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Distinct T Cell Developmental Consequences in Humans and Mice Expressing Identical Mutations in the DLAARN Motif of ZAP-70

Melissa E. Elder,2* Suzanne Skoda-Smith,† Theresa A. Kadlec,§ Fengling Wang,* Jun Wu,† and Arthur Weiss2*§†

The protein tyrosine kinase, ZAP-70, is pivotally involved in transduction of Ag-binding signals from the TCR required for T cell activation and development. Defects in ZAP-70 result in SCID in humans and mice. We describe an infant with SCID due to a novel ZAP-70 mutation, comparable with that which arose spontaneously in an inbred mouse colony. The patient inherited a homozygous missense mutation within the highly conserved DLAARN motif in the ZAP-70 kinase domain. Although the mutation only modestly affected protein stability, catalytic function was absent. Despite identical changes in the amino acid sequence of ZAP-70, the peripheral T cell phenotypes of our patient and affected mice are distinct. ZAP-70 deficiency in this patient, as in other humans, is characterized by abundant nonfunctional CD4+ T cells and absent CD8+ T cells. In contrast, ZAP-70-deficient mice lack both major T cell subsets. Although levels of the ZAP-70-related protein tyrosine kinase, Syk, may be sufficiently increased in human thymocytes to rescue CD4 development, survival of ZAP-70-deficient T cells in the periphery does not appear to be dependent on persistent up-regulation of Syk expression. The Journal of Immunology, 2001, 166: 656–661.

Binding of Ag by the TCR initiates intracellular signaling pathways that lead to transcription of genes required for T cell activation. Propagation of Ag-binding signals from the TCR to the nucleus is dependent on activation of the nonreceptor cytoplasmic protein tyrosine kinases (PTKs),3 Lck and ZAP-70 (reviewed in Refs. 1–3). PTK activation is a rapid and critical event that results in phosphorylation of numerous downstream molecules, including phospholipase Cγ1 (PLCγ1), linker of activated T cells (LAT), Src homology 2 domain-containing 76-kDa leukocyte protein (SLP-76), and Vav (reviewed in Refs. 2–7). These tyrosine phosphorylation reactions are required for mobilization of intracellular free calcium ([Ca2+]i) and activation of the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase pathways, and culminate in T cell activation and initiation of T cell-specific responses (reviewed in Refs. 1 and 3–6).

One PTK critical for induction of Ag-specific T cell responses is ZAP-70. Recruitment of ZAP-70 to the TCR and its subsequent phosphorylation and activation, largely by Lck, is essential for all downstream signaling events (8–11). Activated ZAP-70 phosphorylates SLP-76 and LAT, triggering distal pathways that bring about T cell activation (12–15). Studies in humans and gene-targeted mouse models have confirmed the importance of ZAP-70 to TCR signaling (16–20). An autosomal recessive form of SCID in humans has been described due to mutations within the kinase domain of ZAP-70 that abolish protein expression (16–18). In humans, ZAP-70 deficiency is characterized by absent CD8+ T cells and normal numbers of nonfunctional CD4+ T cells in the peripheral blood. This unusual phenotype suggests that ZAP-70 is critical for CD8+ T cell development but may be dispensable for selection of CD4+ T lymphocytes in the thymus. The presence of peripheral CD4+ T cells in ZAP-70-deficient patients contrasts with their absence in ZAP-70 knockout mice, which are blocked at the CD4+CD8+ double-positive (DP) stage of thymocyte differentiation (20). Recently, a point mutation within the highly conserved DLAARN motif in the zap70 gene that results in the amino acid conversion R464C was described in inbred Strange (ST) mice (21). Although R464C affects ZAP-70 expression, its principal effect is to abrogate ZAP-70 catalytic activity (21). Like zap70 gene-targeted animals, the affected ST mice are devoid of mature T cells. In this report, we describe an infant with SCID who is homozygous for the comparable DLAARN mutation (R465C) but who has normal numbers of circulating CD4+ T cells. The biochemical consequences of this ZAP-70 mutation on T cell activation are examined, and the role of Syk expression in survival of human ZAP-70-deficient T cells in the periphery is assessed.

