Impaired T And B Cell Development in Tcl1 Deficient Mice

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Abstract

TCL1, the overexpression of which may result in T cell leukemia, is normally expressed in early embryonic tissues, the ovary and lymphoid lineage cells. Our analysis of mouse B lineage cells indicates that Tcl1 expression is initiated in pro-B cells and persists in splenic marginal zone and follicular B cells. T lineage Tcl1 expression begins in thymocyte progenitors, continues in CD4⁺CD8⁺ thymocytes, and is extinguished in mature T cells. In Tcl1 deficient mice, we found B lymphopoiesis to be compromised at the pre-B cell stage and T cell lymphopoiesis to be impaired at the CD4⁺CD8⁺ thymocyte stage. A corresponding increase was observed in thymocyte susceptibility to anti-CD3ε induced apoptosis. Reduced numbers of splenic follicular and germinal center B cells were accompanied by impaired production of IgG1 and IgG2b antibodies in response to a T-dependent antigen. The marginal zone B cells and T-cell independent antibody responses were also diminished in Tcl1 knockout mice. This analysis indicates a significant role for Tcl1, a coactivator of Akt signaling, in normal T and B cell development and function.
Introduction

T prolymphocytic leukemias (T-PLL) in humans often have chromosomal translocations that juxtapose the TCR α/δ or β locus to the proximity of the T cell leukemia/lymphoma-1 gene (TCL1) located in the 14q32.1 region or, less frequently, to lie near its mature T cell proliferation 1 (MTCP 1) gene homolog in the Xq23 region. The ensuing aberrant influence of a TCR enhancer element results in overexpression of the TCL1 or MTCP1 genes. Overexpression of either TCL1 or MTCP1 in transgenic mouse models employing a T cell specific promoter may also result in a T cell leukemia that resembles human T-PLL. In addition to the implicit TCL1 involvement in this T cell malignancy, a variety of B lineage tumor cell lines, ranging from pre-B cell to mature B cell phenotype, have also been shown to express high Tcl1 levels. Moreover, TCL1 overexpression under the control of B lineage specific enhancer and promoter elements has been shown to promote B cell chronic lymphocytic leukemia and B cell lymphomas in mice. An important clue to the role of TCL1 in the leukomogenesis process is provided by the functional linkage of Tcl1 to Akt kinase, an intracellular component that participates in the transduction of anti-apoptotic and proliferative signals. In the Akt signaling cascade, Tcl1 acts as an Akt cofactor to enhance kinase activity and nuclear translocation. Tcl1 binding to Akt also facilitates the formation of Akt-Tcl1 hetero-oligomers. The resultant (trans)phosphorylation of Akt1 at Ser473 may thus amplify the PI3-Akt1 pathway to contribute a survival advantage.

Normally TCL1 expression is tightly regulated, being confined to lymphoid and germinal cells in humans and mice. In human B lineage cells, TCL1 expression is initiated in pro-B cells, peaks in the pre-B cells, and persists in IgM-bearing B cells.
High expression levels of \textit{TCL1} transcripts have been found in the mantle zone B cells in the spleen, whereas TCL1 expression is down regulated in germinal center and marginal zone B cells, and is extinguished in terminally differentiated plasma cells \cite{9-11}. In human T lineage cells, \textit{TCL1} expression is seen in the intrathymic CD4\textsuperscript{+}CD8\textsuperscript{-} subpopulation, but not in mature T cells. In the present studies we observed a similar pattern for Tcl1 expression in mouse T and B lineage cells. In order to gain insight into the physiological role(s) that Tcl1 may have in T and B lymphopoiesis, we have examined both pathways of lymphocyte development and their cooperative function in antibody responses of Tcl1 deficient mice. These mice are shown to have modestly compromised T and B lymphopoiesis due either to impaired cellular proliferation or enhanced apoptosis.

