A Population of In Vivo Anergized T Cells with a Lower Activation Threshold for the Induction of CD25 Exhibit Differential Requirements in Mobilization of Intracellular Calcium and Mitogen-Activated Protein Kinase Activation

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A Population of In Vivo Anergized T Cells with a Lower Activation Threshold for the Induction of CD25 Exhibit Differential Requirements in Mobilization of Intracellular Calcium and Mitogen-Activated Protein Kinase Activation

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Chronic exposure of mature T cells with specificity for self-Ags can lead to the induction of a nonfunctional state which is referred to as T cell anergy. It is unclear whether anergic T cells are destined for cell death and thereby harmless or whether they can contribute to the induction of autoimmunity and/or regulation of anti-self reactivity. We have begun to address this issue. In a recent study, we showed that a population of mature CD4\(^+\)CD8\(^-\) T cells that express a transgenic TCR specific for the L\(^d\) MHC class I molecule are rendered anergic in L\(^d\)-expressing mice. In this study, we show that this population of anergic T cells possess a lower activation threshold for the induction of CD25 and CD69 in response to stimulation by antigenic ligands. Furthermore, these anergic T cells undergo extensive proliferation when stimulated with a low-affinity ligand in the presence of an exogenous source of IL-2. Biochemical analysis of the early intracellular signaling events of these in vivo anergized T cells showed that they have a signaling defect at the level of ZAP-70 and linker for the activation of T cell (LAT) phosphorylation. They also exhibit a defect in mobilization of intracellular calcium in response to TCR signaling. However, these anergic T cells demonstrate no defect in SLP-76 phosphorylation and extracellular signal-regulated kinase 1/2 activation. These biochemical characteristics of the anergic T cells were associated with an elevated level of Fyn, but not Lck expression. The potential contributions of these anergic T cells in the induction and/or regulation of autoimmune responses are discussed. The Journal of Immunology, 2000, 164: 2881–2889.

Understanding immune tolerance to self-Ags is essential to attempts to modulate autoimmune pathogenesis. Elucidating the mechanisms of tolerance in the T cell compartment is one aspect of this endeavor. The importance of self-discrimination is underlined by the existence of several mechanisms to ensure its maintenance. Intrathymic mechanisms for the maintenance of tolerance to self-Ags include the deletion of immature thymocytes with specificity for self-Ags (1–4), and the induction of T cell anergy by self-Ags expressed on thymic cortical epithelial cells (5). Mature T cells with specificity for self-Ags can also be deleted extrathymically (6–8) and/or anergized (9, 10). In certain instances, T cell ignorance of extrathymic Ags has also been proposed to be a mechanism for maintaining self-tolerance (11).

Anergy is defined as a cellular state in which a lymphocyte is alive but fails to display certain functional responses when optimally stimulated through both its Ag-specific receptor and any other receptors that are normally required for full activation (12). It is typically characterized by a defect in IL-2 production and T cell proliferation (12). The persistence of anergic T cells specific for self-Ags in vivo is somewhat puzzling. Because these T cells are self-specific, a breakdown of mechanisms to maintain their inactive state could lead to autoimmune reactions. However, it is conceivable that anergic T cells may subserve a regulatory role to prevent the induction of immune responses to self-Ags. We have begun to examine these possibilities using a model of anergy induction centered on a functionally mature CD4\(^+\)CD8\(^-\) T cell population that expresses a transgenic TCR-\(\alpha\)\(\beta\) (herein referred to as DN cells). Development of these DN cells in TCR transgenic mice is thymus dependent (13) but independent of positively selecting MHC molecules (13, 14). They are also resistant to clonal deletion in Ag-expressing mice (14, 15). We have previously found that, based on their inability to produce IL-2 or to proliferate (16), DN T cells in this system are rendered anergic when chronically exposed to their antigenic ligand in vivo. In the present study, we show that these anergic T cells have a lower activation threshold for the induction of CD25. Furthermore, they proliferate vigorously when stimulated with a low-affinity ligand plus an exogenous source of IL-2. The implications of the existence of such a population of anergic T cells in inducing autoimmune reactions or in the suppression of anti-self-reactivity are discussed.

