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Dissection of thymocyte signaling pathways by *in vivo* expression of pertussis toxin ADP-ribosyltransferase

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Stimulation of the T lymphocyte antigen receptor–CD3 complex (TCR–CD3) causes T cell activation by a process associated with increased phosphatidylinositol-specific phospholipase C (PI-PLC) activity. Evidence exists suggesting that GTP-binding (G) proteins, particularly the pertussis toxin (PT)-sensitive G_i proteins, participate in this signal transduction pathway. To clarify the role of G_i proteins in TCR–CD3 signaling, and to investigate other possible functions of G_i molecules in T cells, we expressed the S1 subunit of PT in the thymocytes of transgenic mice using the lymphocyte-specific *lck* promoter. Transgenic thymocytes contained S1 activity and exhibited profound depletion of G_i protein PT substrates in a manner suggesting their inactivation by S1 *in vivo*. Nevertheless, treatment of transgenic thymocytes with mitogenic stimuli provoked normal increases in intracellular free Ca^{2+} concentrations and IL-2 secretion, indicating that G_i proteins are not required for T cell activation. These normal signaling responses notwithstanding, mature thymocytes accumulated in *lck*-PT mice and did not appear in secondary lymphoid organs or in the circulation. Viewed in the context of the known features of *Bordetella pertussis* infection, our results suggest that a PT-sensitive signaling process, probably involving G_i proteins, regulates thymocyte emigration.

Key words: G protein/pertussis toxin/T cell antigen receptor/T lymphocyte/signal transduction.

Introduction

T lymphocytes bear on their surfaces heterodimeric receptor structures that permit recognition of foreign antigens (reviewed in Davis and Bjorkman, 1988). T cell antigen receptor (TCR)-binding of cognate ligand provokes a stereotyped sequence of biochemical events culminating in lymphokine production, cell replication, and the acquisition of a mature effector (cytotoxic or helper) phenotype. The mechanisms responsible for signal transduction from the TCR are enigmatic, but are believed to involve interaction of the TCR with five additional membrane-spanning polypeptides: the γ, δ, ϵ, η, and ζ chains of the CD3 complex (Clevers *et al.*, 1988; Sussman *et al.*, 1988). In response to TCR–CD3 stimulation, the vast majority of mature and immature T cells exhibit increased phosphatidylinositol-specific phospholipase C (PI-PLC) activity leading to accumulation of inositol-1,4,5-trisphosphate and diacylglycerol (reviewed in Weiss *et al.*, 1986). These messengers mediate release of intracellular Ca^{2+} stores and activation of protein kinase C, respectively. Such signaling pathways underlie hormone responses in a variety of cell types (reviewed in Berridge, 1987). In many instances, coupling of the hormone receptor to PI-PLC appears to require a guanine nucleotide binding (G) protein, often a G_i-protein (Fain *et al.*, 1988; Ashkenazi *et al.*, 1989). These and other results implicate G_i proteins in signal transduction from the TCR–CD3 complex (reviewed in Linch *et al.*, 1987).

Like all G proteins, G_i proteins transmit signals from associated cell-surface receptors to specific intracellular effector enzymes by a cycle of GTP binding and hydrolysis (reviewed in Gilman, 1987). In general, the receptor and effector specificities of each G protein are defined by its α subunit, which binds guanine nucleotides and interacts with the effector enzyme system in its activated, GTP-bound form. The homologous α subunits, α_{i1}, α_{i2}, and α_{i3}, define three distinct G_i proteins (Beals *et al.*, 1987; Jones *et al.*, 1987), two of which (α_{i2} and α_{i3}) are present in lymphocytes (Beals *et al.*, 1987; Kim *et al.*, 1988; unpublished results).

G_i proteins are substrates for the S1 subunit mono-ADP-ribosyltransferase of pertussis toxin (PT), the etiological agent of *Bordetella pertussis*. PT modification of a specific cysteine residue in the α subunits of G_i proteins destroys their signal transduction function by preventing guanine nucleotide exchange. PT is therefore an extremely useful reagent for delineating signal transduction pathways dependent on G_i proteins, and has been used to define a role for G_i in receptor-stimulated PI-PLC activation in many cell types (Fain *et al.*, 1988). Such studies also implicate G_i proteins in muscarinic inhibition of adenylyl cyclase (Ashkenazi *et al.*, 1989) and in muscarinic regulation of cardiac atrial K^+ conductance (Yatani *et al.*, 1988).

Similarly, much of the evidence favoring the involvement of G_i proteins in T cell signal transduction derives from studies using PT to block G_i function, although studies using permeabilized T cells (Schrezenmeier *et al.*, 1988) or aluminum fluoride (Ledbetter *et al.*, 1987; O'Shea *et al.*, 1987) also suggest that G proteins regulate T cell behavior. For example, PT application abrogates interleukin-2 (IL-2) secretion from a murine hybridoma (Stanley *et al.*, 1989) and may disrupt other events accompanying T cell activation (Ledbetter *et al.*, 1987). In some cases, however, PT treatment promotes T cell activation (Aussel *et al.*, 1988; Thom and Casnellie, 1989). These apparently conflicting results may be explained by the complex structure of PT. PT holotoxin consists of the S1 polypeptide, which contains ADP-ribosyltransferase activity, and the B oligomer, which

interacts with cell surface structures and facilitates entry of the S1 subunit into the cell interior. Considerable evidence indicates that the B oligomer may provoke mitogenesis in T lymphocytes independently of the S1 subunit (Tamura *et al.*, 1983; Gray *et al.*, 1989). Thus, the effects of exogenously applied PT on lymphocytes are complex and need not necessarily involve G_i proteins.

In order to directly address the role of G_i proteins in T cell signal transduction, we expressed the catalytic S1 subunit of PT in the thymocytes of transgenic mice. This novel approach excludes artifacts resulting from the B oligomer and permits analysis of the role of G_i proteins in normal T-lineage cells, rather than in cell lines. Examination of thymocytes from *lck*-PT transgenic mice reveals that TCR−CD3 signal transduction is relatively insensitive to PT-mediated elimination of G_i protein function. In contrast, transgenic animals exhibit an unusual defect in T cell development resulting in a virtual absence of T cells in peripheral lymphoid compartments. These observations, coupled with previous studies of animals infected with *Bordetella pertussis*, suggest that a PT-sensitive signaling process, almost certainly involving a G_i protein, regulates thymocyte trafficking.

Results

The lck promoter directs expression of the pertussis toxin S1 subunit to the thymocytes of transgenic mice

The S1 sequence from *B.pertussis* was modified to introduce a eukaryotic translation initiation site into the mRNA (see Materials and methods) and inserted into the p1017 expression vector (Figure 1). This vector contains the thymocyte-specific *lck* proximal promoter (Garvin *et al.*, 1988, 1990). In addition to the S1 gene, p1017S1 contains introns and exons of the human growth hormone gene (hGH), included to increase transgene expression (Brinster *et al.*, 1988). Six (C57BL/6 X DBA/2) F_2 founder animals were obtained by injection of the 6.1 kb *Not*I fragment of p1017S1 into fertilized eggs. Five of these exhibited a characteristic phenotype (described below). Three lines of transgenic animals were established; animals from these lines and the original founders were used for experiments described below.

