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Effects of a Constitutively Active Form of Calcineurin on T Cell Activation and Thymic Selection

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Calcineurin is a calcium/calmodulin-dependent phosphatase whose activity is required for the induction of T cell lymphokine production and proliferation. Although its specific role in T cell development is less well defined, studies with the immunosuppressive drugs cyclosporin A and FK-506 suggest that it is involved in both positive and negative selection of immature thymocytes. To more completely characterize a role for calcineurin in T cell development in vivo, we have generated transgenic mice that express an activated form of this enzyme in thymocytes and peripheral T cells. We find that the transgene causes a block in early thymic development, resulting in a reduction in the steady-state number of CD4 and CD8 double positives, but not on the number of mature T cells. We also find that thymocytes and mature T cells expressing this transgene are more sensitive to signals through their TCR. In thymocytes this sensitivity difference is manifested as an increase in positive selection, although negative selection seems to remain unaffected. Therefore, these studies confirm and extend past reports that suggested a role for calcineurin in thymic development and selection.


Upon triggering by specific Ag or Ab, the TCR/CD3 complex initiates a complex series of biochemical processes inside the cell, culminating in the various effector functions including lymphokine production, proliferation, and cytotoxicity (1–5). One of the many pathways known to be important for the induction of these processes is initiated by sustained increases in intracellular calcium, an ubiquitous second messenger (6, 7). In T cells, the increase in cytoplasmic calcium is thought to be accomplished mainly through the inducible activity of the phospholipase isoform PLC-γ (8, 9). Increased calcium concentration couples to a number of downstream pathways in the cell, notably the calcium/calmodulin-dependent (CaM)5 phosphatase calcineurin (10) and the CaM kinases (11, 12).

Calcineurin, or protein phosphatase 2B (P2b), is a ubiquitously expressed serine/threonine phosphatase. It consists of two subunits: the catalytic calcineurin A, a 59-kDa protein, and calcineurin B, a 19-kDa regulatory subunit. A role for calcineurin in T cell activation was discovered when the immunosuppressive compounds cyclosporin A (CsA) and FK-506 were found to inhibit calcium-dependent activation of T cells (13). This effect was traced to specific inhibition of calcineurin by a complex of CsA and an endogenous protein, cyclophilin, a peptidyl prolyl-cis-trans isomerase. FK-506 and its cellular cofactor FK-506 binding protein, which is also an isomerase, appear to inhibit calcineurin in a similar manner. In both cases, exquisite specificity for calcineurin is achieved as a result of an active site formed by the drug/isomerase complex (14). The role of calcineurin has also been substantiated by a constitutively active form of the enzyme that lacks the regulatory domain (15). When such a mutant was expressed in cultured T cells it bypassed the need for calcium elevation in T cell activation and IL-2 transcription. Studies by a number of groups have shown that induction of cytokine transcription depends upon the calcineurin-mediated nuclear transport and activation of the Rel-related family of transcription factors known as NF-AT (5, 16–18). As such, CsA potently inhibits the transcriptional activation of IL-2, IL-3, IL-4, and CD40 ligand.

As with mature T cell activation, T cell development in the thymus is dependent upon signals through the TCR/CD3 complex (3, 19). Such signals appear to regulate both the expansion of cells at the CD4+CD8− double-negative (DN) stage of development (20, 21), and the more extensively studied selection events at the CD4+CD8− double-positive (DP) stage itself (22–24). It is at the DP stage that interaction with peptide Ag/MHC complexes at high affinity leads to negative selection, while interaction with such complexes at low affinity promotes maturation or positive selection (25). Therefore, this step of thymocyte development produces mature T cells that are selected to recognize MHC molecules, but do not recognize self-MHC/peptide complexes so efficiently that they are likely to cause autoimmunity.

A number of groups have investigated the effects of the calcineurin inhibitors CsA and FK-506 on T cell development both in vivo and in vitro. Several studies have shown that CsA administration in vivo leads to partial, although not complete, rescue from deletion of DP thymocytes by the endogenous superantigens (SAgs) present in certain mouse strains (26–29). It has also been shown that CsA can partially inhibit negative selection of DP thymocytes in H-Y TCR-transgenic male mice (30). One study showed that in vitro deletion of thymocytes bearing a particular alloreactive TCR transgene was unaffected by CsA, while deletion of thymocytes bearing a different alloreactive receptor was enhanced (31). Similarly, we saw that the effect of CsA on deletion...
in culture depended on the nature of the TCR-Ag/MHC interaction. Deletion by a weak peptide analogue could be significantly inhibited by CsA, whereas deletion mediated by an antigenic peptide from pigeon cytochrome c (PCC) was not (32, 33). This suggests that calcineurin may not play a role in mediating negative selection because deletion of cells recognizing a high-affinity peptide is not affected by CsA, but calcineurin may play a role in positive selection because CsA can inhibit the selection effects of lower affinity peptides. This is consistent with experiments in which CsA or FK-506 were shown to inhibit thymocyte positive selection in vivo (26, 27, 29, 30). However, all of the in vivo studies had noted profound thymic hypotrophy after immunosuppressant treatment consistent with observed changes in the thymic stromal cells and a potential for disruption of Ag presentation (34, 35). With the exception of this caveat, experiments supporting the idea that CsA can inhibit positive selection seem to be consistent; however, the effects of CsA on negative selection remain controversial.

