James I. Healy,*†§ Ricardo E. Dolmetsch,‡ Luika A. Timmerman,† Jason G. Cyster,* Mathew L. Thomas,‖ Gerald R. Crabtree,†§ Richard S. Lewis,†‡ and Christopher C. Goodnow*†§
*Department of Microbiology and Immunology
†Program in Immunology
‡Department of Molecular and Cellular Physiology
§Howard Hughes Medical Institute
Stanford University School of Medicine
Stanford, California 94305
‖Howard Hughes Medical Institute
Department of Pathology
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

It is not known how immunogenic versus tolerogenic cellular responses are signaled by receptors such as the B cell antigen receptor (BCR). Here we compare BCR signaling in naive cells that respond positively to foreign antigen and self-tolerant cells that respond negatively to self-antigen. In naive cells, foreign antigen triggered a large biphasic calcium response and activated nuclear signals through NF-AT, NF-κB, JNK, and ERK/pp90rsk. In tolerant B cells, self-antigen stimulated low calcium oscillations and activated NF-AT and ERK/pp90rsk but not NF-κB or JNK. Self-reactive B cells lacking the phosphatase CD45 did not exhibit calcium oscillations or ERK/pp90rsk activation, nor did they repond negatively to self-antigen. These data reveal striking biochemical differences in BCR signaling to the nucleus during positive selection by foreign antigens and negative selection by self-antigens.

Introduction

Antigen receptors have the capacity to induce either positive or negative responses in lymphocytes. Following infection or immunization, the binding of foreign antigens to B cell antigen receptors (BCR) elicits positive signals that promote clonal proliferation and differentiation into antibody-secreting cells (Cambier et al., 1994; Cooke et al., 1994; Gold and DeFranco, 1994; Rothstein et al., 1995; Rathmell et al., 1996). The binding of self-antigens, by contrast, induces negative signals that actively enforce self-tolerance by inhibiting self-reactive B cell survival, maturation, proliferation, migration, and antibody secretion (reviewed by Nossal, 1983; Scott, 1993; Goodnow et al., 1995). While the importance of these opposite signaling phenomena has long been recognized (Nossal, 1983), the biochemical distinction between positive and negative signals induced by a single receptor is not known.

Analysis of immunogenic versus tolerogenic responses by mature splenic B cells from immunoglobulin (Ig) gene transgenic mice provides a well-controlled model to explore the basis for positive versus negative signaling. When B cells that bear a uniform BCR specific for the antigen hen egg lysozyme (HEL) have matured in a mouse without being exposed to HEL, these naive cells make a positive initial response upon acute exposure to the foreign HEL antigen (Cooke et al., 1994). BCR signaling in this case up-regulates costimulatory CD86 (B7.2) molecules and promotes mitogenesis and competence to collaborate with helper T cells. By contrast, when B cells with the same BCR specificity mature in mice where HEL is chronically encountered as a self-antigen, antigen binding no longer triggers CD86 expression, mitogenesis, or the capacity to resist Fas-mediated apoptosis (Cooke et al., 1994; Cyster and Goodnow, 1995a; Rathmell et al., 1996). While the loss of these positive responses is consistent with a marked decrease in BCR-induced protein tyrosine phosphorylation in tolerant B cells (Cooke et al., 1994), their BCRs nevertheless retain the full capacity to transmit negative signals that inhibit B cell migration and survival (Cyster et al., 1994; Fulcher and Basten, 1994; Cyster and Goodnow, 1995a; Fulcher et al., 1996) and that block plasma cell differentiation and antibody secretion (Goodnow et al., 1991; J. I. H. et al., unpublished data). Because naive and tolerant B cells are matched for their specificity and maturation stage, they provide a good model to examine the biochemical distinction between positive and negative signaling.

The BCR promotes mitogenesis and immune responses by initiating a branching biochemical cascade of tyrosine kinases and second messengers such as calcium (reviewed by Gold and DeFranco, 1994; Cambier et al., 1994). In turn, these induce gene expression by translocating cytoplasmic transcription factors such as NF-AT and NF-κB to the nucleus (Liou and Baltimore, 1993; Baeuerle and Henkel, 1994; Clipstone and Crabtree, 1994; Rao, 1994; Thanos and Maniatis, 1995; Baldwin, 1996) and by activating mitogen-activated protein kinases (MAPKs) such as extracellular signal–regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) that phosphorylate preexisting nuclear transcription factors (Davis, 1994; Karin and Hunter, 1995).

In principle, the reduced tyrosine phosphorylation elicited by the BCR in tolerant cells could cause a graded reduction in all signals to the nucleus. By differentially inducing target genes with distinct signal thresholds, a purely quantitative model could thus account for the negative rather than positive response. Here we describe the surprising finding that only a subset of BCR signaling pathways to the nucleus is depressed in tolerant B cells responding negatively to self-antigen, while others continue to be activated at levels comparable to that seen in positive mitogenic responses to foreign antigen. This capacity of the BCR to selectively activate distinct nuclear signaling pathways has important implications for the mechanism of self versus nonself recognition.

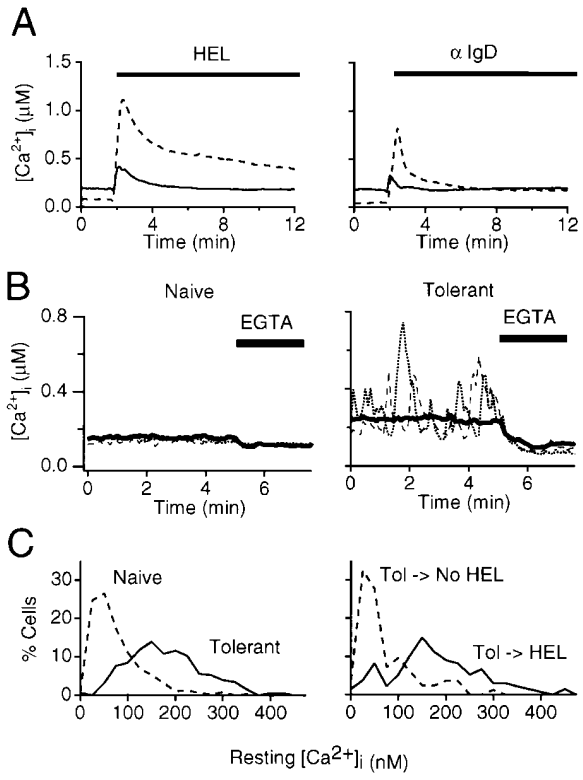

Figure 1. Self-Antigen Stimulates Repetitive Calcium Oscillations in Tolerant B Cells

(A) $[Ca^{2+}]_i$ responses of naive (dashed line) and tolerant (solid line) B lymphocytes stimulated with 500 ng/ml HEL (bar) or 20 μg/ml polyclonal goat anti-IgD (bar). Data are representative of six experiments.

(B) Resting $[Ca^{2+}]_i$ in naive and tolerant B cells before and after addition of 3 mM EGTA (bar). Each panel shows the mean of greater than 250 cells (bold solid line) and tracings from two single cells (dashed and dotted lines). Data are representative of three experiments.