Previous studies of the *lck* proximal promoter demonstrated its activity in the most immature thymocyte subsets and in all thymocyte subsets defined by the cell-surface markers CD4 and CD8, but not in peripheral T lymphocytes or other tissues (Garvin *et al.*, 1990; Sartor *et al.*, 1989; unpublished results). Consistent with these results, immunoreactive S1 protein, visualized by immunoblot analysis (Marchitto *et al.*, 1987), was observed in total transgenic thymocytes but not in spleen, kidney, heart, liver, lung or brain (Figure 2). Additionally, sorted single-positive CD4 and CD8 *lck*-PT thymocytes contained immunoreactive S1 protein (data not shown). These results were confirmed by RNA blot analysis which demonstrated the presence of a complex set of S1-containing transcripts only in the thymocytes of transgenic animals (Figure 3). The amount of S1 detected in transgenic thymocytes varied from 1 to 5 ng S1/mg cell protein, corresponding to approximately 100−500 molecules of S1 per cell. The identity of the immunoreactive M_r 21 kd protein in transgenic thymocytes is unknown, however a 21 kd proteolytic degradation product of S1 has been observed by others (Burns *et al.*, 1987).

The product of the S1 gene is enzymatically active in the absence of other PT subunits (Locht *et al.*, 1987; Nicosia *et al.*, 1987; Barbieri *et al.*, 1987; Runeberg-Nyman *et al.*, 1987). We therefore assayed *lck*-PT thymocyte lysates for the presence of ADP-ribosyltransferase activity towards purified transducin. Whole cell lysates of control and transgenic thymocytes were incubated in the presence of [^{32}P]NAD and bovine transducin for 1 h, then analyzed by SDS−PAGE and autoradiography. Figure 4 shows that a M_r 39 kd protein was labeled when reactions were performed in the presence of transducin and transgenic (lane 2), but not control (lane 5), lysates. This protein co-migrated with purified transducin α subunit labeled by commercial PT (lanes 3 and 6) and with transducin visualized by Coomassie staining of an adjacent lane (arrow). Hence, *lck*-PT thymocytes contain an ADP-ribosyltransferase activity typical of the PT S1 subunit.

G_i α pertussis toxin substrates are depleted in the thymocytes of lck-PT mice

Thymocytes contain G_{i2} and G_{i3} proteins that are well-characterized substrates for the PT ADP-ribosyltransferase (Beals *et al.*, 1987; Kim *et al.*, 1988; Bokoch *et al.*, 1984; unpublished results). To determine if the S1 protein expressed by transgenic thymocytes was active *in vivo*, we examined the ability of thymocyte G_i α subunits to become labeled by PT *in vitro*. *In vitro* labeling by PT should not be possible if these substrates have already been modified *in vivo* by the activity of the endogenous S1 subunit. In addition, we wished to determine the amount of unmodified, and presumably functional, G_i protein remaining in the transgenic thymocytes. Whole cell lysates prepared using wild-type and transgenic thymocytes were incubated at 30°C in the presence of [^{32}P]NAD for 1 h with or without added PT, then analyzed by SDS−PAGE and autoradiography. A M_r 40−41 kd PT substrate was easily detected in thymocytes from the control animal (Figure 5, lane 2). This band co-migrates with the α subunit of G_{i2} and G_{i3} as demonstrated by immunoblotting of human thymus

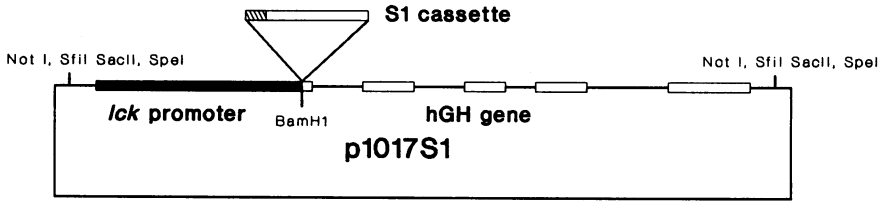

Fig. 1. p1017S1 expression vector. Plasmid p1017S1 contains the S1 cassette (open bar; striped region denotes modified S1 signal peptide) inserted between the proximal murine *lck* promoter (black bar) and the entire coding region (open boxed exons) of the human growth hormone gene (hGH). Polylinker sequences containing rare-cutting restriction enzyme sites (*Not*I−*Spe*I) were introduced as shown.

membranes in an adjacent lane with α_i-specific antibodies (data not shown). In contrast, only a very small amount of ^{32}P was incorporated into this band when transgenic thymocyte lysates were assayed (Figure 5, lane 4). Densitometric analysis reveals that only approximately 10% of the α_i protein capable of being labeled by PT remained in *lck*-PT thymocyte preparations, indicating that these cells contain at most 10% the normal amount of G_i PT substrates.

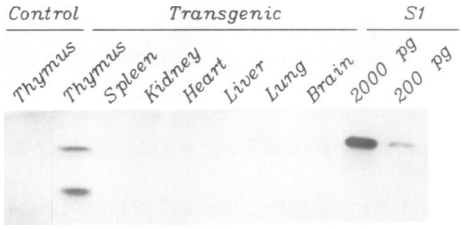

Fig. 2. Expression of S1 protein by *lck*-PT thymocytes. Cells were removed from the thymuses of 5 week old transgenic and non-transgenic littermates, whole cell lysates were prepared, separated by 15% SDS−PAGE, transferred to nitrocellulose, and blotted with a polyclonal rabbit antiserum to PT. Lane 1, 100 μg lysate from control thymus; lanes 2−8, 100 μg lysates from transgenic tissues; lane 9, 1 ng PT (2000 pg S1); and lane 10, 0.1 ng PT (200 pg S1). Lanes 9−10 also contained 4 μg human kidney membranes per lane to permit satisfactory immunoblotting of limiting amounts of PT.

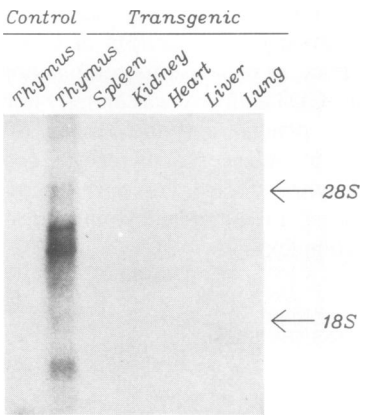

Fig. 3. Expression of p1017S1 transcripts by *lck*-PT thymocytes. RNA (10 μg) from tissues of 3 week old control and transgenic mice was separated by agarose electrophoresis, transferred to nitrocellulose and hybridized with a ^{32}P-labeled S1 probe. A 48 h exposure is shown. The positions of rRNA bands are indicated.

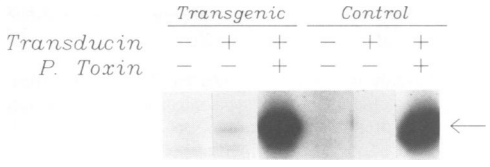

Fig. 4. S1 activity in *lck*-PT thymocytes. Thymocyte lysates were prepared from 7 week old transgenic and littermate control animals. Lysates were incubated in the presence of [^{32}P]NAD for 1 h at 30°C, with or without added activated PT and/or bovine transducin. Reaction products were analyzed by 12% SDS−PAGE and autoradiography. Lanes 1−3, transgenic thymocyte lysates; lanes 4−6, thymocyte lysates from a littermate control animal. Activated PT and/or transducin was added to the reactions in each lane as indicated. The arrow denotes the migration of the transducin α subunit as determined by Coomassie staining of 2 μg analyzed in an adjacent lane.

Next, we ruled out the possibility that endogenous S1 had modified G_i PT substrates after cell lysis but before the assay for PT substrates was performed. In the experiments just described, cells were lysed and kept on ice for 10−15 min before being assayed, we therefore examined the ability of PT to function under these conditions. Normal thymocytes were lysed in the presence or absence of 200 ng activated PT, incubated on ice for 30 min, and then assayed for PT substrates at 30°C in the presence of [^{32}P]NAD and additional PT, as above. Diminution in the radio-labeling of the 40−41 kd PT substrate would indicate that significant modification of these substrates had occurred during the 0°C incubation, however, no such diminution is apparent (Figure 5, compare lanes 2 and 8). Since this experiment involved a 2 to 3-fold longer lag time between cell lysis and performance of the assay than did the experiments shown in lanes 1−4 of Figure 5, and employed 100-fold more S1 than that predicted to be released by lysing a similar number of transgenic thymocytes, the diminished labeling of *lck*-PT thymocytes seen in Figure 5, lane 4 must reflect *in vivo* perturbations of the G_i PT substrates.