To better address the effects of calcineurin on developmental events in vivo, we generated transgenic mice that express a constitutively active calcineurin in their thymocytes and mature T cells. We found that calcineurin caused a block in early DN development, causing a decrease in the steady-state DP population but not in the mature T cell populations. Furthermore, we found that calcineurin can contribute to the efficiency of positive selection of DP thymocytes and the activation of mature T cells. Despite the increased efficiency of signaling through the TCR, under no conditions examined could we detect a conversion of positive selection to negative selection, nor could we detect an enhancement of negative selection.

**Materials and Methods**

**Transgenic constructs**

A 1.6-kb XhoI/BstX1 fragment containing the murine CD4 silencer was blunt-end cloned into the EcoRI site of pK5s (pK5s-sil). The truncated murine calcineurin mutant was removed from pSRα-296 by an EcoRI digest and cloned into the same site in pK5s-sil. A fragment containing the calcineurin cdNA and the silencer was isolated with NotI, and XhoI-linked. This fragment was then ligated into the XhoI site in pTex (36). The entire fragment for injection was isolated by digestion of C57Bl/6 genomic DNA with NotI.

In preparation for injection, transgene fragments were isolated from agarose gels with Qiaex beads (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Integrity of the DNA was verified on an agarose gel. Finally, DNA was diluted to 1.5 μg/ml in sterile injection buffer (5 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

**Mice**

Fragments were injected into CB6 F1 embryos. Founder mice were backcrossed to C57BL/6 mice for further analysis. Founder and F1 mice were also bred to AND TCR-transgenic mice (37, 38). All mice analyzed were rose gel. Finally, DNA was diluted to 1.5 μg/ml in sterile injection buffer (5 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

**Southern blots/PCR-typing of transgenes**

Founder mice were initially identified by Southern blot analysis of DNA isolated from tail biopsies. Tail pieces of ~0.5 cm in length were obtained from mice between 2- and 3-wk of age, and incubated for at least 8 h in buffer containing 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 100 mM NaCl, 0.5% SDS, and 0.5 mg/ml protease K. After incubation, the supernatant was extracted once with phenol/chloroform-isooamyl alcohol, and the DNA was precipitated by the addition of 1.5 vol of 100% ethanol. The DNA pellet was resuspended in 0.1 ml Tris-EDTA, and allowed to resolublize overnight at 4°C. A total of 20 μl of the DNA solution was digested with SstI and run on a 1% agarose gel. The gel was vacuum-transferred to a nylon membrane (Amersham, Arlington Heights, IL), and the DNA was UV crosslinked to the membrane, using a GS Gene Linker from Bio-Rad (Her- cules, CA). Subsequent calcineurin offspring of founder mice were identified by PCR typing of DNA from tail biopsies. The forward primer (TACTTTAGATGTGTACATATAACAGGT) is specific for a portion of murine calcineurin; the reverse primer (ATGGTATCAAGTGACAGTA-CACAC), hybridizes to the murine CD4 silencer. Therefore, this primer combination will only produce products from the transgenic construct.

**Northern blots**

Total RNA from thymocytes was isolated with the Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNA (20 μg) was run on a form- aldehyde-agarose gel and transferred by capillary blotting to nylon membrane. RNA was UV crosslinked to the membrane. Membranes were probed with fragments of truncated calcineurin. After exposure to O-MAT AR film (Eastman Kodak, Rochester, NY), the membrane was stripped by the addition of boiling 0.1% SDS and rocked for 7 min at room temperature. The membrane was reprobed with a fragment of the CD4 silencer and then GAPDH as a control for loading.

**Antibodies**

Directly conjugated Abs to CD4 (YTS 191.1) and CD8α (YTS 169.4) were obtained from Caltag (San Francisco, CA). Directly conjugated Abs to Vε11 (RR8-1) CD69 (1H2.2F3), CD3ε (145-2C11), CD5 (53-7.3), K θ (AF6-88.5), F-E (14-4-4S), Vβ6 (RR4-7), CD44 (IM7), and CD25 (7D4) were obtained from PharMingen (San Diego, CA).

**Flow cytometry**

Lymphocytes were resuspended to 10⁷/0.1 ml in PBS with 1% FCS and 0.1% sodium azide (staining buffer), and stained with 0.5 μg of each Ab, for 20 min at room temperature. Cells were washed with 1.5 ml of staining buffer and finally resuspended in 0.5 ml staining buffer. List mode data was collected on 15,000–20,000 events on a Becton Dickinson (Mountain View, CA) FACScan, using CellQuest software. Four-color analysis was performed on a Becton Dickinson FACScalibur, also using CellQuest software.

**Ab and complement treatment**

Cells were resuspended in appropriate Ab supernatant and Low-Tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), and the suspension was incubated at 37°C for 45 min. To eliminate CD8+ cells, 3.168.36 supernatant (anti-CD8) was used. RL172 or GK1.5 supernatant (anti-CD4) was used to eliminate CD4+ cells.