(C) The distribution of mean basal $[Ca^{2+}]_i$ in single cells. (Left) The distribution of calcium means in freshly isolated naive (dashed line) and tolerant (solid line) B cells. (Right) Distribution of mean calcium among tolerant B cells that were injected intravenously into lymphocyte-deficient *rag2*$^{-/-}$ mice expressing HEL (solid line) or lacking HEL (dashed line) and purified from the spleen 50 hr later for single-cell measurements. Equivalent results were obtained in three separate experiments with transfers from 40–50 hr.

Results

Differential Calcium Signaling in Tolerant B Cells

In previous analyses, tolerant B cells that respond negatively to HEL antigen had a markedly diminished BCR-induced calcium response and diminished tyrosine phosphorylation on Igα, Lyn, and Syk, when compared to naive cells that respond positively to the same antigen (Cooke et al., 1994; M. P. Cooke and C. C. G., unpublished data). Because basal calcium levels appeared to be elevated in tolerant cells (M. P. Cooke and C. C. G., unpublished data), we analyzed the calcium response in more detail by single-cell calcium imaging, which is more sensitive than the flow cytometric analysis used previously.

Naive (nontolerant) B cells expressing BCRs specific for HEL were obtained from IgM/IgD trangenic mice in which the cells developed without HEL antigen exposure (Goodnow et al., 1988). Acute ligation of their BCRs with HEL or anti-IgD antibodies stimulated a biphasic calcium response: a large, transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$) followed by a smaller persistent calcium plateau (Figure 1A, dashed lines).

In tolerant HEL-specific B cells that developed in mice carrying a soluble HEL transgene, the BCRs were chronically engaged by circulating self-antigen (Goodnow, et al., 1988; Mason et al., 1992), and $[Ca^{2+}]_i$ regulation was altered in two respects: in tolerant cells, basal $[Ca^{2+}]_i$ was elevated by 120 nM, and in vitro ligation of their BCRs with HEL or with anti-IgD failed to evoke the large $[Ca^{2+}]_i$ transient (Figures 1A and 1C) even though IgD BCRs were expressed at normal levels. Single-cell analysis revealed that the mean elevation of basal calcium in tolerant B cells resulted from asynchronous calcium oscillations that were rapidly quenched by chelation of extracellular calcium (Figure 1B). To determine if the $[Ca^{2+}]_i$ oscillations were the result of repeated binding of self-antigens, tolerant B cells were transferred to mice that did not express HEL. Within 2 days after transfer, $[Ca^{2+}]_i$ oscillations in the tolerant cells disappeared and the mean basal $[Ca^{2+}]_i$ was reduced to the level of resting naive B cells. $[Ca^{2+}]_i$ oscillations and elevated mean $[Ca^{2+}]_i$ persisted in control transfers of tolerant B cells into HEL-expressing mice (Figure 1C), demonstrating that the oscillations arise through continued stimulation of the self-reactive BCR.

NF-ATp and NF-ATc Are Translocated to the Nuclei of Tolerant B Cells

To determine if the $[Ca^{2+}]_i$ oscillations had consequences for nuclear signaling in tolerant cells, we analyzed the transcription factors NF-ATp (Jain et al., 1993) and NF-ATc (Northrop et al., 1994), which translocate to the nucleus (Flanagan et al., 1991) following dephosphorylation by the calcium-dependent phosphatase calcineurin (reviewed by Clipstone and Crabtree, 1994; Rao, 1994). Western blot analysis revealed that dephosphorylated NF-ATp and NF-ATc were already located in the nuclei of freshly isolated tolerant B cells (Figures 2A–2C; Tolerant lane U), whereas they were dephosphorylated and present in the nuclei of naive B cells only after stimulation with HEL, anti-IgD, or calcium ionophore (Naive lanes H, D, and I). Continued calcium spiking and calcineurin activity were needed to maintain NF-ATp and NF-ATc in the nuclei of tolerant cells because chelation of extracellular calcium or exposure to cyclosporin A, which inhibits calcineurin (Liu et al., 1991; Clipstone and Crabtree, 1992), caused both NF-ATp and NF-ATc to be exported from the nuclei to the cytoplasm of tolerant B cells within 10 min (Figures 2A and 2B; compare lanes U, E, and C). These results show that circulating self-antigens induce low-level $[Ca^{2+}]_i$ oscillations that activate calcineurin and translocate NF-ATp and NF-ATc to the nuclei of tolerant B cells, even though that ligation of their receptors does not induce an initial calcium peak.

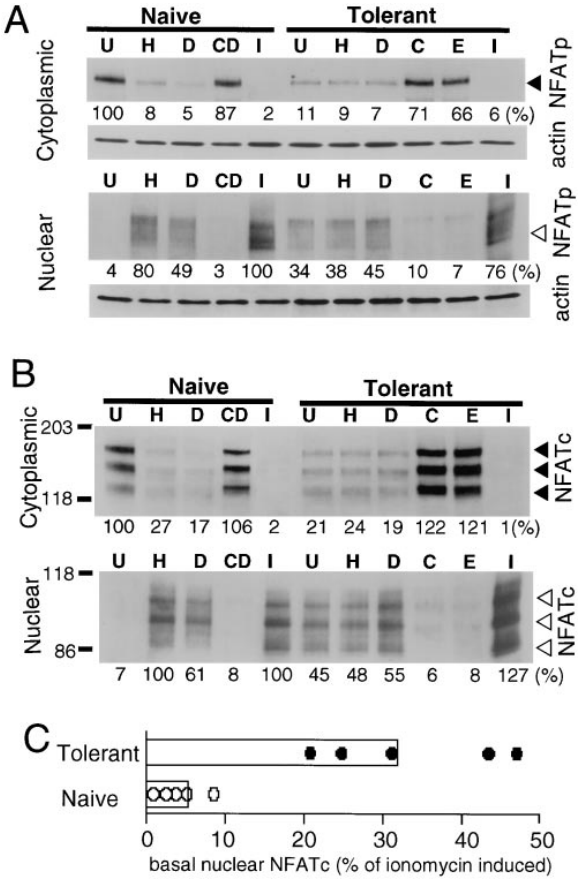

Figure 2. Elevated Nuclear NF-ATp and NF-ATc in Tolerant B Cells Depends on Continued Calcium Spiking and Calcineurin Activity

(A) and (B) Western blot analysis of NF-ATp and NF-ATc in cytoplasmic and nuclear lysates of naive and tolerant B cells. Cells were unstimulated (U) or were stimulated in vitro with 1 μg/ml ionomycin (I), HEL (H), or anti-IgD (D). Cyclosporin at 100 ng/ml (C) was added 10 min before stimulation with anti-IgD (CD). EGTA was added to the media at 3 mM (E). NF-AT cycling also occurs in HEL-stimulated naive B cells (data not shown). Phosphorylated and unphosphorylated NF-AT isoforms are indicated by closed and open arrowheads, respectively. Molecular size markers are given in kilodaltons. Numbers under each lane indicate the percentage of NF-AT immunoreactivity normalized for actin and relative to unstimulated naive cells (cytoplasm) or the ionomycin induced value (nuclear).

(C) Nuclear NF-ATc quantitation from five separate experiments (dots). Bars denote arithmetic means.