To further demonstrate that S1 gains access to G_i PT substrates in *lck*-PT thymocytes *in vivo*, we tested the ability of S1 protein from transgenic thymocytes to label the 40−41 kd PT substrates of non-transgenic thymocytes during incubation of the lysates on ice. Equal numbers of transgenic and control thymocytes were mixed together, lysed, incubated on ice for 30 min, and then assayed for PT substrates at 30°C in the presence of [^{32}P]NAD. Lanes 2 and 6 of Figure 5 demonstrate that no significant modification of 40−41 kd PT substrates occurred during the incubation at 0°C in the presence of S1. We conclude that unmodified, and therefore functional, G_i α subunits are indeed substantially depleted in the intact thymocytes of *lck*-PT mice.

Normal calcium responses by lck-PT thymocytes

To explore the role of PT-sensitive Ca^{2+} responses in T lymphocytes, and to address the regulation of PI-PLC in

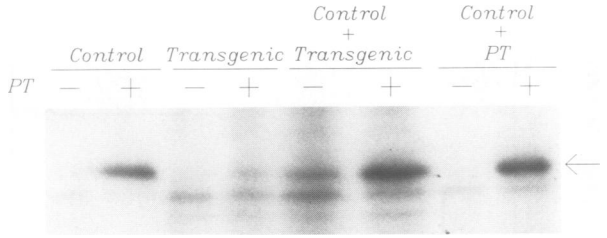

Fig. 5. Pertussis toxin labeling of thymocyte G_i proteins. Thymocytes from 5 week old mice were lysed according to various protocols. Lanes 1−2, whole cell lysates were prepared from control thymocytes as in Figure 4. Lanes 3−4, whole cell lysates were similarly prepared from transgenic thymocytes. Lanes 5−6, equal numbers of transgenic and non-transgenic thymocytes were mixed together, lysed at twice the cell concentration used in Figure 4, and then incubated on ice for 30 min before reactions were performed. Lanes 7−8, lysates from control thymocytes were prepared as in Figure 4, then incubated on ice for 30 min in the presence of 0.2 μg activated PT before reactions were performed. All lysates were then incubated in the presence of [^{32}P]NAD for 1 h at 30°C, with or without added activated PT to a total final concentration of 1 μg/reaction, and reaction products analyzed by 12% SDS−PAGE and autoradiography. Activated PT was added during the 30°C incubations as indicated. The arrow denotes the migration of G_i protein α subunits as determined by immunoblotting of an adjacent lane (data not shown).

these cells, we examined intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in resting and stimulated thymocytes from *lck*-PT mice. Figure 6 shows results of stimulating control and transgenic thymocytes with the mitogenic lectin, concanavalin A (Con A). Despite the presence of PT activity in *lck*-PT thymocytes, and the near-complete inactivation of G_i target proteins, the more mature, single-positive $CD4^+CD8^-$ and $CD4^-CD8^+$ cells from control and transgenic thymuses responded identically to Con A treatment (Figure 6A, 6B), as did the immature $CD4^+CD8^+$ thymocytes (Figure 6C). Figure 6 presents the mean violet/blue (V/B) ratios (which correlate with $[Ca^{2+}]_i$) for these experiments; data analyzed on a cell-by-cell basis similarly demonstrated no differences between responses of comparable transgenic and control thymocyte subsets. Moreover, no differences in responses to either high or low concentrations of stimulating reagent distinguished *lck*-PT responses from those of control thymocytes (data not shown). Con A probably stimulates T cells by interacting with cell-surface CD3 (see Valentine *et al.*, 1985), therefore it is not surprising that both control and transgenic thymocytes also responded with identical $[Ca^{2+}]_i$ increases upon stimulation with anti-CD3 antibodies. Responses of total thymocyte preparations not separated by staining with CD4 and CD8 antibodies correlated with those of stained thymocytes (data not shown).

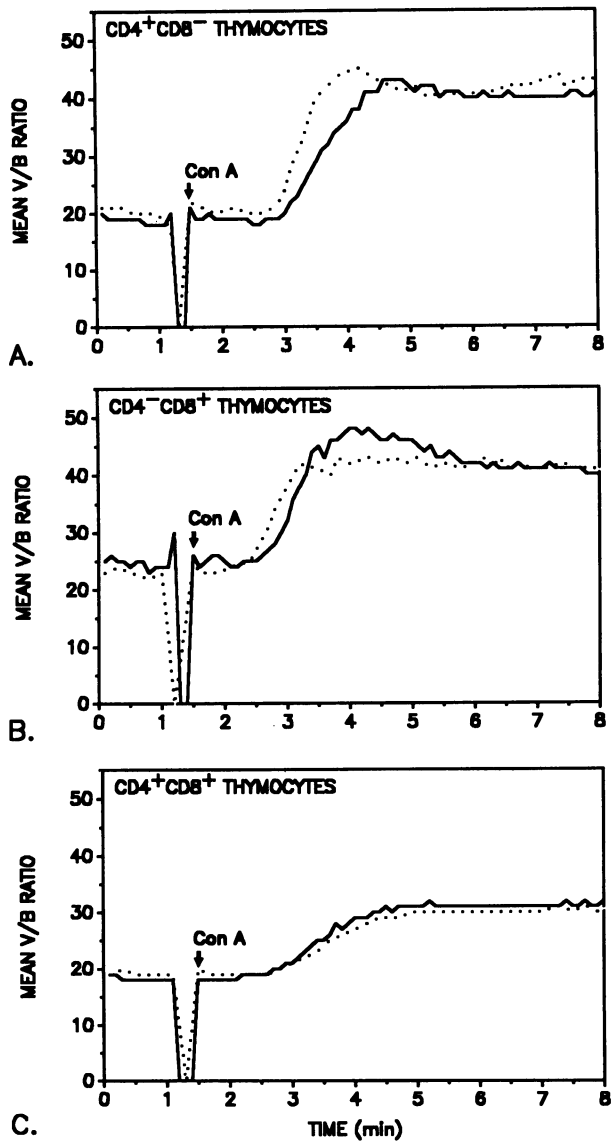

Fig. 6. Ca^{2+} accumulation in stimulated *lck*-PT thymocytes. Thymocytes were isolated from transgenic and littermate control animals, loaded with indo-1, stained with PE-conjugated CD4 and FITC-conjugated CD8, and subsets analyzed for $[Ca^{2+}]_i$ as described in Materials and methods. **Panel A**: $CD4^+CD8^-$ thymocytes; **Panel B**: $CD4^-CD8^+$ thymocytes; **Panel C**: $CD4^+CD8^+$ thymocytes. Con A (50 μg/ml) was added at the time indicated by the arrow, and violet and blue emissions were measured and for each positively-stained CD4/CD8 cell subset. $[Ca^{2+}]_i$ is correlated with the violet/blue emission ratio (V/B ratio), here presented as the mean of all cells analyzed during each time increment. Dashed line, control cells; solid line, transgenic cells.