Materials and Methods

Patient

The patient was a 10-mo-old Caucasian male, the only child of second-degree cousins, who presented for immunologic evaluation after developing Pneumocystis carinii pneumonia at the age of 7 mo. Serum IgG was 63 mg/dl (normal range, 250–690 mg/dl) with normal IgM and IgA for age. Initial blood count revealed 10,600 leukocytes/mm3 and 80% lymphocytes. In vitro lymphocyte-proliferative responses were absent to PHA, PWM, and Con A. At 1 year of age, the patient received a T cell-depleted bone marrow transplant from his mother. He later developed a non-EBV-associated large B cell lymphoma that responded to treatment with combination
chemotherapy. Subsequently, the patient underwent successful retransplantation with mobilized peripheral blood stem cells from his father.

T cell culture and in vitro proliferative assays

Because the availability of the patient’s PBMC for study was limited, many experiments were performed with patient and normal T cell lines (TCL). For production of TCL, PBMC were grown for 2–4 wk in RPMI-C media (RPMI plus 20% human serum; NABI, Boca Raton, FL) with 0.5 mg/ml PMA (Sigma, St. Louis, MO), 1 μM ionomycin (Calbiochem, La Jolla, CA), and 20 IU/ml IL-2 (Boehringer Mannheim, Indianapolis, IN). Immunofluorescence analysis confirmed that the TCL were CD3+. For some experiments, CD8+ T cells were depleted from normal TCL using magnetic beads (Dynal Biotech, Lake Success, NY). In vitro lymphocyte proliferative assays were done as previously described (22).

Immunofluorescence analyses

Cell surface expression of CD3, CD4, CD8, and CD69 was determined using a FACScalibur (Becton Dickinson, San Jose, CA). Leu-4 (anti-CD3)-FITC, Leu-4-PerCP, Leu-2a (anti-CD4)-FITC, Leu-3-PE, and anti-CD69-PE were obtained from Becton Dickinson, and anti-CD3-tricolor (TC) and anti-CD8-TC from Caltag (Burlingame, CA). CD3/4/8 expression on lymph node cells was determined in the University of Florida Clinical Laboratory (Gainesville, FL). CD69 expression was analyzed on resting PBMC and after incubation with PHA (Sigma) for 4 h at 37°C. Intracellular Syk expression was determined as described (23). Briefly, 0.25–2×10^6 PBMC were stained with Leu-3-FITC, washed, and fixed in PBS plus 4% paraformaldehyde. After incubation with 2.4G2 (Fc-blocking mAb), PBMC were permeabilized in PBS plus 1% BSA and 0.1% saponin and stained with FITC-conjugated 4D10.1 (anti-Syk mAb).

Immunoblotting assays

For determination of tyrosine phosphorylons, 2×10^6 TCL or CD8-depleted TCL were incubated for 20 min on ice with Leu-3-biotin plus Leu-4-biotin or PBS alone, washed, and cross-linked with avidin for 4 min at 37°C. Alternatively, 2×10^6 TCL or CD8-depleted TCL were incubated for 4 min with 235 (anti-CD3 IgM mAb; a gift of S. M. Fu, University of Virginia, Charlottesville, VA). Cells were lysed in Nonidet P-40 plus 100 μM Tris (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM NaVO_4 for 30 min at 4°C. Whole lystate lysates were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore, Bedford, MA), blocked, and then probed with 4G10 (anti-phosphotyrosine mAb). Protein blots of ZAP-70 were also done using PBMC, Leu-3-PE, and anti-CD69-PE were obtained from Becton Dickinson, and anti-phospho-MAPK (New England Biolabs, Beverly, MA). Protein blots of ZAP-70 were also done using PBMC extracts.