Previous studies have shown that depending on the cell type and anergy induction protocol, specific biochemical defects have been associated with the anergic state (for review, see Ref. 17). In some models, the block in IL-2 production is attributed to a decrease in IL-2 gene transcription (18) and this block results from a failure to activate p21\(^{ras}\) (Ras) after TCR ligation. This failure leads to a decrease in the kinase activities of both extracellular signal-regulated protein kinase (ERK) and c-Jun NH\(_2\)-terminal kinase, which in turn leads to a failure to activate the IL-2 transcription factor AP-1 (18). In other models based on human T cell clones, an impaired intracellular calcium response has been credited with the reduced IL-2 production by means of impaired binding of the NF-AT transcription factor to distal response elements within the IL-2 enhancer (19).
However, in the latter human T cell model, AP-1 is hardly observed to be affected (20). Although the above-mentioned alterations in AP-1 or NF-AT function provide a distal biochemical basis for the anergic T cell’s inability to produce IL-2, signaling defects more proximal to the TCR also exist in anergic cells and may be causal to the reduced activation of these transcription factors.

Anergic cells exhibit increased expression of p59 \textsuperscript{567} (Fyn) along with increased Fyn protein tyrosine kinase (PTK) \textsuperscript{3} activity (21). Fyn has been reported to be constitutively associated with Cbl in anergic cells (22) and this association has been shown to lead to increased activity of Rap1, a negative regulator of IL-2 transcription (22). More recently, Cbl has been shown to be a negative regulator of ZAP-70 and Cbl\textsuperscript{-}\textsuperscript{-} cells show increased ZAP-70 PTK activity (23, 24). Cbl binds to ZAP-70 and this binding is dependent on phosphorylation of tyrosine 292 on ZAP-70 (25). Anergic T cells have been reported to be unable to activate ZAP-70 upon TCR ligation (26), but it is unclear whether this is due to negative regulation of ZAP-70 by Cbl in anergic T cells.

Previous models of in vivo induced T cell anergy have been based largely on either repeated injections of superantigen (9, 27) or adoptive transfer models of T cells from TCR-transgenic mice (28, 29). However, biochemical analysis of in vivo anergized T cells is very limited, due in part to the difficulty in isolating sufficient numbers of purified anergic cells for these studies. The few studies done to date were performed using heterogeneous CD4 T cells that have been anergized by repeated injections of staphyloccocal enterotoxin A. These studies showed that CD4 T cells anergized in this manner exhibit impaired NF-AT, NF-κB, and AP-1 binding to IL-2 enhancer response elements (30, 31).

In this study, we have examined early intracellular signaling events in in vivo anergized DN cells. We demonstrate that these anergic cells exhibit differential requirements in mobilizing intracellular calcium and in the activation of the ERK mitogen-activated protein (MAP) kinase pathway. These biochemical characteristics were associated with an elevated level of Fyn, but not Lck, expression in these anergic T cells. The inefficient mobilization of intracellular calcium was associated with defects in ZAP-70 and linker for the activation of T cells (LAT) phosphorylation in response to TCR signaling. Efficient ERK1/2 activation was associated with efficient induction of the CD69 activation marker and phosphorylation of SLP-76. A model for the regulation of TCR-signaling pathways in these anergic T cells is proposed.

Materials and Methods

Mice

Breeders for the H-\textsuperscript{2} 2C TCR-transgenic mice (4, 32) were kindly provided by Dr. Denis Loh (then at the University of Washington, St. Louis, MO). The H-\textsuperscript{2} 2C TCR-transgenic mice have been backcrossed to the C57BL/6 background. DBA/2 (H-\textsuperscript{2} 2C) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). H-\textsuperscript{2} 2C TCR-transgenic mice were obtained from the University of British Columbia in the animal facility at the University of British Columbia. H-2\textsuperscript{b} 2C mice were F1 mice obtained from these breeders. H-2\textsuperscript{b} 2C mice were F1 mice obtained from these breeders.