Active ERK and pp90rsk and Expression of the Early Response Gene *Egr-1* in Tolerant B Cells

To determine if negative signaling in response to self-antigens was also accompanied by activation of calcium-independent signals such as the MAPK ERK2 (Izquierdo et al., 1993), we examined ERK2 and its downstream targets and found that tonic signaling by self-antigen also activated this pathway. Fifteen percent of ERK2 was already phosphorylated in freshly isolated tolerant B cells (Figures 3A and 3B; Tolerant lane U), whereas in naive B cells ERK phosphorylation was found only after in vitro stimulation with HEL or anti-IgD (Naive lanes U, H, and D). In vitro stimulation of tolerant cells with anti-IgD induced a greater fraction of ERK to be phosphorylated (lane D), indicating that the pathway from the BCR to ERK was intact. Elevated ERK1 and ERK2 activity in tolerant B cells was confirmed by in-gel-kinase assay (Figure 3C). pp90rsk, a substrate for ERK (Sturgill et al., 1988; Hsiao et al., 1994), was examined to track the ERK pathway to the nucleus. Like ERK1 and ERK2, the phosphorylated active form of pp90rsk was already present in tolerant B cells and was further induced by anti-IgD. In naive B cells, the active form of pp90rsk was detected only after treatment with HEL or anti-IgD (Figure 3D).

Nuclear ERK activity in tolerant cells was further indicated by expression of the early response gene, *Egr-1*, which is induced following BCR engagement via ERK (McMahon and Monroe, 1995). Egr-1 protein was already present in the nuclei of tolerant B cells, and its expression was further induced by anti-IgD antibodies (Figure 3E; Tolerant lanes U and D). In contrast, in naive B cell nuclei, Egr-1 was detectable only after in vitro HEL or anti-IgD stimulation (Figure 3E; Naive lanes U, H, and D). Egr-1 protein disappeared when tolerant B cells were transferred into mice lacking HEL (Figure 3F; lane N) but persisted in control transfers into animals expressing HEL (lane H). This result demonstrates that activation of the ERK/Egr-1 pathway in tolerant B cells, like the calcium oscillations, requires continued exposure to self-antigen.

Self-Antigens Do Not Generate Calcium Oscillations or Activate ERK/pp90rsk Signaling in Self-Reactive B Lymphocytes Lacking CD45

To further substantiate that the continual $[Ca^{2+}]_i$ oscillations and pp90rsk/Egr-1 activation in tolerant B cells depends on BCR signaling, soluble HEL/anti-HEL mice were bred to mice lacking the BCR-associated tyrosine phosphatase CD45 (Cyster et al., 1996). $[Ca^{2+}]_i$ oscillations and elevation in mean basal $[Ca^{2+}]_i$ were not detected in HEL-specific, CD45-deficient B lymphocytes that were chronically exposed to HEL (Figure 4A). Likewise, CD45 was required for the activation of pp90rsk and induction of Egr-1 by self-antigens (Figures 4B and 4C). Since CD45 is required for inhibition of B cell survival by soluble self-antigens (Cyster et al., 1996), these data suggest that chronic signaling through calcium or the ERK pathway or both may be essential for this negative response to self-antigen.

The JNK Pathway Is Not Triggered by the BCR in Tolerant Cells

The finding that BCR activation of NF-AT and ERK was comparable in naive and tolerant lymphocytes despite the opposite functional responses of these cells prompted us to analyze other nuclear signals. The MAPK JNK is homologous to but regulated independently of the ERK pathway (Minden et al., 1994; Sanchez et al., 1994; Yan et al., 1994; Coso et al., 1995; Minden et al., 1995) and is activated by the T cell receptor in a calcium-dependent manner (Su et al., 1994). BCR stimulation of naive B cells induced 10% of JNK1 to migrate more slowly on Western blot analysis, consistent with phosphorylation and activation (Lin et al., 1995), and this effect was augmented by costimulation with phorbol ester (Figures

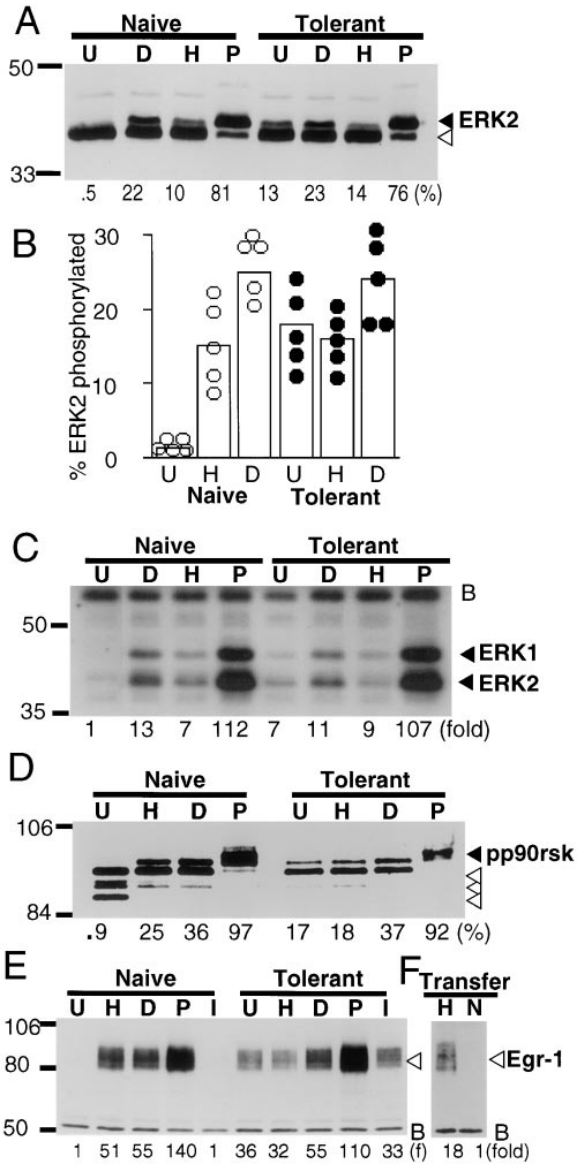

Figure 3. Self-Antigen Continues to Activate the ERK/pp90rsk/Egr-1 Pathway in Tolerant B Cells

(A) Anti-ERK2 Western blot of lysates from purified naive or tolerant B cells either unstimulated (U) or were stimulated in vitro for 5 min at 37°C with 20 μg/ml anti-IgD (D), 500 ng/ml HEL (H), or 50 ng/ml phorbol 12,13-dibutyrate (PdBu) (P). The active phosphorylated form (closed arrowhead) migrates more slowly in SDS-PAGE. The number under each lane indicates the fraction of immunoreactive ERK2 in active form.

(B) Quantitation of ERK2 phosphorylation (Payne et al., 1991; Posada and Cooper, 1992). In each experiment the basal ERK2 phosphorylation in tolerant cells was augmented by anti-IgD.

(C) In-gel-kinase assay for ERK activity in cells stimulated as in (A). ERK1 expression in B cells was confirmed by immunoblot (data not shown). Numbers under each lane indicate the cumulative fold increase in ERK1 and ERK2 activity over that found in unstimulated naive B cells.