**RT-PCR**

Single cell suspensions were made from thymi. Enrichment of thymic subpopulations was done by treating with the appropriate Ab supernatant and Low-Tox rabbit complement for 45 min at 37°C. Next, complement-treated cells were stained with Abs to cell surface receptors and then sorted on a CellSorter (Becton Dickinson). A total of 250,000 cells from 7.8 × 10⁶ thymocytes harvested from one thymus were used. The cDNA was made by Qiagen Total RNA Prep Kit, according to the manufacturer’s instructions. The mRNA was DNase-treated, spun over a Qiagen column to purify the RNA, precipitated, and resuspended in 20 μl RNase-free water containing 5 μg/ml oligo-dT and 1 μg/ml plasmid ribonuclease inhibitor, RNAse (Promega, Madison, WI). The reaction was then incubated for 10 min at 65°C and chilled on ice. Next, the reaction was split into two tubes and 10 μl of a 2X RT mix was added. The 2X RT mix contained 2.5 μl RNasin, 2X reverse transcriptase buffer, 2 μM dNTPs, and 10 U avian myeloblastosis virus reverse transcriptase (Promega). Reactions were incubated for 45 min at 37°C, heat-inactivated 10 min at 85°C, and then quick chilled. The cDNA was titrated for use in the PCR. Primers and reaction conditions were described above and the following β-actin primers were used as a control: 3'CTCTTTGATGTCACGCACGAT and 5'GTGGGCCCCCTTCATAGGCAACAA.

**Luciferase assays**

pNF-AT luciferase (gift of the Weiss Lab, University of California, San Francisco, CA) was cotransfected into T Ag (TAg) Jurkats with plasmids containing the appropriate cDNA and incubated for 36 h at 37°C in 5% CO₂. Cells were then harvested and RT-PCR was performed on a Becton Dickinson FACScan, also using CellQuest software. mRNA expression and reaction conditions were described above and the following β-actin primers were used as a control: 3'CTCTTTGATGTCACGCACGAT and 5'GTGGGCCCCCTTCATAGGCAACAA.
**Proliferation and deletion assays**

Single cell suspensions were made from the thymi or spleens of calcineurin mice or nontransgenic littermates. RBC were lysed by osmotic shock. Cells were washed and resuspended in Eagle Hank’s amino acid medium plus 10% FCS, and aliquoted at 10^5/well in a 96-well plate in the absence of exogenous APC. When mice on a normal background were used, CD8^- T cells were first eliminated by treatment with anti-CD8 Ab (3.168.32 culture supernatant) and Low-Tox rabbit complement (Cedarlane Laboratories). In all cases, cells were cultured 48 h, before an overnight pulse with 1 mCi/well [3 H]thymidine. In vitro thymocyte deletion cultures were conducted exactly as described previously (32, 33, 39).

**Phosphatase assay**

Twenty-five million thymocytes were depleted of all RBC by osmotic shock. Cells were washed in PBS, lysed in 50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM CaCl₂, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM DTT, and subjected to three rounds of freeze and thaw. Protein concentrations were determined by Bradford reagent (Bio-Rad) and then equilibrated. Phosphatase assay was performed as described previously (40, 41).

**Results**

**Generation of transgenic mice**

The cDNA encoding a mutant form of the calcineurin catalytic subunit (15), in which the calmodulin binding domain and the autoinhibitory domain have been deleted (P2bΔ; Fig. 1A), was subcloned into the pTexSil expression vector. Two founders were obtained and were bred to C57BL/6 mice to generate offspring for further analysis. Expression of the calcineurin transgene was determined by Northern blot analysis of total RNA from unseparated thymocytes of first generation mice. One of the lines expressed the transgene (Fig. 1B) and was used for further analysis. To further characterize expression of the transgene, thymocyte subsets were sorted by FACS according to surface staining for CD3, CD4, and CD8. From these sorted cells, RNA was isolated and subjected to RT-PCR analysis (Fig. 1C). All four thymic subsets were found to express the transgene, suggesting that in this founder line, the CD4 silencer did not function. Therefore, mice were examined for effects of the P2bΔ transgene on both early and late stages of thymic development.

We sought to verify the functional activity of the P2bΔ transgene by examining its effect on T cell activation. P2bΔ-transgenic mice were bred to AND TCR-transgenic mice and T cells from AND and AND;P2bΔ littermates were compared for sensitivity to activation by pharmacological agents or Ag. Consistent with cell transfection experiments (15), splenocytes from the double-transgenic mice were sensitive to stimulation with PMA alone, as determined by proliferation (Fig. 2A). The level of stimulation with PMA alone was ~30% of the maximum stimulation achieved with PMA and ionomycin for either AND or AND;P2bΔ mice. Splenocytes from AND;P2bΔ mice were also somewhat more sensitive to stimulation by peptide Ag, as shown by a 2- to 3-fold shift in the dose-response curves and an increase in the maximum response (Fig. 2B). These data indicate that the P2bΔ transgene is sufficient to confer calcium-independent T cell activation and enhance the sensitivity of Ag-mediated activation.

**Phosphatase activity of the P2bΔ transgene**

To quantitate the increase in basal level of phosphatase activity due to the transgene, an in vitro phosphatase assay, measuring serine dephosphorylation of the regulatory subunit of PKA, was used (14, 40, 41). As a control for the activity of the P2bΔ transgene on both early and late stages of thymic development.