The thymocytes of lck-PT mice secrete IL-2 normally in vitro

Like helper T lymphocytes, 'helper' phenotype thymocytes ($CD4^+CD8^-$ cells) secrete IL-2 in response to activating stimuli (Rothenberg *et al.*, 1988). We therefore further investigated whether T cell activating signals were blocked by PT by examining IL-2 production by $CD4^+CD8^-$ *lck*-PT thymocytes. In three separate experiments performed on thymocytes from four different founder animals/lines, $CD4^+CD8^-$ thymocytes in *lck*-PT transgenic thymocyte preparations secreted the same amount of IL-2 in response to Con A and phorbol myristate acetate (PMA) stimulation as did normal $CD4^+CD8^-$ thymocytes (Figure 7). Transgenic thymocytes did not secrete IL-2 without stimulation. Similarly, proliferation of $CD3^{hi}$ thymocytes from *lck*-PT mice in response to combinations of Con A, PMA, and anti-CD3 antibody was entirely normal (data not shown). These results, and the results of the $[Ca^{2+}]_i$ analysis (Figure 6), indicate that thymocytes containing active PT ADP-ribosyltransferase are nevertheless capable of normal responses to mitogenic stimuli mediated by the TCR–CD3 complex.

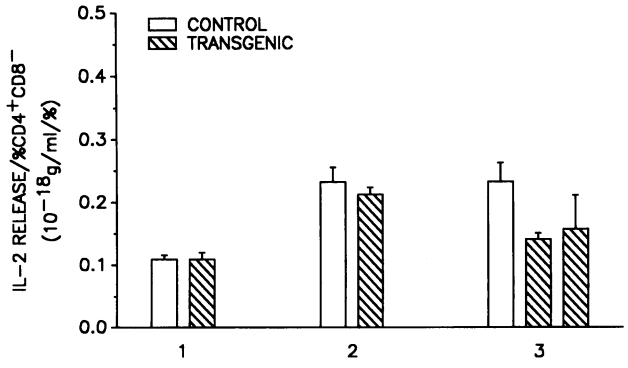

Fig. 7. IL-2 secretion by *lck*-PT thymocytes. Thymocytes were isolated from transgenic and littermate control animals and cultured at 10^7 cells/ml in the presence of 5 μg/ml Con A and 50 ng/ml PMA for 24 h. IL-2 concentrations of cell supernatants were assayed using the CTLL cell line. Three separate experiments are shown. Experiment 1 involved a control animal and a transgenic animal of the 1363 line, 7 weeks of age. Experiment 2 involved a control animal and a transgenic animal of the 1342 line, 6.5 weeks of age. Experiment 3 involved a control animal and two founder transgenic animals, each 4 weeks of age. The data shown compares the amount of IL-2 secreted by the different thymocyte preparations after normalization to the proportion of $CD4^+CD8^-$ thymocytes present (determined by flow cytometry as in Figure 8). Standard deviations of triplicate determinations are indicated by the error bars. Open bars, control animals; hatched bars, transgenic animals.

Abnormal T cell development in lck-PT mice

Despite apparently normal TCR−CD3 signal transduction, defects in T cell development were observed in *lck*-PT animals. The thymuses of transgenic animals were significantly smaller than those of littermate control animals, reflecting an approximately 50% decrease in the number of thymocytes (data not shown). Flow cytometric evaluation of thymocyte subsets revealed a dramatically increased representation of more mature, $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive cells in transgenic thymuses (Figure 8A, 8B). Thymocytes from *lck*-PT transgenic mice contained fewer $CD3^{lo}$ cells (approximately one-half normal) and a much larger percentage of $CD3^{hi}$ cells compared to normal thymocytes (Figure 8C), correlating with the increased representation of more mature thymocytes in *lck*-PT thymuses (see Havran *et al.*, 1987). Analysis of cells doubly-stained for CD3 and CD4 or for CD3 and CD8 revealed that, as in normal thymuses, all $CD3^{hi}$ cells from *lck*-PT thymuses were single-positive for either CD4 or CD8 (data not shown). Although the CD3 surface density of the $CD3^{hi}$ *lck*-PT thymocytes was slightly reduced compared with littermate controls (Figure 8C), these cells were clearly distinct from the $CD3^{lo}$ population. Interestingly, this slightly lower level of CD3 expression is typical for normal peripheral T cells (compare with Figure 9C, below).

Expression of other cell-surface markers by *lck*-PT thymocytes also correlated with the presence of an usually high proportion of mature T cells in transgenic thymuses. Thymocytes expressing high levels of the lymph node homing receptor, Mel-14, were at least 20-fold over-represented in *lck*-PT animals as compared to littermate controls (Figure 8D). In normal mice, high level Mel-14 expression is generally characteristic of peripheral T cells rather than thymocytes (Reichert *et al.*, 1986). Similarly, transgenic thymuses contained disproportionate numbers of $J11d^-$ thymocytes compared to control thymocyte preparations (Figure 8E), although all *lck*-PT thymocytes expressed the T-lineage marker, Thy 1.2 (data not shown). J11d defines a molecule on the surface of immature thymocytes that is invariably absent from mature, functional T cells (Crispe and Bevan, 1987). Thus, analyses of cell-surface markers expressed by *lck*-PT thymocytes consistently demonstrated the increased representation of phenotypically mature T-lineage cells. In fact, the phenotype of the over-represented population closely resembles that of peripheral T lymphocytes.

Remarkably, despite the accumulation of the apparently mature thymocytes described above, thymus-specific expression of the S1 ADP-ribosyltransferase resulted in nearly complete depletion of T cells from spleen, peripheral blood, and lymph nodes, as shown by CD4, CD8, CD3 and Thy 1.2 staining (Figure 9 and data not shown). Peripheral T cell depletion affected both CD4 and CD8 populations to approximately the same extent.

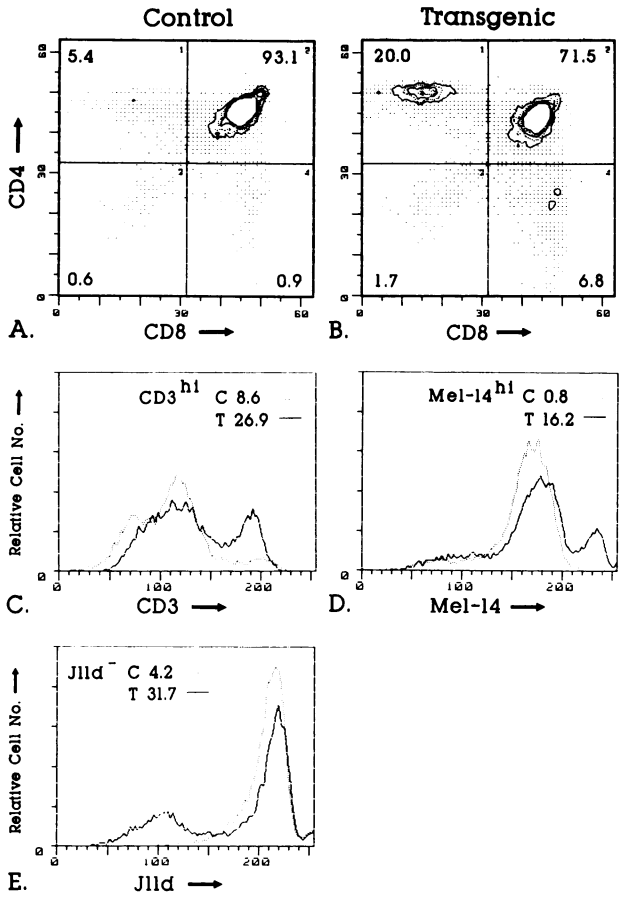

Fig. 8. Flow cytometric analysis of *lck*-PT thymocytes. Thymocytes from 5 week old mice were analyzed for cell-surface markers. **Panel A**: CD4/CD8 staining of control thymocytes. **Panel B**: CD4/CD8 staining of transgenic thymocytes. The percentage of cells in each quadrant is indicated. **Panel C**: CD3 staining. **Panel D**: Mel-14 staining. **Panel E**: J11d staining. The percentages of $CD3^{hi}$, Mel-14^{hi}, and $J11d^-$ cells are indicated; C, control; T, transgenic. For single parameter histograms, the dotted line indicates the tracing of the control animal and the solid line, that of the transgenic animal.