Cells

Lymph node cells were harvested from transgenic mice. Purification of DN cells was as described previously (16). The purified DN cells were typically 70% CD4\textsuperscript{+} CD8\textsuperscript{−} I\textsuperscript{g} and expressed exclusively the 2C TCR, which was detected by the 1B2 mAb (33). The peptide transporter-deficient cell lines T2-L\textsuperscript{4} and T2-K\textsuperscript{b} (34) were derived by transfecting the human T (× B) hybridoma T2 with L\textsuperscript{4} or K\textsuperscript{b}. The T2-L\textsuperscript{4} or T2-K\textsuperscript{b} cells were used as APC for the p2Ca peptide. The p2Ca peptide (LSPPFPDL) was synthesized by the Cbl-Biotechnology (Lake Placid, NY). Anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD3 (145-2C11), and anti-CD25 (PC61) were obtained from American Type Culture Collection (Manassas, VA). Anti-CD69 polyclonal Abs were a kind gift from Dr. Koretsky (University of California, Los Angeles, CA). The anti-CD25 Ab 54.3B is a peptide-specific (N-terminal residues 3–147) rabbit antisera generated in our laboratory (36). The anti-TCR-\textgamma mAb (G3), specific for the cytoplasmic domain of \textgamma, was produced in our laboratory (37).

Intracellular calcium

Flow cytometry was used to measure intracellular calcium levels in cells loaded with the calcium-binding dye fluo-3-acetoxymethyl ester (Molecular Probes, Eugene, OR) using the Chironics software package (Becton Dickinson, Mountain View, CA) as described previously (38).

CD69 and CD25 flow cytometry

Single-cell suspensions of lymph node cells were prepared. Purified DN cells (1 × 10\textsuperscript{5}) were stimulated with 5 × 10\textsuperscript{5} mitomycin C-treated T2-L\textsuperscript{4} or T2-K\textsuperscript{b} cells plus the indicated concentration of the p2Ca peptide in a 24-well plate in a volume of 2.0 ml. No exogenous IL-2 was added. After a culture period of 40 h, the cells were collected and stained with biotinylated anti-CD69 or anti-CD25 mAb followed by streptavidin-Tricolor and analyzed with FACScan flow cytometer using Lysis II software (Becton Dickinson). A total of 15,000 events was analyzed.

Results

Recently, we have described an in vivo system for the generation of anergic T cells (16). This system is based on the 2C TCR-transgenic receptor (4, 32). The 2C TCR is specific for the p2Ca
Anergic H-2^{b/d} 2C DN cells proliferate in response to a low-affinity ligand and exogenous IL-2

The hypoproliferative response of Ag-stimulated anergic T cells has been reported to be reversible in some systems by the addition of IL-2 (17, 43). In agreement with our previous results (16), nonanergic DN cells from H-2^{b} 2C mice exhibit a significant proliferative response to stimulation with the high-affinity p2Ca/L^{d} ligand even without the addition of exogenous IL-2 (Fig. 1). The addition of exogenous IL-2 to these cultures mount a vigorous proliferative response (Fig. 2). These results support the hypothesis that the anergic DN cells have a lower activation threshold than nonanergic DN cells.

The 2C TCR is able to recognize the p2Ca peptide in the context of both the L^{d} and K^{b} MHC class I molecules, but with an 1000-fold lowering in the affinity for the latter interaction (42). Since very low concentrations of the high-affinity p2Ca/L^{d} ligand were able to induce a vigorous proliferative response in anergic DN cells in the presence of exogenous IL-2, we tested the hypothesis that the anergic DN cells may in fact have a lower activation threshold than the nonanergic DN cells. The results in Fig. 2 support such a hypothesis. It was found that nonanergic DN cells were unable to proliferate when stimulated with various concentrations of the low-affinity p2Ca/K^{b} ligand, even in the presence of exogenously added IL-2. In contrast, the anergic DN cells already exhibit a small but significant proliferative response to the p2Ca/K^{b} ligand even in the absence of exogenous IL-2; this small proliferative response was also elicited by the high-affinity ligand (Fig. 1). In the presence of exogenously added IL-2, these cultures mount a vigorous proliferative response (Fig. 2). These results support the hypothesis that the anergic DN cells have a lower activation threshold when compared with nonanergic DN cells.