**FIGURE 1.** Transgenic constructs and expression in transgenic animals. A, Schematic representation of the murine calcineurin A-chain showing the truncated P2bΔ cDNA. The P2bΔ cDNA includes coding sequence for the catalytic domain and the B subunit binding domain, whereas the calmodulin binding domain and the autoinhibitory domain have been deleted. B, Total RNA was extracted from thymocytes from a P2bΔ-transgenic mouse or normal littermate control (NLC). After electrophoresis and transfer to nylon, this blot was probed with DNA fragments complementary to murine calcineurin (P2bΔ), which hybridizes to both endogenous and transgenic message; the CD4 silencer, which hybridizes to both endogenous and transgenic message; and GAPDH, as a control for loading. C, Thymocytes were stained with directly-conjugated anti-CD3 and anti-CD8 and sorted for DN, DP, CD4SP, and CD8SP cells. Total RNA was isolated from each of the sorted populations. PCR using primers specific for the transgene was performed on dilutions of cDNA to determine whether the transgene was being transcribed in each subset, β-actin primers were used as a control. 3′UTR. To control for DNA contamination, PCR was completed on tubes that received no reverse transcriptase.

**FIGURE 2.** Mature T cells from AND;P2bΔ-transgenic mice respond to PMA alone. Splenocytes from H-2bΔ mice were stimulated with PMA in the presence or absence of ionomycin (A) or moth cytochrome c peptide (B). Results are the average cpm (± SD) of triplicate wells from one experiment. A, Cells were stimulated with 2 or 20 ng/ml PMA or 2 ng/ml PMA plus 500 nM ionomycin.
the calcineurin B-chain subunit, the P2bΔ (A-chain) subunit, or both. Cells were divided and half were used to measure luciferase activity as an indicator of NF-AT function, and half were examined for phosphatase activity. The luciferase assay (Fig. 3A) confirmed that P2bΔ can synergize with PMA to cause IL-2 promoter activation in the absence of the induced release of calcium. Furthermore, the activation was suppressed by cyclosporin A. In addition, the luciferase activity was higher when both the constitutively active A subunit and the B subunit were cotransfected. This suggests that the endogenous B subunit is limiting to some extent under these conditions (40, 42–44).

Phosphastase activity was determined in the presence of calcium or the absence of added calcium along with EGTA to chelate cellular calcium. The effects of removing calcium from the in vitro assay are 2-fold. One effect is to eliminate the calcium calmodulin activation of endogenous calcineurin, but a second effect is to inhibit the actual enzymatic phosphatase reaction. In the presence of calcium we found a small increase in phosphatase activity mediated by P2bΔ alone, but a much more significant increase with both the P2bΔ and calcineurin B subunits. This added effect of the B subunit was not seen in the absence of calcium. For comparison, CsA was added as an indication of the calcineurin-independent background of the assay.

Similar experiments were conducted with transgenic thymocytes to correlate the functional response to the corresponding change in the level of phosphatase activity. Thymocytes from transgenic mice and nontransgenic littermates were divided and subjected to proliferation and phosphatase assays. Fig. 3 shows the results of a proliferation assay employing thymocytes from a calcineurin-transgenic mouse or nontransgenic littermate. Although the effect was not as dramatic as that found for splenocytes (Fig. 2), P2bΔ-transgenic thymocytes were sensitive to stimulation by PMA alone. The two populations of cells showed equivalent responses to stimulation with PMA and ionomycin (Fig. 3). Furthermore, a 2- to 3-fold increase in proliferative response to PMA alone (Fig. 3C) is tied to a slight increase in the level of phosphatase activity (Fig. 3D). Although the increase was small it was reproduced in five experiments. Addition of EGTA revealed an increase in the level of calcium-independent activity of the transgenic cells over the littermate level, and the addition of CsA once again shows the calcineurin-independent background of the assay. Therefore, the constitutively active calcineurin expressed in the transgenic thymocytes caused a detectable increase in phosphatase activity that apparently synergizes with PMA to allow cells to proliferate without the need for an increase in intracellular calcium.

We have confirmed that the transgene is producing a constitutively active calcineurin phosphatase, it was found to be expressed in all thymic subsets, and it is sufficient to allow calcium-independent activation of mature T cells. Importantly, the low level of activity allows us to investigate the role of calcineurin in development without a general disruption of cellular physiology. As shown below, higher expression would be predicted to eliminate the thymus entirely. Therefore, the P2bΔ mice were analyzed for changes in development of DN cells and positive and negative selection of DP thymocytes.

Thymic development

Thymocytes, splenocytes, and lymph node cells were isolated from P2bΔ-transgenic mice and nontransgenic littermates and stained with Abs to various cell surface markers. As shown in Fig. 4A, the percentage of mature T cell populations was increased in the thymus and spleen of P2bΔ-transgenic mice. In the thymus, this increase also coincided with an increase in the percentage of CD69high and CD3high cells (Fig. 4), indicating an increase in the proportion of cells being signaled through their TCR (46). When thymocytes were isolated from P2bΔ-transgenic mice, it was also noted that the size of the thymus appeared to be significantly smaller than in nontransgenic littermates. Total numbers of thymocyte subsets from individual 4- to 6-wk-old mice were enumerated (Fig. 4B).