Immunoprecipitation and in vitro kinase assays

Precleared extracts of 2.5×10^7 TCL were incubated with anti-PLCγ2 mAb (Upstate Biotechnology)-conjugated protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Immunoprecipitates were fractionated by SDS-PAGE and incubated first with either 4G10 or anti-PLCγ2 mAb and then HRP-conjugated goat anti-mouse mAb before ECL. In vitro kinase assays, precleared extracts from 2.5×10^7 TCL were immunoprecipitated with rabbit anti-human ZAP-70-coated protein A-Sepharose beads, washed in kinase buffer (10 mM Tris (pH 7.4), with 10 mM MnCl_2), and incubated with [γ-32P]ATP (5000 Ci/mmol; NEN, Life Science Products, Boston, MA) and 3.5 μg GST-band III for 10 min at 25°C. Membranes were treated with 1 M KOH before autoradiography or immunoblotting, as described (24).

[Ca^2+] mobilization

TCL at 2×10^7 cells/ml rested overnight in RPMI-C were loaded with 3 μM Indo-1 (Molecular Probes, Eugene, OR) for 20 min at 37°C, washed, and resuspended at 5×10^6 cells/ml in HBSS plus 1% BSA, 1 mM CaCl_2, and 0.5 mM MgCl_2, as described (17, 19). Cells were stimulated with 235 mAb or Leu-4-biotin plus avidin. Fluorescence measurements were performed using a Hitachi 4500 spectrophotometer (Hitachi, San Jose, CA) at an excitation wavelength of 355 nm and emission wavelengths of 400 and 500 nm. To ensure loading of Indo-1, 1 μM ionomycin was added to the cell suspensions.

DNA isolation, PCR amplification, and sequencing

Genomic DNA was isolated from PBMC using standard SDS/proteinase K digestion and phenol/chloroform extraction. Total RNA was isolated from PBMC using TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized from total RNA using oligo(dT) or random primers. RT-PCR was used to amplify ZAP-70 cDNAs. PCR primers used to generate nearly full length cDNAs (bp 33–1960; stop codon at bp 2067) were 5'-GGAGCTCTAGAGACACCCATG-3' (primer A) and 5'-GGTTACTAGCGGCTGAGCAAGCAGA-3' (primer B), respectively. Nucleotides 1554–2178 of ZAP-70 were amplified separately using primers C (5'-GGGATGAATACCTGGAAGGAAAGG-3') and D (5'-GGTTGCTTCACACAGACGCTG-3'). ZAP-70 sequence from nucleotides 1554–1877 was amplified from genomic DNA using primers C and E (5'-GGCCTATCCAGACGAGCAG-3'). PCR-amplified uncloned cDNA and genomic DNA products or cDNA and genomic DNA clones isolated from independent RT-PCR were sequenced manually using either the T7 Sequenase version 2.0 PCR product or the T7 Sequenase version 2.0 DNA sequencing kits (Amersham Pharmacia Biotech), respectively.

Results

Absent peripheral CD8+ T cells and defective CD4+ T cell proliferation to TCR-mediated stimuli

Flow cytometric analysis of the patient’s PBMC demonstrated decreased percentages (38–42%), but normal numbers (>3500 cells/μl), of circulating CD3+ T cells (data not shown). The percentage of peripheral blood CD4+ T lymphocytes was mildly decreased for age (37–40%), but the absolute number (>3000 cells/μl) was normal. In contrast, the patient had few CD8+ T cells in either his blood (<170 cells/μl) or a femoral lymph node (Fig. 1). The absence of CD8+ T lymphocytes in the peripheral circulation suggested an abnormality of T cell development and, specifically, in thymic selection. CD8 deficiency in humans is commonly associated with defective ZAP-70 expression (16–19). A diagnosis of a T cell signal transduction abnormality such as ZAP-70 deficiency was suggested by findings that the patient’s T cells were refractory to anti-CD3 stimulation in vitro (425 cpm (patient) vs 92,993 cpm (control)) but responded normally to PMA plus ionomycin (38,713