Anergic but not nonanergic DN cells express CD25 and CD69 in response to the low-affinity ligand

We sought independent support for the hypothesis that anergic DN cells have a lower activation threshold than nonanergic DN cells. The expression of activation markers associated with T cell activation following stimulation of these cells with either the high- or low-affinity ligand was assessed. DN cells from H-2^{b} or H-2^{b/d} 2C mice were activated with either the high- or low-affinity ligand in the absence of exogenously added IL-2 and the expression of CD25 and CD69 on these cells was determined after 40 h of stimulation. The results in Fig. 3 show that the anergic DN cells were able to undergo blastogenesis (increase in forward scatter) and up-regulate CD25 and CD69 in response to either the high- or low-affinity ligand. In contrast, only the high-, but not low-, affinity ligand was able to induce blastogenesis, CD25 and CD69 expression in the nonanergic DN cells. These results are consistent with...
the proliferation data in Figs. 1 and 2 and further support the hypothesis that the anergic DN cells have a lower activation threshold in comparison to the nonanergic DN cells. Furthermore, the vigorous proliferation that ensued when exogenous IL-2 was added to these stimulated cultures indicated that these anergic DN cells retain the biochemical machinery that is required for normal proliferation.

**Impaired tyrosine phosphorylation of LAT in anergic DN cells**

The above findings suggest that the anergic DN cells were able to mediate signaling from the TCR which led to effective induction of CD25 and CD69 expression. We have shown in a recent study that Ag-activated anergic DN cells were defective in IL-2 production (16). Stimulation of anergic and nonanergic DN cells with an anti-CD3ε mAb led to similar findings. Thus, anti-CD3ε-stimulated anergic DN cells (8 × 10^5/well in 0.2 ml with 10 μg/ml of immobilized 2C11 mAb for 18 h) produced less IL-2 on a per cell basis (3.5 U/ml) when compared with similarly stimulated nonanergic DN cells (10 U/ml). The proliferative response of anti-CD3ε-stimulated anergic DN was also lower when compared with similarly stimulated nonanergic DN cells (Fig. 4). To determine the extent of the signaling defects in the anergic cells, we first compared whole-cell lysate phosphorylation profiles from anti-CD3ε-stimulated H-2^b 2C and H-2^bd 2C DN cells. We noted that a major difference in the phosphorylation of a 36-kDa protein was observed (Fig. 5A). The apparent molecular mass and rapid phosphorylation of this protein in the H-2^b DN cells suggested this protein to be the LAT adaptor molecule (44).

Immunoprecipitation studies confirmed the identity of this 36-kDa protein to be LAT (Fig. 5B). In this experiment, LAT was also hypophosphorylated in anti-CD3ε-stimulated anergic DN cells. This hypophosphorylation of LAT could be accounted for in part by the lower amount of LAT that was precipitated from the anergic DN cells (Fig. 5B). Densitometric quantitation of the data indicated that when the lesser amount of immunoprecipitated LAT was taken into consideration, LAT phosphorylation was reduced by about 60% relative to that observed in the nonanergic DN cells. The level of expression of LAT in anergic and nonanergic DN cells was also determined by Western blotting of whole-cell lysates, and the data were quantified by densitometric analysis (Fig. 5C). This analysis showed a 34% reduction in LAT expression in the anergic population. These data indicate that the hypophosphorylation of LAT in the anergic DN cells upon TCR stimulation is
To explain the LAT phosphorylation defect observed in the H-2 b/d 2C DN cells, one representative experiment of three are shown.