(D) Anti-pp90rsk Western blot of cells stimulated for 10 min as in (A). Open arrowheads indicate unphosphorylated pp90rsk isoforms; closed arrowhead indicates phosphorylated active pp90rsk (Chen et al., 1991). Numbers under each lane indicate the fraction of immunoreactive pp90rsk in the active form.

(E) Anti-Egr-1 Western blot of nuclear lysates from cells stimulated for 60 min as above. Ionomycin (I) was used at 500 ng/ml. Numbers under each lane indicate the fold induction of Egr-1 expression over the background level found in unstimulated naive B cell nuclei. B indicates the nonspecific staining background band used to quantitate loading.

(F) Anti-Egr-1 Western blot of nuclear lysates from tolerant B cells transferred for 12 hr into recipient mice lacking (N) or expressing HEL (H) as in Figure 1C.

5A–5C; lanes U, H, D, P, and DP). As measured by mobility shift, JNK1 was not phosphorylated in freshly isolated tolerant B cells, and BCR-induced phosphorylation was markedly depressed relative to naive B cells (lanes U, H, D, and DP). Comparable results were obtained by assaying the in vivo phosphorylation state of activating transcription factor–2 (ATF2), a nuclear substrate for JNK (Gupta et al., 1995) (Figure 5D). ATF2 can also be phosphorylated by the related stress-activated protein kinase p38 (Raingeaud et al., 1995), so that we cannot exclude the possibility that p38 may account for ATF2 phosphorylation induced in naive cells. In tolerant cells, phosphorylation of JNK1 and ATF2 was normal when the BCR was bypassed by phorbol ester and ionomycin (Figures 5C and 5D; lane PI), indicating that signaling was disrupted between the BCR and JNK. Thus, the MAPKs ERK and JNK are both activated in naive B cells that mount a positive response, whereas they are activated differentially in tolerant B cells that make a negative response.

The NF-κB Pathway Is Not Induced by the BCR in Tolerant Cells

The NF-κB proteins c-Rel and Rel-A are important calcium-responsive transcriptional regulators in lymphocytes that are sequestered in the cytoplasm bound to the translocation inhibitor IκBα until immune signals trigger their release (Liou and Baltimore, 1993; Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995; Baldwin, 1996). In naive B cells, BCR stimulation induced IκBα degradation and nuclear translocation of c-Rel and RelA within 15 min (Figures 6A–6C and data not shown) by a mechanism that was blocked by calcium chelation or cyclosporin A (lanes E and C). In freshly isolated tolerant cells, IκBα degradation and c-Rel/RelA translocation were not apparent nor were they induced by in vitro stimulation with HEL (lane H), and they were only weakly stimulated by anti-IgD (lane D). Signaling appears to be blocked between the BCR and IκBα in tolerant cells because IκBα degradation and c-Rel/RelA translocation were induced normally by phorbol ester and ionomycin (lanes PI). Therefore, neither JNK nor NF-κB is activated by the BCR in tolerant B cells.

Discussion

These data reveal remarkable plasticity in signaling by BCRs and suggest how a single receptor type can signal either positively to promote immunity or negatively to enforce self-tolerance (Figure 7). During positive signaling in naive B cells, acute BCR ligation by foreign HEL antigen stimulates a biphasic calcium response and activates nuclear signals through NF-AT, NF-κB, JNK, and

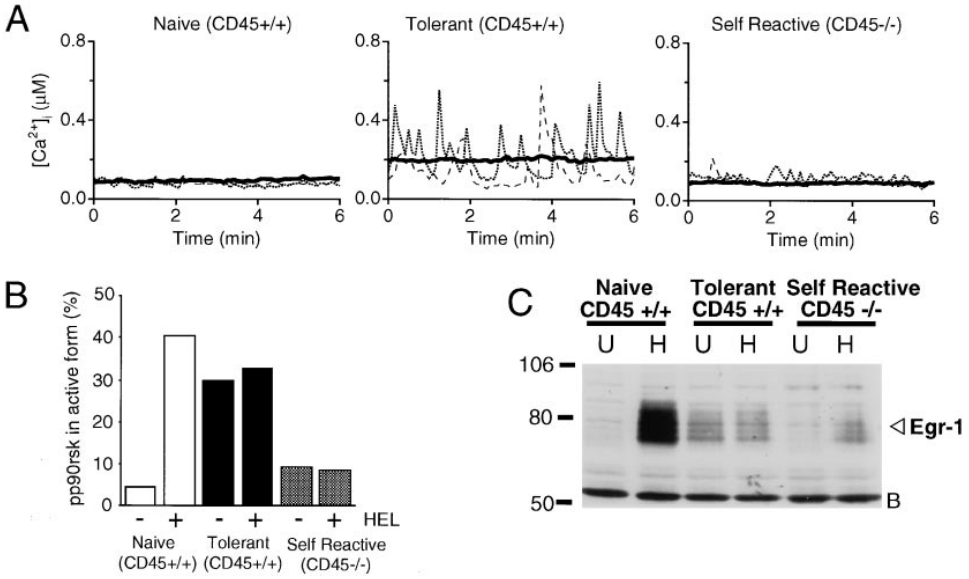

Figure 4. Self-Antigen Does Not Stimulate $[Ca^{2+}]_i$ Oscillations or Activate the ERK Pathway in CD45-Deficient Self-Reactive B Cells

(A) Resting $[Ca^{2+}]_i$ in CD45-deficient B cells that develop in the presence of self-antigen compared to $CD45^{+/+}$ tolerant or naive B cells. Each panel shows the mean resting calcium (bold solid line) and tracings from two single cells (dashed and dotted lines).

(B) Data from Western blot analysis of pp90rsk in CD45-deficient self-reactive B cells. Purified B cells were unstimulated (−) or were stimulated for 10 min with 500 ng/ml HEL (+), and lysates were analyzed by Western blot for pp90rsk as in Figure 3.

(C) Egr-1 expression in the nuclei of self-reactive B cells that lack CD45. Purified cells of each genotype were stimulated for 1 hr with either media alone (U) or with 500 ng/ml HEL (H).

ERK. By contrast, BCR stimulation with the same ligand has a negative effect in self-reactive B cells that have been chronically exposed to HEL expressed as a self-antigen, and this is accompanied by a different calcium pattern and activation of only the NF-AT and ERK pathways. B cells lacking CD45 do not detectably activate calcium or ERK signaling and lack a negative reponse to chronic autoantigen exposure. These findings have important implications for self versus nonself discrimination and raise two key issues. First, how are diverse nuclear signals activated in concert by the BCR within naive cells, but activated differentially by the same receptor during negative signaling in self-tolerant cells? Second, how might differential activation of nuclear signals explain the phenomenon of positive versus negative cell responses?