**FIGURE 3.** P2bΔ transactivates the NF-AT promoter in synergy with PMA. A, TAg Jurkat cells were transfected with 20 μg NF-AT luciferase alone (■), 5 μg pCMV-Cn B (□), 5 μg pSRo-P2bΔ (■), or 5 μg pCMV-Cn B and 5 μg pSRo-P2bΔ (□) and incubated for 36 h. Cells were harvested and then incubated 7 h with indicated treatments. Cells were then lysed and assayed for luciferase activity. Treatment concentrations were as follows: 5 ng/ml PMA, 0.1 μg/ml calcium ionophore A23187, or 1.5 nM CsA. RLU, Relative light units. B, Transfected TAg Jurkat cells described above were also assayed for phosphatase activity in the presence or absence of 1 mM EGTA or 0.5 nM CsA. Reactions containing EGTA/CsA and CsA alone were not performed for cells transfected with the Cn B subunit alone. C, Thymocytes were treated with the indicated concentrations of PMA for 3 days. Each bar represents the average cpm for three wells (± SD) of one experiment. Responses with 5 ng/ml PMA and 500 nM ionomycin were 456,276 (± 32,510) for NLC and 362,817 (± 31,902) for P2bΔ. Data is representative of five experiments. D, P2bΔ thymocytes and NLC cells were assayed for calcineurin phosphatase activity in the presence or absence of 1 mM EGTA or 0.5 nM CsA. Data are representative of three experiments.
CD3 high represented 11.1% NLC and 20.0% bers are reduced by about 60% in scatter. Thymocytes and splenocytes from affected (Fig. 4) contrary to what might be predicted in the face of negative selection. First, the absolute numbers of mature CD4SP and CD8SP thymocytes were not correspondingly increased in negative selection. However, several additional observations are not consistent with a decrease in cell death, but rather it is a result of a partial arrest in DN development. This could be due to the increased signaling through calcineurin that in some way diminishes expansion, or it could be indirect due to a feedback of excess positive selection (47). To examine this issue, we looked at the progression of development in embryonic thymuses. At embryonic day 18, before the appearance of CD4SP or CD8SP there was already an apparent decrease in overall cellularity, an increase in CD4+CD25+ cells, and a decrease in CD4+CD25− cells (data not shown). Because a population of positively selected cells had not yet appeared, we propose that the P2bΔ transgene directly affects the rate of progression through the CD4+CD25+ stage of maturation.

**The effect of activated calcineurin on negative selection**

To examine the effect of the P2bΔ transgene on negative selection, we investigated three experimental models; in vivo negative selection in TCR-transgenic mice, age-dependent progression of the SAg-mediated Vβ6-positive T cell deletion, and the apoptosis of cultured thymocytes mediated by the presentation of peptide Ags. We bred P2bΔ mice to AND TCR-transgenic mice in which the cells express a receptor specific for PCC in association with H-2Eβ. The TCR expressed by the majority of T cells consists of Vβ3 and Vα11 (37). Previous studies have determined that no selection occurs in H-2d or H-2m mice, positive selection occurs in H-2Aβ mice, and a dominant, late-stage negative selection occurs in mice that express H-2Aα (32, 38). Mice were bred to express the two transgenes and different MHC haplotypes to determine the effects of the P2bΔ transgene on positive and negative selection.

In the first model we assessed AND;P2bΔ mice that expressed H-2b/+. In these mice, we have shown that CD4− development is blocked such that there are less than 10% CD4SP thymocytes and 3% CD4−Vα11high thymocytes (Fig. 5), whereas H-2b or H-2bi mouse have 20–40% CD4− cells (32). The negative selection in this model occurs at the DP−SP transition because there is no other selection in the DP cells (data not shown). Second, to demonstrate that thymocyte development is blocked such that there is no selection in the DP cells (data not shown). Third, the proportion and number of T cells in the peripheral lymphoid organs was not significantly diminished in P2bΔ-transgenic mice. The alternative was addressed by analyzing thymic DN cells to determine whether there was a halt in DN development.

According to the expression of CD25 and CD44 surface molecules, there is a maturational sequence that divides the DN cells into four distinct subpopulations. Thymic precursors enter the thymus and are reduced by about 60% in scatter. CD69 (FL-3 channel) of live cells gated by forward light scatter vs side light scatter. Shown are two parameter dot plots of CD4 (FL-2 channel) vs CD8.

**FIGURE 4.** Cell subset analysis of thymocytes and splenocytes from P2bΔ-transgenic mice. A. Thymocytes and splenocytes were stained directly with conjugated Abs to CD4, CD8, and either CD69 or CD3ε (2C11). Shown are two parameter dot plots of CD4 (FL-2 channel) vs CD8 (FL-3 channel) of live cells gated by forward light scatter vs side light scatter. The CD69− cells represented 13.1% NLC and 17.2% bers. The CD3ε− cells represented 11.1% NLC and 20.0% P2bΔ. B. Thymocyte subsets were enumerated for P2bΔ and NLC. Each circle represents one mouse. C, DN cells from a nontransgenic littermate (−/−), hemizygous (+/−), or homozygous (+/+) transgenic mouse were isolated and stained with directly conjugated Abs to CD44 and CD25. Percentages of cells falling in each quadrant are shown.