![FIGURE 1. Lack of peripheral CD8+ T cells in a patient with SCID. PBMC and isolated lymph node cells obtained from the patient and normal individuals were stained with Leu-3 (CD4)-PE and Leu-2a (CD8)-FITC, and analyzed by flow cytometry. Percentages of CD4+ and CD8+ T cells are indicated in italics.]
Deficient tyrosine phosphorylation is not due to absent ZAP-70 protein in the patient’s T cells

To confirm that the patient had a functional defect in coupling of the TCR to distal signaling pathways, we analyzed whether his TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-mediated cross-linking of CD3 or both CD3 and TCL was capable of triggering cytoplasmic PTK reactions on TCR engagement. Ab-med...
TCL, ZAP-70 protein from the patient’s TCL could not phosphor- 
ylate GST-band III or undergo autophosphorylation after TCR li-
gation (Fig. 4). This finding suggested that, like the comparable 
R464C mutation in ST mice, the principal effect of the R465C 
mutation in humans is to abolish ZAP-70 kinase function.

We subsequently confirmed that the mutant ZAP-70 was cata-
lytically inactive by evaluating ZAP-70-dependent phosphoryla-
tion events in the patient’s T lymphocytes. In contrast to a TCR-
stimulated normal TCL, tyrosine phosphorylation of PLCγ1 and 
the MAPKs, Erk1/2, was significantly decreased in the patient’s 
TCL (Fig. 5A). Consistent with a defect in PLCγ1 phosphoryla-
tion, no increase in [Ca$^{2+}$], was observed in the patient’s TCL after 
incubation with two different CD3 mAbs (Fig. 5B). Moreover, the 
patient’s T cells did not inducibly express the activation marker, 
CD69, after TCR stimulation by PHA (Fig. 5C). CD69 expression 
is dependent on activation of the Ras/MAPK pathway. These 
results demonstrate that, despite its expression in the patient’s T 
lymphocytes, the mutant ZAP-70 could not phosphorylate down-
stream substrates required for activation of the calcium and Ras/
MAPK pathways.

Survival of the patient’s T cells in the circulation is not due to 
up-regulation of Syk expression

Findings in previous ZAP-70-deficient patients have led to the sug-
gestion that Syk may compensate for loss of ZAP-70 in the thy-
mus, thereby rescuing development of some CD4$^+$ T cells (23). To 
determine whether Syk plays a role in the survival of ZAP-70-de-
deficient T cells in the peripheral circulation, we analyzed intra-
cellular Syk expression in PBMC at the single-cell level. Immu-
nofluorescence analysis revealed that ~5% of the patient’s peripheral 
CD4$^+$ T cells had detectable Syk expression, in comparison with ~2% of normal CD4$^+$ T cells (Fig. 6). The level of 
Syk observed in this CD4$^+$ T cell subpopulation was approxi-
ately one-tenth that demonstrated in human B cells. The percentage 
of CD4$^+$ Syk$^{\text{intermediate}}$ T cells increased to 13% in the pa-

ten’s TCL but remained unchanged in the normal TCL (data not shown). However, despite increased Syk expression, the patient’s 
cultured CD4$^+$ T cells were unresponsive to TCR-mediated stimul-
i. Although not identical, similar findings were reported recently in 
cultured T cells from two siblings homozygous for the mutation 
A507V, which abolishes both ZAP-70 expression and function

FIGURE 4. R465C abrogates ZAP-70 catalytic activity. Whole cell lys-
ates of patient (Pt) and normal (Ni) TCL stimulated with 235 (anti-CD3 
IgM mAb) were immunoprecipitated with rabbit anti-human ZAP-70-
coated beads and incubated with GST-band III and [γ-32P]ATP before 
autoradiography in an in vitro kinase assay. Blots were stripped and re-
probed with 2F3.2 (anti-ZAP-70 mAb) or anti-GST Ab.