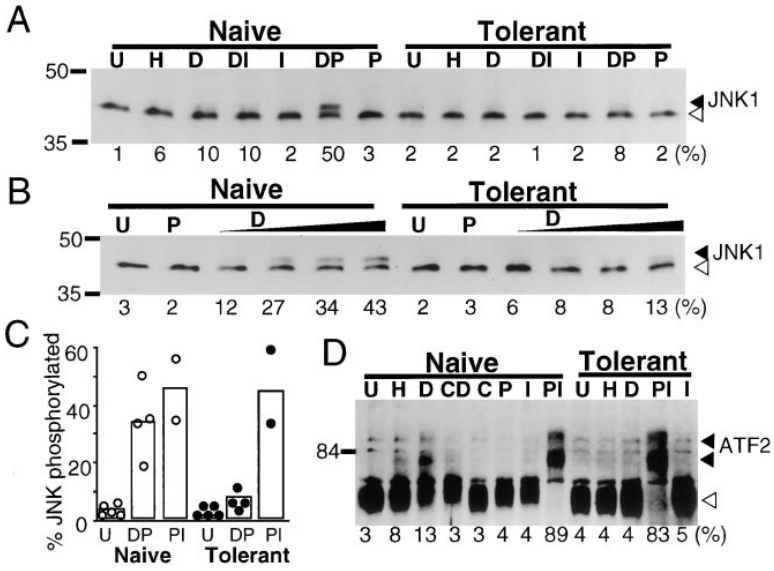

Figure 5. Neither JNK nor ATF2 Is Phosphorylated in Tolerant B Cells

(A) and (B) Western blot analysis of JNK1 in B cells stimulated for 10 min with media alone (U), 500 ng/ml HEL (H), 50 μg/ml anti-IgD (D), 500 ng/ml ionomycin (I), anti-IgD plus ionomycin (DI), 5 ng/ml PdBu (P), or anti-IgD plus PdBu (DP).

(B) Costimulations with 5 ng/ml PdBu and anti-IgD at 5.5, 16.5, 50, and 150 μg/ml for 10 min.

(C) Quantitation of JNK phosphorylation measured as in (A). Dots represent data from individual experiments; bars indicate means.

(D) Western blot analysis of phosphorylation of the transcription factor ATF2. Cells were stimulated as in (A) with the following exceptions: 20 μg/ml polyclonal anti-IgD (D), 150 ng/ml cyclosporin A and 20 μg/ml anti-IgD (CD), 150 ng/ml cyclosporin A alone (C), or 5 ng/ml PdBu and 500 ng/ml ionomycin (PI) combined. Numbers under each lane indicate the percentage of immunoreactive JNK1 or ATF2 in its phosphorylated form.

Phosphorylated and unphosphorylated isoforms are indicated by closed and open arrowheads, respectively.

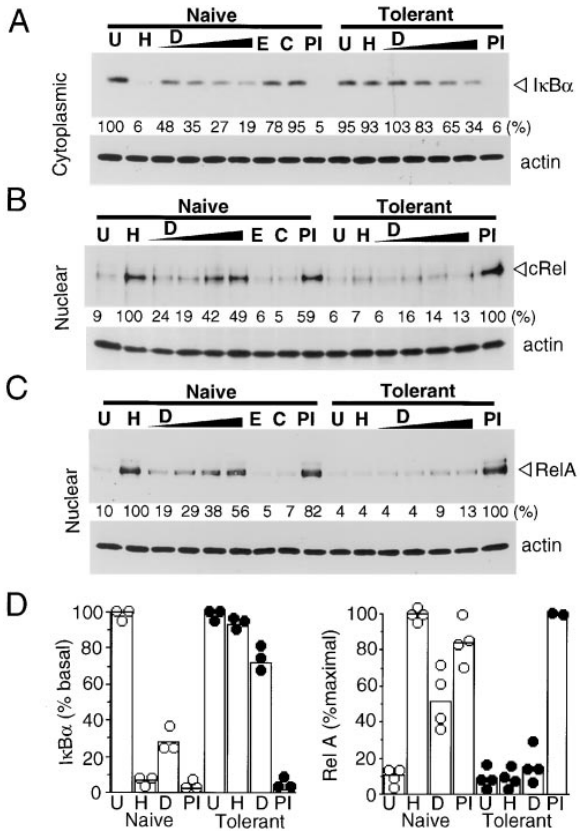

Figure 6. Self-Antigen Does Not Induce IκBα Degradation or c-Rel and Rel-A Nuclear Translocation in Tolerant B Cells

(A–C) Western blot analysis of extracts from naive or tolerant B cells stimulated for 60 min in vitro with media alone (U), 500 ng/ml HEL (H), 5 ng/ml PdBu and 1 μg/ml ionomycin (PI) or anti-IgD at 5.5, 16.5, 50, and 150 μg/ml (D). Addition of 3 mM EGTA (E) or 25 ng/ml cyclosporin (C) was done 10 min before stimulating with 50 μg/ml anti-IgD. Cytoplasmic (A) and nuclear (B and C) proteins were immunoblotted with anti-IκBα (A), anti-c-Rel (B), anti-p65 Rel-A (C), and anti-actin. Similar results were seen with anti-IgD treatments for 15 min. Numbers under lanes represent the percentage immunoreactive target, after correcting for actin, relative to unstimulated naive cells (A) or relative to HEL-stimulated naive B cells (B and C). (D) Quantitation of IκBα degradation and nuclear Rel-A induction in individual experiments (dots). Unlike NF-ATc, Rel-A did not exit the nucleus of HEL-stimulated naive B cells after chelation of extracellular calcium for 40 min (data not shown).

Differential Versus Concerted Activation of Nuclear Signaling Pathways

Differences in the amount of BCR-induced protein tyrosine phosphorylation and calcium signaling are likely to explain the differential activation of transcriptional pathways in tolerant cells. Previous studies have established that the net tyrosine phosphorylation of many proteins, including Igα and the tyrosine kinases Lyn and Syk, is much lower during BCR stimulation in tolerant cells compared to naive cells (Cooke et al., 1994; M. P. Cooke and C. C. G., unpublished data). Because of the importance of these events for activating phospholipase C-γ and stimulating calcium release and entry (Cambier et al., 1994; Gold and DeFranco, 1994; Takata et al., 1994), their diminution presumably accounts for the absence of the peak and sustained-phase calcium responses in tolerant cells (Figure 1). The low calcium oscillations that are induced by the self-reactive BCR in tolerant cells are in principle sufficient to explain the selective activation of NF-AT over NF-κB and JNK even though all three of these signals are shown here to be calcium- and calcineurin-responsive in B cells (Figures 2, 4, and 5) as they are in T cells (Clipstone and Crabtree, 1994; Rao, 1994; Su et al., 1994; Baldwin, 1996). Elevated intracellular calcium is sufficient on its own to activate calcineurin-mediated NF-AT dephosphorylation and nuclear translocation in T cells (Clipstone and Crabtree, 1994; Rao, 1994) and in B cells (Figure 2). Moreover, NF-AT dephosphorylation and translocation is triggered by low calcium increases in the range observed in tolerant cells, whereas the higher calcium concentrations achieved during the initial spike in naive cells are necessary for NF-κB and JNK activation (R. E. D. et al., submitted). In addition, NF-κB and JNK cannot be activated by calcium elevation alone but require other, less well-defined second messenger systems that may also fail to be triggered efficiently by the BCR in tolerant cells.