Compared with nontransgenic littermates, total thymocyte numbers are reduced by about 60% in P2bΔ hemizygous mice and nearly 90% in homozygous mice (Fig. 4). Nearly all of the decrease was accounted for in the DP population (Fig. 4B). We considered two possibilities to explain the decrease in cell numbers: there is an inhibition in cellular expansion and differentiation in early thymocyte development, or there is an increase in cell death that may be associated with negative selection at the DP stage. However, several additional observations are not consistent with an increase in negative selection. First, the absolute numbers of mature CD4SP and CD8SP thymocytes were not correspondingly affected (Fig. 4B) contrary to what might be predicted in the face of a more stringent negative selection. Second, attempts to demonstrate increased thymocyte cell death by the use of in situ TUNEL staining revealed no detectable increase in apoptotic cells (data not shown). Third, the proportion and number of T cells in the peripheral lymphoid organs was not significantly diminished in P2bΔ-transgenic mice. The alternative was addressed by analyzing thymic DN cells to determine whether there was a halt in DN development.

According to the expression of CD25 and CD44 surface molecules, there is a maturational sequence that divides the DN cells into four distinct subpopulations. Thymic precursors enter the thymus as CD4loCD44+CD25− cells, called early lymphoid progenitor cells. As cells mature, they down-regulate CD4 and are called triple negative because they have no CD4, CD8, or TCR on their cell surface. These cells begin to undergo TCR-β chain rearrangement and β-selection (reviewed in Ref. 20), coupled with the regulated expression of CD44 and CD25. The precursor cells progress from CD44−CD25− → CD44+CD25+ (pro-T cell) → CD44+CD25+ (early pre-T cell) → CD44+CD25− DN (late pre-T cell). The CD44+CD25− cells begin to up-regulate the CD4 and CD8 coreceptors as well as intermediate levels of the TCR. Flow cytometric analyses of DN thymocytes revealed an apparent DN cell arrest at the CD44−CD25− stage of development, where 65% of the DN cells were CD44−CD25− vs 35% in nontransgenic littermates (Fig. 4C). This effect was enhanced in mice homozygous for the transgene where 74% of the DN cells were found to be CD44+CD25−.

These observations suggest that the decrease in the size of the DP thymocyte population does not appear to result from a large increase in cell death, but rather it is a result of a partial arrest in DN development. This could be due to the increased signaling through calcineurin that in some way diminishes expansion, or it could be indirect due to a feedback of excess positive selection (47). To examine this issue, we looked at the progression of development in embryonic thymuses. At embryonic day 18, before the appearance of CD4SP or CD8SP there was already an apparent decrease in overall cellularity, an increase in CD4−CD25+ cells, and a decrease in CD4+CD25− cells (data not shown). Because a population of positively selected cells had not yet appeared, we propose that the P2bΔ transgene directly affects the rate of progression through the CD4+CD25+ stage of maturation.
FIGURE 5. No increase in negative selection in AND-H-2AΔm mice. Cells from the thymus and lymph nodes (LN) of AND;P2bΔ double-transgenic mice of MHC haplotype H-2^b/k were examined for reactive cells (CD4^+Vα11^+). The total cellularity from thymus and LN (A), the percentage of cells that stain CD4^+ (B), and the percentage of cells that are CD4^+Vα11^+bc (C) were determined (NLC; P2b; P2bΔ). This comparison is representative of a total of four AND and four AND;P2bΔ mice analyzed on three separate days.

mouse mammary tumor virus 7. In a normal D1.LP mouse, deletion of SAg-reactive, Vβ6-bearing T cells is incomplete but progressive with age (48). Even in adult mice, the Vβ6 deletion is not as high as that seen in DBA-2 mice. This is presumably due to a requirement for H-2E molecules for optimum mouse mammary tumor virus 7 SAg presentation. In these analyses we did not see a P2bΔ-dependent difference in the number of mature Vβ6^+CD4^+ cells in the thymus or periphery, suggesting again that the transgene did not enhance the negative selection of these cells (Fig. 6).

In a third model, we examined the Ag-dependent deletion of thymocytes in culture (32, 33, 39). As a background for these studies, we previously showed that the calcineurin-specific inhibitor, CsA, inhibited deletion mediated by an altered peptide, cytochrome c fragment 88–104 (K99A), but not deletion mediated by cytochrome c peptide itself (PCC). Both of these peptides cause calcium mobilization and cell death, although only the antigenic peptide is capable of activating mature T cells (33, 39). Therefore, we were interested in whether or not thymocytes from AND;P2bΔ-transgenic mice were more sensitive to this form of negative selection, and whether P2bΔ would confer a differential sensitivity to the altered peptide vs the antigenic peptide. Depicted in Fig. 7 are the results of three such experiments. Deletion caused by K99A was virtually unchanged in AND;P2bΔ as compared with AND controls. There was a trend toward increased deletion using PCC, though the variability was such that there was no statistically significant difference. Certainly, there was no preferential sensitivity of AND;P2bΔ mice to peptide K99A over PCC peptide, as we would have predicted from the effects of CsA deletion on in vitro negative selection. Finally, it is likely that deletion mediated by PCC, but not K99A, is mediated in part by the cytokines secreted by the mature thymocytes (49, 50). Thus, we do not view the differences between AND and AND;P2bΔ in the response to PCC to reflect a meaningful effect on physiological negative selection. We conclude from an analysis of three experimental models that an active form of calcineurin does not enhance negative selection.