(27). Despite increased Syk expression and rescue of SLP-76/ 
LAT/PLCγ1 phosphorylation and [Ca$^{2+}$], mobilization after long 
term culture, induction of downstream signaling events, including 
Ras/MAPK activation and CD3-mediated IL-2 production, re-

mained markedly defective in TCL from those patients (27).

Discussion

Novel human ZAP-70 mutation

We describe an infant with SCID due to a novel mutation in the 
zap70 gene that abrogates enzymatic function without appreciably 
ffecting either expression or phosphorylation of ZAP-70. With the 
exception of one child who lacked ZAP-70 mRNA by Northern 
 blot in whom the underlying mutation is not known (19) and an-
other child with temperature-sensitive P80Q and M572L mutations 
(28), ZAP-70-deficient patients have inherited mutations within a 
small region of the ZAP-70 kinase domain that significantly affect 
both protein stability and catalytic activity. These included a 13-bp 
deletion resulting in a frame-shift after residue 503 and premature 
termination at amino acid 538 (16), two amino acid conversions, 
S185R (17) and A507V (27), and a splicing error that inserts LEQ 
to the protein after residue 541 (17, 18).

Role of Syk in CD4$^+$ thymocyte selection in human ZAP-70 
deficiency

The development of peripheral CD4$^+$ T cells in ZAP-70-deficient 
patients, which is not seen in mice deficient in ZAP-70, has been 
attributed in part to the nature of the underlying mutations that 
conceivably may allow for some residual ZAP-70 activity in vivo. 
However, our patient provides the first description of identical mu-

nations in zap70 having disparate effects on T cell development in 
humans vs mice. To explain these findings, we suggest instead that 
the ability of Syk to contribute to pre-TCR and TCR signaling is 
substantially different in humans as compared with mice. Our con-
clusion is supported by recent observations that Syk is expressed at 
highest levels in human and murine thymocytes during stages of 
development in which pre-TCR signaling is required (23). In con-
trast to mice, which down-regulate Syk expression to peripheral T 
cell levels at the CD44 CD25$^+$ checkpoint, human DP thymo-
cyes express significant levels of Syk and do not completely 
down-regulate this PTK to levels seen in peripheral T lymphocytes 
until after positive selection has commenced (23). Consistent with 
these observations, TCR-stimulated HTLV-1-transformed DP thy-
mocytes, but not peripheral T cells, from a ZAP-70-deficient pa-

\[\begin{align*}
\text{T-cell line:} & \\
\text{TCR:} & \\
\text{Pt} & \quad \text{Ni} \\
\text{ZAP-70} & \\
\text{GST-Band III} & \\
\text{ZAP-70} & \\
\text{GST-Band III} & \\
\end{align*}\]

Findings in our patient support the notion that the fundamental 
role of ZAP-70 in TCR signaling is phosphorylation of down-
stream substrates, including PLCγ1, SLP-76, and LAT. Although 
ZAP-70 is most important for initiation of downstream signaling 
events in peripheral T cells, findings in ZAP-70-deficient patients 
suggest that Syk, at levels detectable in the thymus, is capable of 
transducing signals from both the pre-TCR and TCR. However, 
under normal circumstances, Syk appears to play a role for the 
most part in early αβ T cell differentiation, whereas ZAP-70 func-
tion is critical for positive and negative selection in the thymus and 
T cell activation in the periphery. Our results differ from a recent
observation that a subset of cultured primary T cells from SCID patients with a ZAP-70 null mutation are capable of proximal TCR signaling as a result of markedly increased Syk levels (27). We suggest that, despite increased Syk expression in a subset of our patient’s TCL, the catalytically inactive ZAP-70 mutant is able to compete favorably for immunoreceptor tyrosine-based activation motif binding, thus inhibiting Syk phosphorylation of downstream effectors critical to activation of the calcium and Ras/MAPK pathways.