**ZAP-70 phosphorylation is reduced in H-2<sup>bd</sup> 2C DN cells**

To explain the LAT phosphorylation defect observed in the H-2<sup>bd</sup> 2C DN cells, we examined signaling events known to be upstream of LAT phosphorylation. ZAP-70 has LAT as a major substrate and phosphorylation of LAT by ZAP-70 is a major event in T cell activation (44). We first determine the expression level of ZAP-70 in anergic and nonanergic DN cells and found it to be fairly equivalent (Fig. 6A). Immunoprecipitation studies of ZAP-70 found it to be tyrosine phosphorylated in nonanergic DN cells upon TCR ligation (Fig. 6B). Previous studies have shown that phosphorylation of ZAP-70 is associated with its activation (45, 46). In contrast, tyrosine phosphorylation of ZAP-70 upon TCR ligation was found to be impaired in the H-2<sup>bd</sup> 2C DN cells (Fig. 6B). In this experiment, we observed more tyrosine phosphorylation of ZAP-70 despite the fact that slightly less ZAP-70 was precipitated from TCR-stimulated nonanergic cells. This observation strengthens the conclusion that ZAP-70 is hypotyrosine phosphorylated in anergic DN cells upon TCR stimulation. However, precipitation of less ZAP-70 from TCR-stimulated nonanergic cells was not a consistent finding since in repeat experiments similar amounts of ZAP-70 were precipitated from nonstimulated and stimulated cells (data not shown).

Activation of ZAP-70 requires the immunoreceptor tyrosine-based activation motifs on the TCR-ζ chain to be doubly phosphorylated to allow binding of the tandem Src homology 2 domains present in ZAP-70 (45). These phosphorylation events occur via activation of the Src kinases, Lck and Fyn, and their subsequent recruitment to the TCR-ζ chains, their autophosphorylation, and then subsequently their phosphorylation of the immunoreceptor tyrosine-based activation motifs within the TCR chains (45, 47). We examined whether the hypophosphorylation of ZAP-70 in anergic DN cells is related to the less efficient phosphorylation of TCR-ζ chains upon TCR stimulation. Fig. 7 indicates the fairly equivalent level of expression of the TCR-ζ chain in anergic and nonanergic DN cells. Quantitation of p21 and p23 by densitometric analysis yielded the following results: percent decrease in p21 tyrosine phosphorylation in H-2<sup>bd</sup> 2C DN cells relative to H-2<sup>b</sup> 2C DN cells at 3, 10, and 15 min after TCR stimulation was 31%, 5%, and 37%, respectively; the corresponding values for p23 were decreases of 55%, 30%, and 50%, respectively. These data suggest that there is less p23 in anergic DN cells after TCR stimulation.

The reduced induction of p23, which represents fully phosphorylated TCR-ζ chains (48), may explain in part the less efficient recruitment and activation of ZAP-70 to the TCR-CD3 signaling complex in TCR-stimulated anergic DN cells.

Previous studies have shown that the protein expression level of Fyn is increased in anergic T cell clones (21). In this study, we showed that the protein expression level of Fyn, as determined by Western blot analysis, was increased in anergic DN cells (Fig. 8). Such an increase in expression was not observed for Lck (Fig. 8).

**Anergic DN cells have impaired mobilization of intracellular calcium**

One major consequence of LAT phosphorylation is the TCR-mediated activation of phospholipase C (PLC)-γ1 and the Ras pathway (49, 50). Having observed a defect in the phosphorylation of LAT in TCR-stimulated anergic DN cells, and since PLC-γ1 activation is required for the induction of a sustained increase in intracellular calcium, we examined the ability of these cells to mobilize intracellular calcium in response to TCR ligation. In this respect, the anergic DN cells were found to be inefficient in mobilizing intracellular calcium when compared with nonanergic DN cells (Fig. 9). This observation supports the notion that one consequence of defective LAT phosphorylation in anergic DN cells is a failure to mobilize intracellular calcium efficiently in response to TCR ligation.