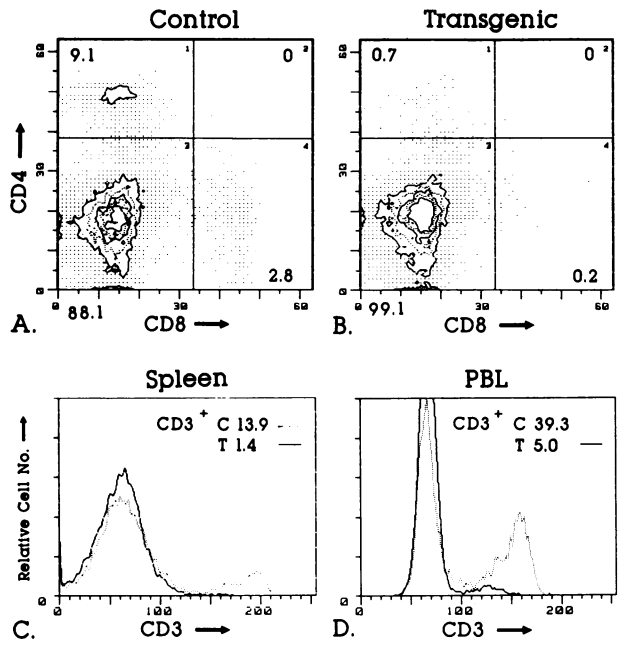

Fig. 9. Flow cytometric analysis of *lck*-PT peripheral lymphocytes. Peripheral blood lymphocytes (PBL) and splenocytes from the animals described in Figure 8 were analyzed. **Panel A**: CD4/CD8 staining of control splenocytes. **Panel B**: CD4/CD8 staining of transgenic splenocytes. The percentage of cells in each quadrant is indicated. **Panel C**: CD3 staining of splenocytes. **Panel D**: CD3 staining of peripheral blood lymphocytes. The percentages of $CD3^+$ cells are indicated; C, control; T, transgenic. For single parameter histograms, the dotted line indicates the tracing of the control animal and the solid line, that of the transgenic animal.

Discussion

Bacterial toxin-derived ADP-ribosyltransferases affect the function of many different eukaryotic signal transduction pathways (reviewed in Simpson *et al.*, 1989), and the use of these toxins has provided a powerful means for dissecting molecular mechanisms of signal transduction. In particular, the use of cholera and pertussis toxins has revealed the pivotal role of G proteins in catecholamine receptor signaling (reviewed in Gilman, 1987). We have employed a novel strategy to examine toxin-sensitive signaling elements by directing the expression of the PT ADP-ribosyltransferase in thymocytes of transgenic mice.

This strategy offers several advantages over conventional methods for examining PT effects. First, by engineering the expression of PT in normal cells, we avoid potential artifacts that may result from the use of culture-adapted cell lines that no longer maintain stringent signaling requirements. Secondly, the transgenic mouse system allows the analysis of complex signaling pathways for which no satisfactory tissue culture models exist, e.g. it enables investigation of mechanisms involved in lymphocyte differentiation. Finally, by directing expression of the catalytic subunit of PT intracellularly, confounding effects of the non-catalytic B oligomer are eliminated. Such effects are of particular concern in the study of T lymphocytes, where interaction of the B oligomer of PT with the antigen receptor complex appears to stimulate mitogenesis in the complete absence of the catalytically active S1 moiety (Gray *et al.*, 1989). From this perspective, *lck*-PT mice offer a relatively unambiguous assessment of the importance of PT-sensitive signaling elements in thymocyte responses.

Our S1 expression construct includes a modified version of the bacterial secretion signal sequence (see Materials and Methods). Bacterial expression studies make clear that retention of a signal sequence does not interfere with the enzymatic function of S1 (Locht *et al.*, 1987; Nicosia *et al.*, 1987; Barbieri *et al.*, 1987; Runeberg-Nyman *et al.*, 1987), and suggest that such a sequence may assist S1 synthesis in heterologous systems (Burnette *et al.*, 1988; Nicosia *et al.*, 1987). While the p1017S1 construct including the residual signal sequence successfully directs S1 expression in *lck*-PT thymocytes, it is possible that this sequence may cause some amount of the S1 protein to be targeted to the endoplasmic reticulum (Wickner and Lodish, 1985). Since the S1 subunit probably does not enter this compartment when applied in the form of the holotoxin, ADP-ribosylation may be affecting previously unexamined pathways in *lck*-PT thymocytes. Nevertheless, some fraction of the endogenously-synthesized S1 toxin does gain access to the G_i protein PT substrates (Figure 5) which are presumably associated with the plasma membrane (Buss *et al.*, 1987). Since even the wild-type S1 signal sequence functions poorly in bacterial expression systems (Burnette *et al.*, 1988; Barbieri *et al.*, 1987; Nicosia *et al.*, 1987), p1017S1-derived protein would at best be inefficiently targeted to the endoplasmic reticulum in thymocytes. Analysis of S1 expression constructs lacking a signal sequence will, however, be required to assess whether an important S1 target resides within the secretory compartments of lymphoid cells.

Normal IL-2 release and calcium responses by lck-PT thymocytes

TCR−CD3 stimulation of mature T lymphocytes typically provokes increased $[Ca^{2+}]_i$ and PKC activity and culminates in T cell proliferation and lymphokine secretion. Since direct provision of these two intracellular signals by application of ionomycin and phorbol ester activates T lymphocytes, it has frequently been suggested that TCR−CD3 signal transduction leading to T cell activation involves, and may require, activation of PI-PLC (reviewed in Weiss *et al.*, 1986). Interestingly, PT blocks $[Ca^{2+}]_i$ accumulation (Ledbetter *et al.*, 1987) and IL-2 release (Stanley *et al.*, 1989) in two *in vitro* models of T cell stimulation. These results suggested that a PT-sensitive element might mediate signaling from the antigen receptor to PI-PLC in mature thymocytes and T cells.

In other signaling systems, the PT-sensitive G_i and G_o proteins participate in ligand-stimulated activation of PI-PLC (Fain *et al.*, 1988; Ashkenazi *et al.*, 1989; Moriarty *et al.*, 1990) and a similar role for G_i proteins in T cell signal transduction has been suggested (Linch *et al.*, 1987). The primary PT substrates evident in T lymphocytes by *in vitro* PT labeling and cDNA cloning are the 40−41 kd α subunits of the G_{i2} and G_{i3} proteins (Stanley *et al.*, 1989; Beals *et al.*, 1987; Kim *et al.*, 1988; unpublished results). Additional G protein PT substrates have been detected in lymphocytes by other means (Wang *et al.*, 1988; unpublished results); the presence of G_o (39 kd) has not been reported in these cells.