It will be important in future work to identify the biochemical steps that allow BCRs on tolerant cells to continue to trigger the observed calcium oscillations and ERK/Egr-1 activity. By removing the tolerant cells from antigenic stimulation, these signals were shown to depend on repeated BCR engagement by HEL rather than on an irreversible change in calcium homeostasis (Figures 1 and 3). Moreover, stimulation with polyclonal anti-IgD, which elicits a greater ERK response than HEL, did so equally in naive and tolerant cells, thus indicating that communication from the BCR to ERK was intact. It is important that BCR-induced accumulation of phosphate on tyrosines in Igα, Lyn, Syk, and other proteins is markedly blunted but not absent in tolerant cells (Cooke et al., 1994; M. P. Cooke and C. C. G., unpublished data). It is thus conceivable that tyrosine kinase activity is still induced but is offset by increased activity of negative regulatory tyrosine phosphatases such as SHP-1 (Cyster and Goodnow, 1995b; Plas et al., 1996). Indeed, the calcium oscillations and ERK activity that are stimulated by the BCR in tolerant cells depend on the presence of CD45 (Figure 4), suggesting that these signals are elicited by *src*-family kinases such as Lyn that are positively regulated by CD45 (Pingel et al., 1989; Koretzky et al., 1990; Justement et al., 1991; Shiroo et al., 1992; Volarevic et al., 1992; Chui et al., 1994; Benatar et al., 1996). In light of the association between this pattern of recurrent signaling and negative signaling in tolerant cells, it is particularly intriguing that *lyn*-deficient mice are predisposed to autoimmunity (Hibbs et al., 1995; Nishizumi, et al., 1995).

Role of Different Nuclear Signals in Positive Versus Negative B Cell Responses

The failure of NF-κB activation in tolerant B cells may on its own explain the absence of a mitogenic response to BCR engagement in these cells. NF-κB is a key positive regulator of inducible immune response genes in mice and humans (reviewed by Baldwin, 1996) and in invertebrates (Ip et al., 1993). Murine B and T lymphocytes lacking even a single copy of the c-*Rel* gene have

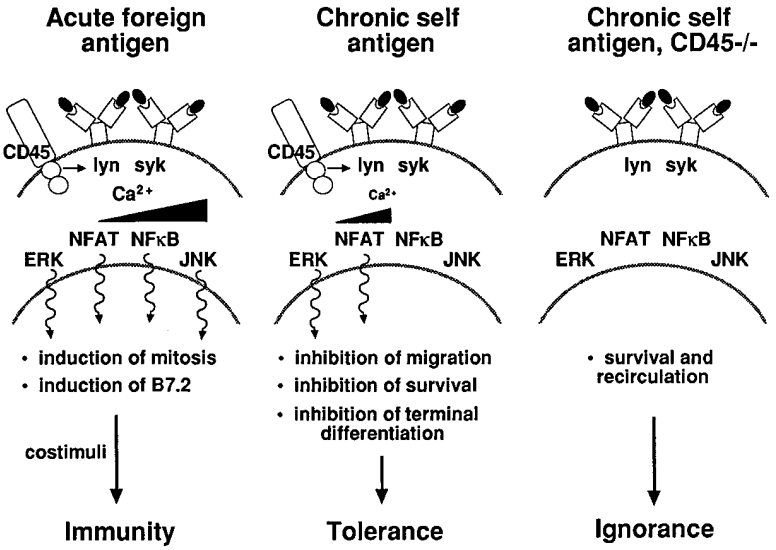

Figure 7. Model Summarizing the Biochemical Changes in Nuclear Signals and Functional Responses to Antigen That Occur in Naive Versus Tolerant B Cells

(Left) Following acute stimulation with foreign antigen, naive B cells exhibit a large intracellular calcium response, activation of a broad spectrum of nuclear signals, and expression of cell surface B7.2 protein, and subsequently make a mitogenic response in the presence of costimuli from T cells or endotoxin. These positive responses promote active immunity. (Middle) By contrast, following chronic stimulation by self-antigens, tolerant B cells exhibit only a low oscillatory calcium response and activation of a subset of nuclear signals, and fail to respond by B7.2 induction or mitogenesis. Instead they respond negatively to self-antigen, exhibiting antigen-dependent inhibition of migration, recirculation, and survival within lymphoid tissues and antigen-dependent inhibition of terminal differentiation into antibody-secreting cells.

(Right) These negative responses to chronic autoantigen exposure are not seen in self-reactive B cells that lack CD45, where BCR signaling is less efficient and chronic calcium signaling and NF-AT/ERK activation are not induced.

a severe deficit in mitogenesis induced by their antigen receptors (Kontgen et al., 1995). Interestingly, $CD4^+$ T cells rendered anergic by repeated exposure to the superantigen staphylococcal enterotoxin also have diminished nuclear RelA (Sundstedt et al., 1996). Because of the importance of NF-κB as a proimmunity signal in diverse cell lineages and phyla, it is striking that selective uncoupling of this pathway from BCRs and TCRs is associated with tolerance.

The activation of the NF-AT and ERK pathways in tolerant B cells, on the other hand, raises the possibility that their activity—in the absence of NF-κB or JNK—is responsible for negative cell responses. This recurrent signaling was absent in CD45-deficient B cells (Figure 4), in which self-antigen no longer inhibits B cell survival (Cyster et al., 1996). In particular, recurrent activation of the ERK pathway in tolerant B cells may block terminal differentiation into autoantibody-secreting plasma cells, because both phenomena are induced by phorbol ester, are resistant to cyclosporin A, and are inhibited by a dominant negative *ras* transgene (J. I. H. et al., unpublished data). Consistent with the notion that NF-ATp negatively regulates immune responses, NF-ATp deficient B lymphocytes have elevated proliferative responses (Hodge et al., 1996; Xanthoudakis et al., 1996).

Differential cellular responses are also triggered by antigen receptors in T cells, for example in T cell anergy (reviewed by Mueller and Jenkins, 1995) or in the negative versus positive effects of partial T cell agonists (reviewed by Kersh and Allen, 1996). In both circumstances, antigen receptor engagement triggers only a subset of lymphocyte responses, such as interleukin-4 secretion or cytotoxicity without cell proliferation. In anergic T cells, loss of TCR-induced mitogenesis is associated with a lack of IL-2 gene induction (reviewed by Schwartz, 1990) and a failure to activate JNK and ERK (Fields et al., 1996; Li et al., 1996) or NF-κB (Sundstedt et al., 1996). These signaling deficits in anergic T cells may explain the lack of mitosis, but alone they do not elucidate how lymphocyte antigen receptors induce differential responses.

The present findings demonstrate that signaling by the BCR is plastic, allowing nuclear signals to be activated independently of one another. Throughout development, this plasticity may allow the antigen receptor to activate different combinations of signals and thereby induce alternate cell fates. Furthermore, the distinct signaling patterns associated with positive versus negative reponses identify pharmacologic targets for manipulating immune responses to self or foreign antigens. Genetic differences in the tuning and balancing of these different nuclear signals represent important candidates for inherited susceptibility to immunodeficiency or autoimmunity.

Experimental Procedures

Animals and Lymphocyte Purification

Splenic B lymphocytes were purified by depleting non–B cells from the spleens of MD4 × ML5 transgenic mice that were bred and typed as described (Cyster et al., 1996).

Calcium Imaging

Single-cell calcium imaging was performed as described (Dolmetsch and Lewis, 1994) except that purified B cells were loaded with 1 mM fura-2 AM for 15 min at 37°C, settled onto glass slides, and stimulated as indicated.