**Normal phosphorylation of SLP-76 and ERK1/2 activation in TCR-stimulated anergic DN cells**

The adaptor molecule SLP-76, like LAT, is a linker molecule closely associated with proximal TCR signaling (reviewed in Ref. 51). SLP-76 also undergoes tyrosine phosphorylation upon TCR engagement and is also a substrate of ZAP-70 (52). SLP-76 has been shown to associate with the Src homology 3 domain of Grb2...
via proline-rich motifs and is essential for the coupling of TCR-regulated PTKs to downstream signaling pathways (53). Previous studies have also shown that tyrosine phosphorylation of PLC-γ1 and the Ras-signaling pathway are defective in SLP-76−/− T cells (52). The anergic DN cells exhibit efficient induction of CD69 (Fig. 3), which has been shown to be dependent on the Ras-signaling pathway (52). The anergic DN cells exhibit efficient induction of CD69, and the Ras-signaling pathway is defective in SLP-76−/− T cells. However, induction of calcium mobilization in TCR-stimulated anergic DN cells was shown to be defective (Fig. 9). To reconcile these findings, we hypothesize that LAT and SLP-76 phosphorylation occur normally in anergic DN cells. To test this hypothesis, we examined SLP-76 phosphorylation in anergic and nonanergic DN cells upon TCR ligation (Fig. 10). SLP-76 was immunoprecipitated from these two cell types before and after TCR stimulation. It was found that similar amounts of SLP-76 were precipitated from H-2b and H-2bd DN cells (Fig. 10). Quantitation of the data in Fig. 10 indicates that SLP-76 tyrosine phosphorylation in H-2bd 2C DN cells after TCR stimulation was decreased by 12% relative to H-2b 2C DN cells. This observation is consistent with the notion that phosphorylation of SLP-76 is less dependent on activated ZAP-70. Alternatively, it is conceivable that other uncharacterized pathways are responsible for SLP-76 phosphorylation in anergic DN cells.

Our observation of efficient CD69 induction in anergic DN cells after Ag stimulation (Fig. 3) suggest that the Ras-signaling pathway is intact in these cells. Since ERK1/2 phosphorylation and activation occurs downstream of the Ras-signaling pathway, we sought independent confirmation of activation of the Ras-signaling pathway by examining the phosphorylation of ERK1/2 in nonanergic and anergic DN cells upon TCR ligation. The results in Fig. 11 indicate that ERK1/2 phosphorylation occurs normally in anergic DN cells upon TCR stimulation. Quantitation of the data in Fig. 11 showed that the level of phosphorylation of ERK1/2 in H-2bd DN cells was within 10% of that observed in H-2b DN cells. These data are consistent with the hypothesis that the Ras-signaling pathway and ERK1/2 are activated normally in TCR-stimulated anergic DN cells.

Discussion

In this study, we have provided functional evidence for the lowering of activation threshold in a population of in vivo anergized T cells. We found that these anergic cells are able to express CD25 and CD69 and proliferate extensively when stimulated with a low-affinity ligand in the presence of an exogenous IL-2 source. In contrast, nonanergic T cells that expressed equivalent levels of the 2C TCR (16) were not activated by the low-affinity ligand. We have also provided biochemical analysis of these in vivo anergized T cells that was aimed at increasing our understanding of the inherent signaling defects of in vivo anergized T cells. Our results indicate that TCR-induced phosphorylation of LAT is deficient in these anergic T cells and that this may be due to their failure to fully activate ZAP-70 upon TCR ligation. The inefficient mobilization of intracellular calcium in TCR-stimulated anergic cells may be a consequence of deficient LAT phosphorylation. The defect in LAT phosphorylation is associated with an elevated basal level of the Fyn PTK. In contrast to defective LAT phosphorylation, SLP-76 phosphorylation occurs normally in TCR-stimulated anergic cells. The induction of CD69 in Ag-stimulated cells and the efficient phosphorylation of ERK1/2 MAP kinases also suggest that the Ras-signaling pathway is unaffected in these anergic T cells.