Findings in ST and ZAP-70-knockout thymocytes suggest that another important function of ZAP-70 is to promote immunoreceptor tyrosine-based activation motif phosphorylation of TCR subunits in DP cells limited in their expression of Lck (29). Although not equivalent cell types, reduced ζ phosphorylation was also observed in our patient’s TCL, despite the presence of considerable Lck and mutant ZAP-70 protein.

In summary, we suggest that human and murine DP thymocytes have different dependencies on ZAP-70 function during development, based on the T cell phenotypes of comparable ZAP-70 mutations. Despite similar effects on TCR signaling, a patient with a catalytically inactive ZAP-70 protein is able to produce large numbers of nonfunctional peripheral CD4⁺ T cells. The elevated levels of Syk in human DP thymocytes, in contrast to those in mice, may explain the differential consequences of identical ZAP-70 mutations.

Syk expression in ZAP-70-deficient peripheral T cells
Recent studies in mice demonstrate that survival of circulating T cells is diminished in the absence of TCR ligands (30–32), suggesting that TCR-mediated signals are required for maintenance of the peripheral T cell pool. However, survival of CD4⁺ T cells in

**FIGURE 5.** Distal signaling pathways are poorly activated in the patient’s T cells. A. Phosphorylation of ZAP-70 substrates. Patient (Pt) and normal (Nl) whole cell lysates were immunoprecipitated (IP) with anti-PLCγ1 mAb-coated beads. Immunoblots were probed with 4G10 (anti-phosphotyrosine) or anti-PLCγ1 mAb. Stripped whole cell lysate 4G10 blots were reprobed with anti-phospho-MAPK mAb to detect phospho-Erk1/2. B. Mobilization of [Ca²⁺]i after TCR stimulation. Indo-1-loaded TCL were stimulated with 235mAb(anti-CD3IgM) or Leu4(anti-CD3)-biotin plus avidin (Av). Ionomycin (Iono) was added to ensure that the cells were loaded properly with Indo-1. Changes in [Ca²⁺]i in nanomols over time (s) were determined by fluorometry. C. CD69 expression by immunofluorescence. PBMC were stained with anti-CD19 (B cell) mAb or Leu-3 (anti-CD4)-PE plus anti-CD3-tricolor, permeabilized, and stained with 4D10.1-FITC (anti-Syk); 4D10.2-FITC plus competitor peptide (anti-Syk + pep); or an isotype-matched staining control (control Ig), before analysis by flow cytometry. Percentages of gated CD3⁺CD4⁺ Syk⁺ cells are indicated in italics.

**FIGURE 6.** Immunofluorescence analysis of intracellular Syk expression. Patient and normal PBMC were stained with either anti-CD19 (B cell) mAb or Leu-3 (anti-CD4)-PE plus anti-CD3-tricolor, permeabilized, and stained with 4D10.1-FITC (anti-Syk); 4D10.2-FITC plus competitor peptide (anti-Syk + pep); or an isotype-matched staining control (control Ig), before analysis by flow cytometry. Percentages of gated CD3⁺CD4⁺Syk⁺ cells are indicated in italics.
the blood of ZAP-70-deficient patients would not appear to depend on ZAP-70-dependent TCR signaling or to up-regulation of Syk expression. It is doubtful that the presence of a Sykintermediate sub-set can account entirely for survival of the patient’s considerable number of circulating CD4$^+$ T cells. Alternatively, the level of Syk activity required for peripheral T cell survival may be less than that required for propagation of TCR signals. Further investigation is required to determine whether viability of ZAP-70-deficient T cells is dependent on residual Syk function and whether these CD4$^+$ T cells have a normal life span in the peripheral circulation.

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References