The effect of calcineurin on positive selection

Using the AND-transgenic mice, we also wanted to determine the effect of the P2bΔ transgene on positive selection. As described above, previous studies have determined that this transgenic TCR is not selected in the presence of H-2^d or H-2^q, whereas there is a large population of Vα11^+Vβ3^+CD4^+ thymocytes that develop in the presence of H-2^d (32, 38). Presented in Fig. 8 is a representative comparison of the steady-state cell thymus populations under two conditions that promote positive selection. If the percentage of CD4SP thymocytes is used as a measure of positive selection, one copy of H-2Ab present in H-2^b/q mice is suboptimal when compared with the selection in H-2^b mice (Fig. 8A). As shown in this experiment, the presence of the P2bΔ transgene correlates with a substantial increase in the CD4 SP population, and thus positive selection in the presence of H-2^b/h or H-2^b/d but not H-2^b.

In contrast to non-TCR-transgenic mice (Fig. 3B), overall thymus size was not consistently different between AND and AND; P2bΔ mice (Fig. 8B, upper panels), although there was increased variability in H-2^b/d mice. The accumulation of data for the percentage of CD4SP thymocytes showed that indeed there was a significant enhancement of positive selection in H-2^b/d and H-2^b/q mice. In H-2^b/d mice the average number of CD4SP thymocytes increased from 21% to 45%. We note that there was no enhancement of positive selection in H-2^b mice by this measure (Fig. 8B). If anything, there was a small, although probably not significant, decrease in the number of CD4SP cells. We also noted that the increased efficiency of positive selection in H-2^b/h and H-2^b/d mice was manifest in the periphery by an increased percentage of CD4^+Vα11^+ T cells in the lymph nodes (data not shown). These
mice (H-2 b/q and H-2 b/d) are significant (3). For H-2 b thymocytes, different differentiation of MHC class II positive DP thymocytes.

AND, P2b

mean. SDs were as follows: 1) for H-2 b/q thymocytes (left panels), or H-2 b/d (middle panels), or H-2 b (right panels) were stained and analyzed as indicated. The percentages of CD4+ thymocytes from AND;P2bΔ vs AND mice (H-2 b/q and H-2 b/d) are significant (p < 0.01) as determined by Student’s t test. B, Quantitation of total thymocytes and relative number of CD4+ thymocytes in AND;P2bΔ-transgenic mice. Total thymocytes from AND or AND;P2bΔ-transgenic mice of MHC haplotype H-2 b/q (left panels), H-2 b/d (middle panels), or H-2 b (right panels) were counted and the percentage of CD4+ thymocytes was determined by FACS analysis. Each dot represents one mouse. The black bar in each plot represents the mean. SDs were as follows: 1) for H-2 b/q thymocytes (× 10^7), ± 3.8 AND, ± 1.2 P2bΔ; %CD4, ± 7.0 AND, ± 6.5 P2bΔ; 2) for H-2 b/d thymocytes (× 10^7), ± 1.4 AND, ± 3.5 P2bΔ; %CD4, ± 10.4 AND, ± 9.2 P2bΔ; and 3) for H-2 b thymocytes (× 10^7), ± 1.2 AND, ± 1.2 P2bΔ; %CD4, ± 3.9 AND, ± 2.4 P2bΔ.

experiments suggest that under conditions of suboptimal positive selection, the P2bΔ transgene can enhance the selection and differentiation of MHC class II positive DP thymocytes.

Discussion

The purpose for conducting these studies was to determine how the calcineurin (P2bΔ) phosphatase affects T cell development and activation, and to resolve the inconsistent data obtained using the calcineurin inhibitors CsA and FK-506. Although studies on T cell activation events in mature cells have been conducted using calcineurin inhibitors (15, 51–54) as well as calcineurin overexpression (15, 55, 56), only the former approach had been used to study the potential roles of calcineurin in T cell development. Not only were the results from studies using immunosuppressants to look at calcineurin’s role in thymic negative selection inconsistent, the interpretation of the results was clouded by the effects CsA or FK-506 have on thymic architecture and the differentiation of CD4SP and CD8SP thymocytes. A genetic approach, presented in this report, allowed us to determine the effects of calcineurin activation in a cell-autonomous manner.

Despite the relatively low level of expression of the P2bΔ-transgenic mice, for the purposes of these studies it appears to be optimal. The homozygous transgenic mice have a profound deficiency in thymic expansion, and thus any further increase in expression would abrogate thymic development entirely. In the founder line examined in this report, we could examine selection in the heterozygotes and homozygotes and thus see a dosage effect of this form of calcineurin. One unexpected finding was that the CD4 silencer did not appear to restrict expression in the way we had previously found for a test transgene with identical control elements (A. Wurster and L.P. Kane, unpublished observations).

Although this early expression of calcineurin did have the effect of inhibiting progression through the CD4+ CD25+ stage in otherwise wild-type mice, it did not seem to diminish expansion in AND-H-2b+/D-2+/b/-transgenic mice. We were thus able to examine its effect on positive and negative selection.