Thymocytes of *lck*-PT mice contain less than 10% the normal amount of 40−41 kd PT substrates, indicating that ADP-ribosylation has inactivated at least 90% of G_i proteins in these cells (Figure 5). Since G_i PT substrates are limiting in other signal transduction systems (Backlund *et al.*, 1985; Bokoch and Gilman, 1984), G_i-dependent signal transduction should be severely disrupted in *lck*-PT transgenic thymocytes. Our results demonstrate that thymocytes expressing endogenous S1 and grossly deficient in PT targets can nevertheless be stimulated to release IL-2 by a combination of mitogen and phorbol ester. In fact, transgenic $CD4^+CD8^-$ thymocytes secreted an amount of IL-2 not detectably different from that secreted by normal $CD4^+CD8^-$ thymocytes (Figure 7). Similarly, transgenic $CD3^+$ thymocytes proliferated normally in response to combinations of mitogen, phorbol ester, and antibody to CD3. Moreover, *lck*-PT thymocytes stimulated with mitogen or antibody to CD3 accumulated intracellular Ca^{2+} in a normal manner (Figure 6). These results indicate that G_i proteins are not required for CD3-mediated augmentation of $[Ca^{2+}]_i$, or for T cell activation in general. The increased $[Ca^{2+}]_i$ associated with stimulation of T lymphocytes derives partly from Ca^{2+} released from intracellular stores by IP3 and partly from extracellular Ca^{2+} (reviewed in Weiss *et al.*, 1986). Entry of extracellular Ca^{2+} is thought to depend on IP3 either directly (Kuno and Gardner, 1987) or indirectly (Weiss *et al.*, 1986). Therefore, our results argue strongly that TCR−CD3-stimulated PI-PLC catalysis in thymocytes is not mediated by any PT-sensitive element. We conclude that PT holotoxin exerts its effects on TCR−CD3 signal transduction exclusively via its surface-active B oligomer, and not through ADP-ribosylation of a G protein.

An S1-sensitive target modulates thymocyte development

Mice bearing the *lck*-PT expression construct exhibit remarkable alterations in T cell distribution. Thymocyte

numbers are reduced by half, due primarily to depletion of $CD4^+CD8^+$ immature thymocytes. Although this effectively increases the proportion of the more mature, $CD3^{hi}$, single-positive CD4 or CD8 cells in *lck*-PT thymuses, it does not adequately account for the unusual expression of other maturational markers by *lck*-PT thymocytes. In fact, the increased prevalence of Mel-14^{hi} and J11d^- T-lineage cells suggests that rather than mature thymocytes, cells similar to peripheral T lymphocytes are over-represented in *lck*-PT thymuses. These effects are the direct result of S1 expression; mice bearing other transgenes under the control of the *lck* promoter do not exhibit comparable abnormalities (Garvin *et al.*, 1990, unpublished data).

Interestingly, the deficit in peripheral T cell representation in *lck*-PT mice is even more dramatic than the thymic phenotype: spleens and lymph nodes of these animals contain few T lymphocytes despite the presence of near-normal numbers of 'mature' single-positive thymocytes. Although defective maturation of thymocytes could lead to a failure in peripheralization (or the destruction of 'mature' cells after thymic export), studies of humans and mice exposed to systemic PT suggest a more comprehensive explanation for the altered thymocyte profiles of *lck*-PT mice. Human infection with *B. pertussis* is associated with the accumulation of non-proliferating B and T lymphocytes in the blood (Bernales *et al.*, 1976). Similarly, mice injected with PT exhibit lymphocytosis (Morse and Riester, 1967), an effect mediated by enzymatically active S1 subunit (Black *et al.*, 1988). PT-generated lymphocytosis in the mouse occurs concomitantly with a significant decline in thymus weight and the inability of lymphocytes to home to lymph nodes (Morse, 1964; Morse and Barron, 1970). Additionally, *in vitro* PT treatment of lymphocytes interferes with their subsequent migration from the bloodstream to lymph nodes and Peyer's patches (Spangrude *et al.*, 1984), despite the continued expression of adhesion/homing molecules, e.g. Mel-14 and LFA-1, at normal levels (Huang *et al.*, 1989).

In the context of these results, we propose that a PT-sensitive pathway, probably involving a G_i protein, regulates lymphocyte and thymocyte trafficking. PT reportedly blocks the association of thymocytes with thymic stromal elements *in vitro* (Sugimoto *et al.*, 1983). Since thymocyte maturation is crucially dependent on such interactions, the developmental abnormalities observed in *lck*-PT thymuses, and the defective peripheralization of mature T cells in these mice, could both be explained by disruption of a signaling process which mediates effective adhesion of lymphocytes to endothelial cells. Interestingly, preliminary results indicate that *lck*-PT thymocytes perform poorly in *in vivo* homing assays. The *lck*-PT animals described here should prove especially useful in defining this process and in elucidating its molecular features.

A general strategy for analysis of specialized signaling pathways

We have successfully generated animals that express endogenously synthesized PT in a tissue-specific fashion, and have defined in this way a PT-specific phenotype with quite distinctive characteristics. These experiments encourage the view that *in vivo* synthesis of appropriately modified bacterial toxin catalytic subunits will provide a general method for analysis of eukaryotic signaling pathways. Our strategy should prove particularly valuable for examining signaling pathways that function during development, providing access to cell populations that have no *in vitro* counterparts.

Materials and methods

Expression construct

The expression vector, p1017 (Figure 1), was made by inserting the 3.2 kb proximal murine *lck* promoter (Garvin *et al.*, 1990) between the *Eco*RI and *Sma*I sites of Puc19. 2.1 kb of human growth hormone gene sequence extending from the *Bam*HI site in the first exon to the *Eco*RI site after the poly-A addition signal (Seeburg, 1982) was juxtaposed downstream of the promoter between the *Bam*HI and *Sal*I sites of the polylinker. Additionally, p1017 contains an oligonucleotide ligated into the *Sph*I and *Eco*RI sites of pUC19, thereby introducing *Spe*I, *Sac*II, *Sfi*I, and *Not*I recognition sequences.

To improve the translation efficiency of the bacterial S1 gene in mammalian cells, a eukaryotic translation initiation consensus sequence (Kozak, 1986) was engineered in the S1 cassette by inserting the oligomer, AATTCTAGACCATGGATCCCGGGT. The *Eco*RI-*Xba*I fragment containing the S1 ADP-ribosyltransferase (Nicosia *et al.*, 1986) was first subcloned into the polylinker of pUC18, and the oligonucleotide inserted at the amino terminus of the S1 coding sequence by cutting the plasmid with *Sst*II and *Bsp*MII, partially digesting with *Exo*III, and then with *Eco*RI, and ligating the annealed oligonucleotides between the *Eco*RI site and the blunt ends from the *Exo*III digestion. After subcloning, the presence of the modified translation initiation site was confirmed by DNA sequencing. The 800 bp *Xba*I fragment containing the modified S1 cassette was removed from the pUC vector and cloned into the *Bam*HI site of the p1017 vector to form p1017S1. The cloning junctions were confirmed by sequencing, and *Not*I digestion was used to remove the fragment of interest. The final sequence of the 5′ end of the S1 gene used in the experiments described here was TCTAGACCATGGATCCCGGGTTTCGC (the initiating ATG is underlined). This encodes a signal sequence beginning with MRPGFR, in contrast with the wild-type signal sequence, which begins, MRCTRAIR (corresponding amino acids are underlined).

Transgenic mice

Purified linear DNA molecules (2 ng/μl) were injected into the pronuclei of (C57BL/6J X DBA/2J) F2 hybrid mouse zygotes. Subsequent generation and detection of transgenic mice was as previously described (Garvin *et al.*, 1990; Hogan *et al.*, 1986). Transgene-positive lines, detected using probes for either hGH or S1, were established by backcrossing founder animals with C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME).

RNA blots

RNA was isolated by homogenization of tissues in guanidinium isothiocyanate and centrifugation through CsCl as described (Marth *et al.*, 1985). RNA samples were fractionated by electrophoresis in 1% agarose–formaldehyde gels, blotted onto nitrocellulose membranes, hybridized with an 800 bp *Xba*I fragment containing the S1 gene labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983), and washed in three changes of 1 × SSC, 0.1% SDS at 59°C for 90 min prior to autoradiography.