The biochemical changes observed in the anergic DN cells following TCR stimulation are remarkably similar to the biochemical phenotype of T cells that have been stimulated by partial agonists. TCR partial agonist ligands were shown to activate the Ras/MAP kinase pathway by an alternative pathway that is independent of ZAP-70 kinase activity (54) and LAT phosphorylation (55). In this model, Grb2-SOS complexes were found to associate with incompletely phosphorylated p21 phospho-TCR-ζ; this observation provides a possible explanation for the activation of the Ras/MAP kinase pathway in the absence of ZAP-70 activation and phosphorylation of LAT (55). Interestingly, we found that despite the
incomplete activation of all of the TCR-signaling pathways, these signals were sufficient to promote the induction of CD25 in anergic cells and support their proliferation in the presence of exogenous IL-2.

A critical defining characteristic of T cell anergy is an inability to produce IL-2 upon TCR ligation. We have shown previously that Ag-stimulated anergic DN cells are defective in IL-2 production (16). Distinct regulatory regions exist in the S′ promoter region of the IL-2 gene, and these distinct regions can be bound by varied nuclear factors to initiate IL-2 transcription. These factors include the AP-1 and NF-AT proteins. The AP-1 family of nuclear factors include members of the Fos and Jun families. Fos and Jun can bind DNA as the AP-1 complex and have been implicated in the control of IL-2 transcription. AP-1 dependent DNA binding and IL-2 gene transcription are deficient in some forms of anergy (18). In conjunction with AP-1, NF-AT is also involved in IL-2 transcription and can bind the IL-2 promoter at an NF-AT site when complexed with AP-1. In contrast to Fos and Jun, which are regulated by ERK and c-Jun NH2-terminal kinase activation (56, 57), NF-AT exists in the cytoplasm in an inactive phosphorylated form. TCR-mediated activation of calcium signaling leads to activation of calcineurin, a phosphatase able to dephosphorylate NF-ATp, allowing NF-AT to enter the nucleus and bind DNA in the presence of AP-1 (58). Thus, the nature of IL-2 transcriptional regulation is such that different mechanisms may exist to control the production of IL-2. As mentioned above, a failure to activate the AP-1 complex has been reported in many forms of anergy. As well, a failure to activate NF-AT has also been reported (20) and this form of anergy has been referred to as calcium-blocked anergy to contrast it from Ras-blocked anergy (reviewed in Ref. 17). Our findings are consistent with the form of anergy we have studied to be calcium blocked and not Ras blocked.

The failure of anergic DN cells to mobilize intracellular calcium upon TCR ligation is likely due to their inability to phosphorylate LAT, and this is most likely due to their failure to optimally activate ZAP-70 (Fig. 5). Similar reductions in phosphorylation of a 38-kDa molecule, possibly LAT, have previously been reported in anergic Th1 cells (59). LAT is phosphorylated by ZAP-70 upon TCR ligation, leading to recruitment of multiple signaling molecules that culminates in the activation of calcium- and Ras-dependent pathways (50). In this study, we have shown that whereas defective LAT phosphorylation affected calcium-dependent pathways in anergic DN cells, it does not seem to affect the Ras-signaling pathway in these cells.

We also observed efficient tyrosine phosphorylation of SLP-76 upon TCR ligation in the anergic cells (Fig. 9). Because SLP-76 is a substrate for ZAP-70 (52), this observation suggests that either suboptimally activated ZAP-70 is sufficient to phosphorylate SLP-76 but not LAT, or alternatively SLP-76 may be phosphorylated by other mechanisms that remain to be defined. Previous studies have shown that SLP-76 tyrosine phosphorylation is required for optimal SLP-76 function including Vav recruitment to SLP-76 (reviewed in Ref. 51). SLP-76-deficient Jurkat T cells exhibit a marked reduction in PLC-γ1 tyrosine phosphorylation, intracellular calcium mobilization, and ERK activation (52). Since ERK activation, but not calcium mobilization, is normal in TCR-stimulated anergic DN cells, these findings support the notion that phosphorylated SLP-76 alone, in the absence of phosphorylated LAT, may be sufficient to activate the Ras pathway. However, it is insufficient to activate intracellular calcium mobilization.