Cell Lysates and Western Blot Analysis

For NF-AT, cell suspensions at approximately 10^7 cells/ml were stimulated at 37°C; stimulations were stopped on ice; cells were centrifuged at 3°C and resuspended in ice-cold buffer Hx (20 mM HEPES pH 7.5, 5 mM NaCl, 10 mM NaF, 2 mM EDTA, 6 mM pNPP, 1 mM $NaVO_4$, 2.5 mM PMSF, 40 μg/ml each aprotinin and leupeptin); and an equal volume Hx containing 0.8% NP40 was added. After 2 min on ice, nuclei were centrifuged at 600 × g, and the supernatant (cytosol) was added to boiling SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and frozen on dry ice. Nuclei were rinsed once in 0.2 ml Hx, boiled with sample buffer, and centrifuged

for 8 min at 70,000× g 4°C in a Beckman airfuge to remove chromatin. Fractions from 2 × 10^6 cells per lane were resolved by SDS-PAGE. Sequential immunoblots were performed with anti-NF-ATp (4G10G5), anti-NF-ATc (7A6), and anti-actin (Sigma, AC-40) using enhanced chemiluminescence (Amersham). Quantitation of exposed films was performed with a Molecular Dynamics Computing Densitometer. The quantitation of immunoreactive NF-AT is underestimated under conditions in which a significant fraction is partially phosphorylated, such as during HEL or anti-IgD stimulation, because these partially phosphorylated forms migrate hetergenously between the fully phosphorylated and dephosphorylated forms. These minor bands are detected inefficiently because of the nonlinearity of X-ray film to weak signals. For the same reason, cyclosporin A, EGTA, or ionomycin treatment causes an apparent increase in NF-AT by driving all of the protein into fully phosphorylated or dephosphorylated species. Analysis of ERK2, pp90rsk, and Egr-1 was performed as described (Cyster et al., 1996). In-gel-kinase assay was performed as described (Samuels and McMahon, 1994). Lysates for JNK assays were prepared and analyzed as described for ERK. Antibodies to JNK1 were from Santa Cruz Biotechnology and Pharmingen. For ATF2, rinsed nuclei prepared as for NF-AT were extracted with 400 mM NaCl in buffer Hx on ice for 20 min. Antibodies to ATF2 were kind gifts from Dr. J. Hoeffler and Dr. M. Green. Cytosolic lysates for IκBα were performed as NF-AT. Nuclear extracts for c-Rel and RelA were prepared as for ATF2. Anti-IκBα, anti-c-Rel, and anti-Rel-A were from Santa Cruz Biotechnology, and anti-rabbit HRP was from Zymed.

Acknowledgments

Correspondence should be addressed to C. C. G. (e-mail: goodnow@cmgm.stanford.edu). The authors thank Dr. John Blenis, Dr. James Hoeffler, Dr. Fred Finkelman, and Dr. Michael Green for their gifts of antisera. J. I. H. is a Beckman Scholar supported by the Medical Scientist Training Program and the Program in Molecular and Genetic Medicine. R. E. D. and R. S. L. are supported by the American Heart Association and a grant from the National Institutes of Health, respectively. M. L. T., G. R. C., and C. C. G. are investigators of the Howard Hughes Medical Institute.

Received December 9, 1996; revised March 6, 1997.

References

Baeuerle, P.A., and Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. Annu. Rev. Immunol. *12*, 141–179.

Baldwin, A. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. *14*, 649–681.

Benatar, T., Carsetti, R., Furlonger, C., Kamalia, N., Mak, T. and Paige, C. (1996). Immunoglobulin-mediated signal transduction in B cells from CD45-deficient mice. J. Exp. Med. *183*, 329–334.

Cambier, J.C., Pleiman, C.M., and Clark, M.R. (1994). Signal transduction by the B cell antigen receptor and its coreceptors. Annu. Rev. Immunol. *12*, 457–486.

Chen, R.H., Chung, J., and Blenis, J. (1991). Regulation of pp90rsk phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester- and cyclic AMP-mediated signal transduction. Mol. Cell. Biol. *11*, 1861–1867.

Chui, D., Ong, C., Johnson, P., Teh, H., and Marth, J. (1994) Specific CD45 isoforms differentially regulate T cell receptor signaling. EMBO J. *13*, 798–807.

Clipstone, N.A., and Crabtree, G.R. (1992). Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. Nature *357*, 695–697.

Clipstone, N.A., and Crabtree, G.R. (1994). Signal transduction between the plasma membrane and the nucleus of T lymphocytes. Annu. Rev. Biochem. *63*, 1045–1083.

Cooke, M.P., Heath, A.W., Shokat, K.M., Zeng, Y., Finkelman, F.D., Linsley, P.S., Howard, M., and Goodnow, C.C. (1994) Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. J. Exp. Med. *179*, 425–438.

Coso, O.A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137–1146.

Cyster, J.G., and Goodnow, C.C. (1995a). Antigen-induced exclusion from follicles and anergy are separate and complementary process that influence peripheral B cell fate. Immunity *3*, 691–701.

Cyster, J.G., and Goodnow, C.C. (1995b). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. Immunity *2*, 13–24.

Cyster, J.G., Hartley, S.B., and Goodnow, C.C. (1994) Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. Nature *371*, 389–395.

Cyster, J., Healy, J., Kishihara, K., Mak, T., Thomas, M., and Goodnow, C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. Nature *381*, 325–328.

Davis, R.J. (1994). MAPKs: new JNK expands the group. Trends Biochem. Sci. *19*, 470–473.

Dolmetsch, R., and Lewis, R. (1994). Signaling between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels generates $[Ca^{2+}]i$ oscillations in T lymphocytes. J. Gen. Physiol. *103*, 365–388.

Fields, P., Gajewski, T., and Fitch, F. (1996). Blocked Ras activation in anergic CD4+ T cells. Science *271*, 1276–1278.

Flanagan, W., Corthesy, B., Bran, R., and Crabtree, G. (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature *352*, 803–807.

Fulcher, D.A., and Basten, A. (1994). Reduced life span of anergic self-reactive B cells in a double-transgenic model. J. Exp. Med. *179*, 125–134.

Fulcher, D., Lyons, A., Korn, S., Cook, M., Koleda, C., Parish, C., Fazekas de St. Groth, B., and Basten, A. (1996). The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. J. Exp. Med. *183*, 2313–2338.

Gold, M.R., and DeFranco, A.L. (1994). Biochemistry of B lymphocyte activation. Adv. Immunol. *55*, 221–295.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., and Raphael, K., et al. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676–682.

Goodnow, C.C., Brink, R., and Adams, E. (1991). Breakdown of self-tolerance in anergic B lymphocytes. Nature *352*, 532–536.

Goodnow, C.G., Cyster, J.G., Hartley, S.B., Bell, S.E., Cooke, M.P., Healy, J.I., Akkaraju, S., Rathmell, J.C., Pogue, S.P., and Shokat, K.P. (1995). Self-tolerance checkpoints in B lymphocyte development. Adv. Immunol. *59*, 279–368.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science *267*, 389–393.