ADP-ribosylation assays

Thymuses were removed from transgenic and littermate control mice, lymphocytes were released by teasing, and were washed and counted. Cells were lysed as follows: $1-5 \times 10^7$ thymocytes were washed twice with PBS (4°C), and resuspended in cold, hypotonic lysis buffer (20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF) at a concentration of $1-2 \times 10^7$ cells/30 ml. Cells were allowed to swell on ice for 10–15 min, and then homogenized. *In vitro* PT substrates of cell lysates were assayed using a modification of the procedure of Bokoch *et al.* (1984). 10–20 μl of cell lysates containing 25–50 μg protein (determined by Bradford assay) were used per reaction. Reactions were started by adding reaction buffer to a final vol of 20–50 μl. The composition of the reaction buffer was adjusted for each experiment so that final reaction concentrations were 0.1 M Tris pH 8.0, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, and 0.01 mM NAD, plus 4 μCi (10–80 pmol) [^{32}P]NAD (New England Nuclear, Wilmington, DE). Selected reactions also contained 1 μg of activated commercial PT (List Biological Laboratories, Campbell, CA) or 0.5 μg bovine transducin (a gift from Dr. James Hurley, Univ. of Washington). Commercial PT was resuspended in water to a concentration of 1 mg/ml, and activated just before use by adding DTT to 0.1 M and incubating at 25°C for 10 min. Reactions were performed at 30°C for 30–60 min and were stopped by the addition of 1/4 vol sample buffer (200 mM Tris pH 6.8, 4% SDS, 40% glycerol, 4% 2-mercaptoethanol, 0.05% bromophenol blue) and immersion in a boiling water bath for 7 min. Reaction products were separated by 12% SDS–PAGE

at 4°C. Gels were stained with Coomassie blue, destained, dried, and exposed to X-ray film overnight. Quantitation of selected autoradiograms was performed by computer densitometry (BioImage, Ann Arbor, MI).

Immunoblots

Proteins separated by 12 or 15% SDS–PAGE were transferred to nitrocellulose and immunoblotted as described (Marth *et al.*, 1987). Rabbit anti-PT serum (a gift from Dr Witold Cieplak, NIH) was used at a dilution of 1:500, and the secondary reagent, goat anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN), was used at 1:3000.

Flow cytometry

Cells were removed from lymphoid organs of control and transgenic animals by teasing, and splenocyte and blood cell preparations were depleted of erythrocytes by alkaline lysis (Mishell and Shiigi, 1980). For flow cytometric analysis, cells were stained with saturating concentrations of antibodies at 4°C as described (Garvin *et al.*, 1990). Monoclonal antibodies used were: fluorescein isothiocyanate (FITC)-conjugated 53–6.71 (CD8) (Becton Dickinson, Mountain View, CA), phycoerythrin (PE)-conjugated GK 1.5 (CD4) (Becton Dickinson) or biotinylated GK 1.5, biotinylated 30-H12 (Thy1.2) (Becton Dickinson), biotinylated 500AA.2 (CD3) (Havran *et al.*, 1987), Mel-14 (Gallatin *et al.*, 1983), and J11d hybridoma supernatants (Bruce *et al.*, 1981). Mel-14 supernatant was kindly provided by Dr W. M. Gallatin (Fred Hutchinson Cancer Research Center), and J11d supernatant was a gift from Dr Andrew Farr (University of Washington). PE-conjugated streptavidin (Caltag, San Francisco, CA) was used to detect binding of biotinylated reagents, and PE-conjugated goat anti-rat IgG absorbed against mouse IgG (Caltag) was used to detect Mel-14 and J11d binding. Multiparameter flow cytometry was carried out on a FACSTAR instrument (Becton-Dickinson). A total of 10,000 list-mode lymphocyte-gated events were collected for each sample and data were analyzed using FACSTAR Consort 30 software.

T cell stimulation and IL-2 assay

Thymocytes were prepared as above, and cultured in 1 ml of medium at a density of 10^6 cells/ml in 24-well plates with either 5 μg/ml Con A (Sigma #C-2010, St. Louis, MO), 50 ng/ml PMA (Sigma), both Con A and PMA, or nothing added. Medium consisted of either RPMI 1640 (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 3 μM glycine, 200 U/ml penicillin, 60 μg/ml streptomycin, and 2 mM L-glutamine, or the same additionally supplemented with 1 mM oxaloacetate, 0.2 U/ml insulin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids (GIBCO). After 24 h, supernatants were removed and frozen for later IL-2 assay, which was performed by measuring CTLL cell proliferation as previously described (Gillis *et al.*, 1978). Results of different experiments were standardized against responses obtained with recombinant human IL-2 (a gift from Dr Kenneth Grabstein, Immunex Corp., Seattle, WA).

$[Ca^{2+}]_i$ analysis

Cytoplasmic free calcium concentrations were measured with indo-1 using an Ortho Cytofluorograph 50HH with a Model 2150 computer, as described (Rabinovitch *et al.*, 1986). Briefly, thymocyte suspensions prepared in 5%FCS/RPMI media were loaded with the acetoxy-methyl ester of indo-1 (indo-AM) (Molecular Probes, Inc., Portland, OR) at 3.5 μg indo-AM/ml for 45 min at 37°C at a concentration of 5×10^7 cells/ml. Washed cells were further stained in azide-free PE-conjugated CD4 and FITC-conjugated CD8 antibody solution (Becton-Dickinson) at a concentration of 1.8×10^8 cells/ml for 20 min at 23°C. Flow cytometry was performed at 37°C at typical rates of 1000 cells/s, and data collected on each cell included the ratio of violet to blue emissions, which parallels $[Ca^{2+}]_i$. Baseline calcium levels were established after 2 min of cell flow, then Con A was added to a final concentration of 10 or 50 μg/ml and flow was resumed. Data from positively-stained thymocyte subsets was analyzed as described (Rabinovitch *et al.*, 1986).

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References

Ashkenazi,A., Peralta,E.G., Winslow,J.W., Ramachandran,J. and Capon,D.J. (1989) *Cell*, **56**, 487–493.

Aussel,C., Mary,D., Peyron,J.-F., Pelassy,C., Ferrua,B. and Fehlmann,M. (1988) *J. Immunol.*, **140**, 215–220.

Backlund,P.S.,Jr., Meade,B.D., Manclark,C.R., Cantoni,G.L. and Aksamit,R.R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2637–2641.

Barbieri,J.T., Rappuoli,R. and Collier,R.J. (1987) *Infect. Immun.*, **55**, 1321–1323.

Beals,C.R., Wilson,C.B. and Perlmutter,R.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7886–7890.

Bernales,R., Eastman,J. and Kaplan,J. (1976) *Pediat. Res.*, **10**, 965–967.

Berridge,M.J. (1987) *Annu. Rev. Biochem.*, **56**, 159–193.

Black,W.J., Munoz,J.J., Peacock,M.G., Schad,P.A., Cowell,J.L., Burchall,J.J., Lim,M., Kent,A., Steinman,L. and Falkow,S. (1988) *Science*, **240**, 656–659.

Bokoch,G.M., Katada,T., Northup,J.K., Ui,M. and Gilman,A.G. (1984) *J. Biol. Chem.*, **259**, 3560–3567.

Bokoch,G.M. and Gilman,A.G. (1984) *Cell*, **39**, 301–308.

Brinster,R.L., Allen,J.M., Behringer,R.R., Gelinas,R.E. and Palmiter,R.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 836–840.

Bruce,J., Symington,F.W., McKearn,T.J. and Sprent,J. (1981) *J. Immunol.*, **127**, 2496–2501.

Burnette,W.N., Mar,V.L., Cieplak,W., Morris,C.F., Kaljot,K.T., Marchitto,K.S., Sachdev,R.K., Locht,C. and Keith,J.M. (1988) *Bio/Technology*, 6, 699–706.