We have shown that the p23 form of the TCR-γ chain is phosphorylated to a lesser extent in TCR-stimulated anergic DN cells. It is unclear whether this is a consequence of the increased expression of Fyn relative to Lck. The less efficient phosphorylation of p23 may lead to less efficient recruitment and activation of ZAP-70. Alternatively, and/or in addition to this mechanism, the failure to fully activate ZAP-70 and hence phosphorylate LAT may be due to the presence or activation of a negative regulator of ZAP-70. Previously, constitutive association of Cbl with Fyn has been observed in anergic T cells (22). We found that anergic DN cells have an increased basal level of Fyn expression and this may lead to more efficient phosphorylation of Cbl. Recent studies have shown that Cbl acts as a negative regulator of ZAP-70 (23, 24). Furthermore, tyrosine phosphorylation of LAT and SLP-76 was also found to be sustained in TCR-stimulated Cbl−/− thymocytes (60, 61). It is therefore conceivable that the suboptimal phosphorylation of ZAP-70 in TCR-stimulated anergic DN cells is due in part to increased Fyn expression and recruitment of Cbl to the TCR-signaling complex. In this scenario, the hypophosphorylation of ZAP-70 is a consequence of negative regulation by Cbl. This possibility is currently under investigation.

The observation that anergic DN cells proliferated extensively in response to stimulation with a low-affinity ligand and an exogenous IL-2 source (Fig. 2) has important implications in the role of anergic T cells in the induction or regulation to anti-self-responses. This observation indicates that the anergic cells have a significantly lower activation threshold than their nonanergic counterparts. In this regard, the anergic DN cells behave like “memory” cells. The high expression level of CD44 and CD45RB on these cells is also consistent with the conclusion that they have been activated by Ag in vivo (16). However, the expansion of Ag-stimulated anergic DN cells is dependent on an exogenous IL-2 source. One can envision a scenario where the anergic DN cell and a normal T cell both bind to the same APC. The anergic DN cell would come in contact with IL-2 produced by the activated bystander T cell. This scenario could potentially lead to autoimmune consequences. We have begun to investigate this possibility and our preliminary experiments indicate that even after activation with Ag and IL-2, the activated anergic DN cells are unable to produce their own IL-2. Thus, the expansion of these activated DN cells is dependent on a constant supply of exogenous IL-2. This may limit the autoimmune potential of this population of anergic T cells. This consideration raises the interesting possibility that such a form of T cell anergy may serve to down-regulate anti-self-immune responses. One can envision a scenario whereby the anergic DN cell and a self-reactive conventional T cell bind to the same APC. The lower activation threshold of the DN cell will lead to more efficient induction of CD25 on the anergic DN cell and in this activated state the DN cell serves as a “sponge” to soak up IL-2 in the vicinity. We propose that competition for the limited amount of IL-2 will inhibit proliferation of the self-reactive T cells. Importantly, the anti-self-immune response mediated by the activated DN cells is self-limiting since its maintenance is dependent on exogenous sources of IL-2.

The anergic state that we have described for the DN cells differ from that described for conventional CD4 and CD8 T cells that have been anergized in vivo in that the latter is irreversible by the addition of exogenous sources of IL-2 (27, 62, 63). However, in vivo anergized CD4 T cells can also serve to down-regulate neighboring immune responses through the release of IL-10 (64). This observation further emphasized the potential importance of anergic T cells in the down-regulation of autoimmune responses. The relevance of this population of anergic DN cells in normal mice remains to be determined. It has been suggested that the αβ T cells in TCR-transgenic mice may result from the premature expression of the α and β TCR transgenes in the γδ lineage (65). Therefore, we need to entertain the possibility that γδ T cells may potentially

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provide a source of regulatory T cells for the prevention of autoimmunity. We hypothesize that these γδ T cells may perform this important function using mechanisms that are similar to the ones that we have proposed. This possibility is currently under investigation.

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References