\textbf{Materials and Methods}

\textit{Mice, cell preparation, cell counting, and statistical analysis}

\textit{Tcl1}\textsuperscript{−/−} and \textit{Tcl1}\textsuperscript{+/−} mice were generated as described previously \cite{20}. Bone marrow (BM) cells were obtained by flushing the cavities of both femoral and tibial bones with media. Thymus and spleen samples were minced between frosted ends of glass slides. Cells suspended in media were then filtered through fine metal screens or Nylon membranes (Fisher, Pittsburgh, PA) to remove cellular debris. Erythrocytes were lysed in a 0.1 M ammonium chloride buffer at pH 7.4 (Sigma-Aldrich) for 1 - 2 min at room temperature and the cells washed in FACS buffer (1% fetal calf serum in PBS) before enumeration of the nucleated cells by light microscopy. For isolation of peripheral blood mononuclear cells (PBMC), blood collected from \textit{Tcl1}\textsuperscript{+/+}, \textit{Tcl1}\textsuperscript{+/−}, and \textit{Tcl1}\textsuperscript{−/−} mice was layered over lymphocyte separation medium (density 1.078 g/ml; Cellgro, Mediatech Inc, Herndon,
VA) and centrifuged to remove leukocytes and erythrocytes. Since there were no differences between wild type and heterozygous mice in lymphoid organ cellularity, we grouped wild type and heterozygous mouse data together. The total number of cells in each population was estimated by enumerating the nucleated cells by light microscopy and determining the percentage for each population by immunofluorescence. The statistical significance of population differences was calculated by a Student’s 2-tailed t-test using the Excel program.

**Antibodies**

PerCP (peridinin chlorophyll protein)-labeled anti-B220, APC (allophycocyanin)-labeled anti-B220, FITC (fluorescein isothiocyanate)-labeled anti-S7/CD43, PE (phycoerythrin)-labeled anti-S7/CD43, FITC-labeled anti-CD4, PE-labeled anti-CD4, FITC-labeled anti-CD8, PE-labeled anti-CD8, FITC-labeled anti-CD25, PE-Cy5-labeled anti-CD44, PE-labeled anti-IgD, FITC-labeled anti-IgD, Biotin-labeled anti-IgM; PE-labeled anti-CD5, PE-labeled anti-B220, and PE-Cy5 (CyChrome)-labeled anti-CD19, were obtained from PharMingen (San Diego, CA). FITC-labeled goat anti-mouse IgM, biotin-labeled goat anti-rat Ig, streptavidin (SA)-Cy-Chrome, SA-PE, and SA-APC were obtained from Southern Biotechnology Associates (Birmingham, AL). FITC-labeled anti-peanut agglutinin (PNA) was obtained from Vector Laboratories, Inc, Burlingham, CA)

**Flow cytometric analysis and cell sorting**

Cells ($10^6$) were incubated with FcR blocker (PharMingen) before staining with FITC-, PE-, PerCP-, PE-Cy5-, APC-labeled monoclonal antibodies against cell surface antigens.
After washing, the stained cells were analyzed using a FACS Calibur instrument (Becton-Dickinson, Franklin Lakes, NJ) and WINMDI 2.8 software [The Scripps Research Institute Cytometry Software Page (http://facs.scripps.edu/software.html)]. Lymphocyte subpopulations were purified by differential immunofluorescence cell sorting with a MoFlo instrument (Cytomation, Fort Collins, CO).