Hibbs, M.L, Tarlinton, D.M., Armes, J., Grail, D., Hodgson, G., Maglitto, R., Stacker, S.A., and Dunn, A.R. (1995). Multiple defects in the immune system of *Lyn*-deficient mice, culminating in autoimmune disease. Cell *83*, 301–311.

Hodge, M.R., Ranger, A.M., Charles de la Brousse, F., Hoey, T., Grusby, M.J., and Glimcher, L.H. (1996). Hyperproliferation and dysregulation of IL-4 expression in NF-ATp–deficient mice. Immunity *4*, 397–405.

Hsiao, K.M., Chou, S.Y., Shih, S.J., and Ferrell, J.E. (1994) Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer in vivo. Proc. Natl. Acad. Sci. USA *91*, 5480–5484.

Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., González-Crespo, S., Tatei, K., and Levine, M. (1993). *Dif*, a *dorsal*-related gene that mediates an immune response in Drosophila. Cell *75*, 753–763.

Izquierdo, M., Leevers, S.J., Marshall, C.J., and Cantrell, D. (1993). p21ras couples the T cell antigen receptor to extracellular signal-related kinase 2 in T lymphocytes. J. Exp. Med. *178*, 1199–1208.

Jain, J., McCaffrey, P., Miner, Z., Kerppola, T., Lambert, J., Verdine, G., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature *365*, 352–355.

Justement, L.B., Campbell, K.S., Chien, N.C., and Cambier, J.C. (1991). Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science *252*, 1839–1842.

Karin, M., and Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr. Biol. *5*, 747–757.

Kersh, G., and Allen, P. (1996). Essential flexibility in the T-cell recognition of antigen. Nature *380*, 495–498.

Kontgen, F., Grumont, R.J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., and Gerondakis, S. (1995). Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev. *9*, 1965–1977.

Koretzky, G., Picus, J., Thomas, M., and Weiss, A. (1990) Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. Nature *346*, 66–68.

Li, W., Whaley, C., Mondino, A., and Mueller, D. (1996). Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. Science *271*, 1272–1276.

Lin, A., Minden, A., Martinetto, F., Claret, F., Lange-Carter, C., Mercurio, F., Johnson, G., and Karin, M. (1995). Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science *268*, 286–290.

Liou, H.C., and Baltimore, D. (1993). Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system. Curr. Opin. Cell Biol. *5*, 477–487.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.

Mason, D.Y., Jones, M., and Goodnow, C.C. (1992) Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. Int. Immunol. *4*, 163–175.

McMahon, S.B., and Monroe, J.G. (1995). Activation of the p21 ras pathway couples antigen receptor stimulation to induciton of the primary response gene Egr-1 in B lymphocytes. J. Exp. Med. *181*, 417–422.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R., Johnson, G.L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen activated protein kinases by Raf-1 and MEKK. Science *266*, 1719–1723.

Minden, A., Lin, A., Claret, F.-X., Abo, A., and Karin, M. (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell *81*, 1147–1157.

Mueller, D., and Jenkins, M. (1995). Molecular mechanisms underlying functional T-cell unresponsiveness. Curr. Opin. Immunol. *7*, 375–381.

Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., and Yamamoto, T. (1995). Impaired proliferation of peripheral B cells and indications of autoimmune disease in *lyn*-deficient mice. Immunity *3*, 549–560.

Northrop, J., Ho, S., Chen, L., Thomas, D., Timmerman, L., Nolan, G., Admon, A., and Crabtree, G. (1994). NF-AT components define a family of transcription factors targeted in T-cell activation. Nature *369*, 497–502.

Nossal, G.J. (1983). Cellular mechanisms of immunologic tolerance. Annu. Rev. Immunol. *1*, 33–62.

Payne, D.M., Rossomando, A., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. *10*, 885–892.

Pingel, J.T., and Thomas, M.L. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. Cell *58*, 1055–1065.

Plas, D.R., Johnson, R., Pingel, J.T., Matthews, R.J., Dalton, M., Roy, G., Chan, A.C., and Thomas, M.L. (1996). Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. Science *272*, 1173–1176.

Posada, J., and Cooper, J.A. (1992). Requirements for phorphorylation of MAP kinase during meiosis in Xenopus Oocytes. Science *255*, 212–215.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Proinflammatorycytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. *270*, 7420–7426.

Rao, A. (1994). NF-ATp: a transcription factor required for the coordinate induction of several cytokine genes. Immunol. Today *15*, 274–281.

Rathmell, J.C., Townsend, S.E., Xu, J.C., Flavell, R.A., and Goodnow, C.C. (1996). Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. Cell *87*, 319–329.

Rothstein, T.L., Wang, J.K.M., Panka, D.J., Foote, L.C., Wang, Z., Stanger, B., Cui, H., Ju, S., and Marshak-Rothstein, A. (1995). Protection against Th1-mediated apotosis by antigen receptor engagement in B cells. Nature *374*, 163–165.

Samuels, M.L., and McMahon, M. (1994). Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3T3 cells expressing delta Raf-1:ER, an estradiol-regulated form of Raf-1. Mol. Cell. Biol. *14*, 7855–7866.

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodget, J.R., Avruch, J., Kyriakis, J.M., and Zon, L.I. (1994). Role of SAPK/ERK kinase-1 in regulating the stress activated pathway regulating transcription factor c-Jun. Nature *372*, 794–798.

Schwartz, R.H. (1990). A cell culture model for T lymphocyte clonal anergy. Science *248*, 1349–1356.

Scott, D.W. (1993). Analysis of B cell tolerance in vitro. Adv. Immunol. *54*, 393–425.

Shiroo, M., Goff, L., Biffen, M., Shivnan, E., and Alexander, D. (1992). CD45 tyrosine phosphatase-activated p59fyn couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. EMBO J. *11*, 4887–4897.

Sturgill, T.W., Ray, L.B., Erikson, E., and Maller, J.L. (1988) Insulin stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature *334*, 715–718.

Su, B., Jacinto, E., Masahiko, H., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727–736.

Sundstedt, A., Sigvardsson, M., Leanderson, T., Hedlund, G., Kalland, T., and Dohlstein, M. (1996). In vivo anergized CD4+ T cells express perturbed AP-1 and NF-κB transcription factors. Proc. Natl. Acad. Sci. *93*, 979–984.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. EMBO J. *13*, 1341–1349.

Thanos, D., and Maniatis, T. (1995). NF-κB: a lesson in family values. Cell *80*, 529–532.

Volarevic, S., Niklinska, B., Burns, C., Yamada, H., June, C., Dumont, F., and Ashwell, J. (1992). The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern

of late T cell receptor-induced Ca^{2+} oscillations. J. Exp. Med. *176*, 835–844.

Xanthoudakis, S., Viola, J., Shaw, K., Luo, C., Wallace, J., Bozza, P., Curran, T., and Rao, A. (1996). An enhanced immune response in mice lacking the transcription factor NFAT1. Science *272*, 892–895.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodget, J.R., and Templeton, D.J. (1994). Activation of stress activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature *372*, 798–800.

Note Added in Proof

The data referred to as R.F.D. et al., submitted, are now in press: Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C., and Healy, J.I. (1997). Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. Nature *386*, in press.