Burns,D.L., Hausman,S.Z., Lindner,W., Robey,F.A. and Manclark,C.R. (1987) *J. Biol. Chem.*, **262**, 17677–17682.

Buss,J.E., Mumby,S.M., Casey,P.J., Gilman,A.G. and Sefton,B.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7493–7497.

Clevers,H., Alarcon,B., Wileman,T. and Terhorst,C. (1988) *Annu. Rev. Immunol.*, 6, 629–662.

Crispe,I.N. and Bevan,M.J. (1987) *J. Immunol.*, **138**, 2013–2018.

Davis,M.M. and Bjorkman,P.J. (1988) *Nature*, **334**, 395–402.

Fain,J.N., Wallace,M.A. and Wojcikiewicz,R.J.H. (1988) *FASEB J.*, **2**, 2569–2574.

Feinberg,A. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.

Gallatin,W.M., Weissman,I.L. and Butcher,E.C. (1983) *Nature*, **304**, 30–34.

Garvin,A.M., Pawar,S., Marth,J.D. and Perlmutter,R.M. (1988) *Mol. Cell. Biol.*, **8**, 3058–3064.

Garvin,A.M., Abraham,K.M., Forbush,K.A., Farr,A.G., Davison,B.L. and Perlmutter,R.M. (1990) *Int. Immunol.*, **2**, 173–180.

Gillis,S., Ferm.,M., Ou,W. and Smith,K. (1978) *J. Immunol.*, **120**, 2027–2032.

Gilman,A.G. (1987) *Annu. Rev. Biochem.*, **56**, 615–619.

Gray,L.S., Huber,K.S., Gray,M.C., Hewlett,E.L. and Engelhard,V.H. (1989) *J. Immunol.*, **142**, 1631–1638.

Havran,W.L., Poenie,M., Kimura,J., Tsien,R., Weiss,A. and Allison,J.P. (1987). *Nature*, **330**, 170–173.

Hogan,B., Constantin,F. and Lacy,E. (1986) *Manipulating the mouse embryo: a laboratory manual.* Cold spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Huang,K., Im,S.-Y., Samlowski,W.E. and Daynes,R.A. (1989) *J. Immunol.*, **143**, 229–238.

Jones,D.T. and Reed,R.R. (1987) *J. Biol. Chem.*, **262**, 14241–14249.

Kim,S., Ang,S.-L., Bloch,D.B., Bloch,K.D., Kawahara,Y., Tolman,C., Lee,R., Seidman,J.G. and Neer,E.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4153–4157.

Kozak,M. (1986) *Cell*, **44**, 283–292.

Kuno,M. and Gardner,P. (1987) *Nature*, **326**, 301–304.

Ledbetter,J.A., June,C.H., Grosmaire,L.S. and Rabinovitch,P.S. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1384–1388.

Linch,D.C., Wallace,D.L. and O'Flynn,K. (1987) *Immunol. Rev.*, **95**, 138–159.

Locht,C., Cieplak,W., Marchitto,K.S., Sato,H. and Keith,J.M. (1987) *Infect. Immun.*, **55**, 2546–2553.

Marchitto,K.S., Munoz,J.J. and Keith,J.M. (1987) *Infect. Immun.*, **55**, 1309–1313.

Marth,J.D., Peet,R., Krebs,E.G. and Perlmutter,R.M. (1985) *Cell*, **43**, 393–404.

Marth,J.D., Lewis,D.B., Wilson,C.B., Gearn,M.E., Krebs,E.G. and Perlmutter,R.M. (1987) *EMBO J.*, **6**, 2727–2734.

Mishell,B.B. and Shiigi,S.M. (1980) *Selected methods in cellular immunology.* W.H.Freeman, San Fransisco.

Moriarty,T.M., Padrell,E., Carty,D.J., Omri,G., Landau,E.M. and Iyengar,R. (1990) *Nature*, **343**, 79–82.

Morse,S.I. (1964) *J. Exp. Med.*, **121**, 49–68.
Morse,S.I. and Riester,S.K. (1967) *J. Exp. Med.*, **125**, 401–408.
Morse,S.I. and Barron,B.A. (1970) *J. Exp. Med.*, **132**, 663–672.
Nicosia,A., Perugini,M., Franzini,C., Casagli,M.C., Borri,M.G., Antoni,G., Almoni,M., Neri,P., Ratti,G. and Rappuoli,R. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 4631–4635.
Nicosia,A., Bartoloni,A., Perugini,M. and Rappuoli,R. (1987) *Infect. Immun.*, **55**, 963–967.
O'Shea,J.J., Urdahl,K.B., Luong,H.T., Chused,T.M., Samelson,L.E. and Klausner,R.D. (1987) *J. Immunol.*, **139**, 3463–3469.
Rabinovitch,P.S., June,C.H., Grossmann,A. and Ledbetter,J.A. (1986) *J. Immunol.*, **137**, 952–961.
Reichert,R.A., Weissman,I.L. and Butcher,E.C. (1986) *J. Immunol.*, **136**, 3521–3528.
Rothenberg,E.V., McGuire,K.L. and Boyer,P.D. (1988) *Immunol. Rev.*, **104**, 29–53.
Runeberg-Nyman,K., Engstrom,O. Lofdahl,S., Ylostalo,S. and Sarvas,M. (1987) *Microb. Pathogenesis*, 3, 461–468.
Sartor,O., Gregory,F.S., Templeton,N.S., Pawar,S., Perlmutter,R.M. and Rosen,N. (1989) *Mol. Cell. Biol.*, **9**, 2983–2988.
Schrezenmeier,H., Ahnert-Hilger,G. and Fleischer,B. (1988) *J. Exp. Med.*, **168**, 817–822.
Seeburg,P.H. (1982) *DNA*, **1**, 239–249.
Simpson,L.L., Stiles,B.G., Zepeda,H. and Wilkins,T.D. (1989) *Infect. Immun.*, **57**, 255–261.
Spangrude,G.J., Braaten,B.A. and Daynes,R.A. (1984) *J. Immunol.*, **132**, 354–362.
Stanley,J.B., Gorczynski,R.M., Delovitch,T.L. and Mills,G.B. (1989) *J. Immunol.*, **142**, 3546–3552.
Sugimoto,M., Nakanishi,Y., Otokawa,M., Uchida,N., Yasuda,T., Sato,H. and Sato,Y. (1983) *J. Immunol.*, **130**, 2767–2774.
Sussman,J.J., Bonifacino,J.S., Lippincott-Schwartz,J., Weissman,A.M. Saito,T., Klausner,R.D. and Ashwell,J.D. (1988) *Cell*, **52**, 85–95.
Tamura,M., Nogimori,K., Yajima,M., Ase,K. and Ui,M. (1983) *J. Biol. Chem.*, **258**, 6756–6761.
Thom,R. and Casnellie,J.E. (1989) *FEBS Lett.*, **244**, 181–184.
Valentine,M.A., Tsoukas,C.D., Rhodes,G., Vaughan,J.H. and Carson,D.A. (1985) *Eur J. Immunol.*, *184*, 365–385.
Wang,P., Toyoshima,S. and Osawa,T. (1988) *J. Biochem.*, **103**, 137–142.
Weiss,A., Imboden,J., Hardy,K., Manger,B., Terhorst,C. and Stobo,J. (1986) *Annu. Rev. Immunol.*, **4**, 593–620.
Wickner,W.T. and Lodish,H.F. (1985) *Science*, **230**, 400–407.
Yatani,A., Mattera,R., Codina,J., Graf,R., Okabe,K., Padrell,E., Iyengar,R., Brown,A.M. and Birnbaumer,L. (1988) *Nature*, **336**, 680–682.

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