We did not find evidence for the participation of calcineurin in negative selection. We were not able to detect any increase in apoptotic thymocytes in thymic sections, although if there was a subtle increase in the rate of apoptosis associated with negative selection it might be difficult to detect by a TUNEL. We have been successful in using TUNEL to detect negative selection in TCR and PCC double-transgenic mice (57), but in this case a large fraction of the cells died due to the recognition of a “self” Ag. Nonetheless, the rate of macrophage engulfment of dying thymocytes is very high (58), so a negative result with TUNEL may not be meaningful. Another prediction is that if there was a considerable increase in negative selection at the DP stage, this should have resulted in a significant decrease in the number of mature, CD4+ and CD8+ thymocytes and T cells. Again, we did not observe a significant decrease in the number of mature thymocytes, nor did we observe a decrease in the proportion of T cells in the peripheral lymphoid organs. In fact, we consistently saw an increase in the proportion of mature thymocytes and T cells in the spleen and lymph nodes.

To further clarify the effects of the active phosphatase on negative selection, we measured the number of mature CD4SP thymocytes in AND TCR-transgenic mice that expressed H-2 b/d. Because deletion is not complete in these mice, we should be able to detect an enhancement of negative selection by a loss of CD4+Vα11 positive cells. In contrast, the representative experiment presented in Fig. 5 shows an increase in the percentage of Vα11 cells that survive, supporting the idea that calcineurin does not enhance negative selection and may instead aid in positive selection and survival. Further evidence against a role for calcineurin in negative selection was provided by examining a model of SAg-mediated deletion. We specifically choose a model in which deletion is suboptimal so that an enhanced negative selection could be detected. In a comparison of 17 mice equally distributed between P2bΔ+/− and P2bΔ−/− genotypes, there was no difference in the age-dependent SAg-mediated thymic deletion. If calcineurin was involved in this type of negative selection, we would expect that the number of Vα11−/− SAg-reactive cells surviving would be diminished compared with the nontransgenic controls.

In contrast to the lack of evidence for an effect on negative selection, the analysis indicated that the P2bΔ transgene promoted positive selection. Thymocytes expressing high levels of CD5, CD69, and CD3, putative indicators of positive selection, were
always present in increased proportions. This observation is consistent with the increased positive selection seen in AND;P2bΔ double-transgenic mice expressing either H-2β2d or H-2β2d (Fig. 8). Although there was considerable variability in the efficiency of positive selection when the data from different experiments were pooled, we consistently noted a higher percentage of CD4+ thymocytes in AND;P2bΔ double-transgenic mice, when compared with AND littermates. We were also able to observe an increase in the percentage of CD4+ T cells in the peripheral lymphoid organs. However, we did not observe an increase in steady state numbers of CD4+ thymocytes when we examined AND;P2bΔ mice of MHC haplotype H-2b. We interpret this result to show that there is not a general increase in the survival of CD4SP thymocytes, but rather there is an increase in the efficiency of suboptimal positive selection. Thus, we did not see evidence for the conversion of a strong positive selection signal in H-2b mice to negative selection.

The possibility exists that the P2bΔ transgene affects negative selection, but the magnitude of the effect is too small to measure. Because we can easily measure effects of the transgene on PMA-induced proliferation, Ag-induced proliferation, and positive selection, at the least we can say that calcineurin is substantially more important for the signal transduction involved in these responses as compared with negative selection. Based on these results we argue that negative selection is not simply an increase in the signaling associated with positive selection. This interpretation is consistent with studies conducted using FK-506 (59). In vivo, FK-506 treatment blocked the development of mature SP thymocytes as well as the induction of CD69 expression, two indicators of positive selection. However, no apparent effect on negative selection was found when looking at deletion due to a negative selection in TCR-transgenic mice or when analyzing deletion mediated by SAGs. This concept that positive and negative selection use distinct signaling pathways has been suggested by work on other thymic selection signaling pathways as well. Loss and gain-of-function genetic studies indicate that the mitogen-activated protein (MAP) kinase pathway preferentially affects positive selection (60–62), whereas the use of MAP kinase inhibitors seemed to result in the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. 63:1045.

Conversely, dominant-negative forms of nur77 orphan steroid receptor in thymocytes suppressed negative selection without affecting positive selection (64). These examples support the idea that positive and negative selection can emanate from distinct signaling pathways.

With these mice, we were also able to confirm, for the first time with naive T cells, that a constitutively active calcineurin is sufficient to synergize with a suboptimal concentration of 2 ng/ml FK-506 to induce cellular proliferation. Significantly, we also saw an increased sensitivity to antigenic stimulation. These experiments show that the major (if not the only) calcium-dependent enzyme required for T cell proliferation is calcineurin. This is consistent with the observation that calcineurin is the predominant calmodulin-binding protein in T cells (65). In all cases the mature T cells from calcineurin-transgenic mice appeared phenotypically normal as determined by FACs analysis of a number of different markers, such as CD3, CD4, CD8, and CD69. Thus, the increased proliferation of calcineurin-transgenic T cells in response to PMA or Ag (Fig. 8) is most likely attributable to calcineurin itself and is not a by-product of aberrant selection processes.

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