**Analysis of Tcl1 gene family transcripts**

RNA was isolated from FACS-sorted subpopulations (96 – 99 % purity) of lymphocytes from tissue samples using the TRI Reagent (Molecular Research Center, Cincinnati, OH) and dissolved in 20 µl of RNase-free distilled water (Ambion, Austin, TX). First-strand cDNA was synthesized from the isolated RNA (10 µl) using oligo(dT)$_{15}$ primers and RNase H$^{-}$ reverse transcriptase (Superscript II, GIBCO/BRL, Carlsbad, CA) in a total volume of 40 µl. 2 µl of cDNA were used for PCR. The first round of PCR for Tcl1 amplification was carried out with 5’orf (5’-ATGGCTACCCAGCGGGCACAC-3’) and 3’ orf (5’-GTTATTCATCGTTGGACTCCGAG-3’) primers. After denaturation at 94$^{\circ}$ C for 4 min, 30 cycles of PCR were performed with the following conditions: 94$^{\circ}$ C for 1 min, 61$^{\circ}$ C for 1 min, 72$^{\circ}$ C for 30 sec. 2 ul of first PCR reaction were used in a nested PCR of 30 cycles using 5’ sense (5’-ACACCCCAACCGCCTGATGATC-3’) and 3’ reverse (5’-GATATGGTACAGGATCTGCCAATAC-3’) primers. As a cDNA quality control, murine β-actin cDNA was amplified (single round of PCR) under the same PCR conditions except for an annealing temperature of 58$^{\circ}$ C using 5’ oligo (CCTAAGGCCAAAAGGTGAAAG) and 3’ oligo (5’-TCTTCATGGTGCTAGG-AGCCA-3’) primers. To verify the murine Tcl1 cDNA PCR product, a Southern blot
analysis was performed with 5 µl of 10 times diluted nested PCR products using a specific internal Tcl1 probe (5‘-GGGAGAAGCAGCAGTTGGATGAG-3’). The β-actin cDNA internal probe was 5’-CACCCCAGCCATGTACGTAGCCATCC-3’ . PCR products were separated on an agarose gel and transferred to a Nylon membrane (Amersham, Buckinghamshire, UK). The gene specific probes were labeled using 20 U of T4 polynucleotide kinase in 40 ul labeling mixture (40 µCi of [γ-32P]ATP and 1X kinase buffer (Gibco BRL). PCR primers used for evaluation of the Tcl1b1-b5 genes were: Tcl1-b1 F: GCA GCT TTT GAT CCC CTG GGG C and R: 5’ GAG AAC GGT CAG GAC CCA AAC C with annealing of 70° C; Tcl1b2 F: TGC AGG TTT TTA TCC TCC GA and R: CCT TTT ACT CCA GCA TCA GGA TC with annealing at 55°C; Tc11 B4 F: AGT CCC GAC TCT CTC AAG ACT TT and R: CAA AGG CAC AAA GTG AGC AAG AG with an annealing temperature of 60°C ; Tcl1B5 F: CTG TGT CTG TTG ATC CCC AG and R: TCA TCC TCG CCT ATT ATT ATG TC with annealing at 55 °C. All the reactions were denatured at 94°C for 30”; annealed at the specific temperature for 30” and elongated at 72°C for 1’.

**Anti-CD3 antibody treatment and immunizations**

Mice, 8 – 12 weeks old, received a single intraperitoneal injection of 25 µg of anti-CD3ε antibody. The hamster anti-mouse CD3ε antibody (clone 145-2C11) was kindly provided by Dr. Chander Raman (University of Alabama at Birmingham). Mice, 11 – 13 weeks old, were immunized intravenously with 2-4 x 10⁸ sheep red blood cells (SRBC; Colorado Serum Company, Denver, Co) to elicit SRBC specific antibody immune responses or immunized intraperitoneally to evaluate germinal center (GC) responses.
Pre-immune blood samples were obtained 2 days before immunization, and blood samples were drawn 1 week after immunization. Spleens were obtained 4 days after an intraperitoneal immunization to evaluate GC formation. To evaluate T independent antigen responsiveness, mice (11 – 16 weeks old) were immunized intraperitoneally with $1 \times 10^8$ heat-inactivated S. pneumoniae organisms (a gift from Dr. John Kearney, UAB).

**In vitro proliferation and survival assays**

Cell suspension of spleen and thymus samples were depleted of red cells and macrophages and cultured at $1 \times 10^5$ cells per well in 96-well plates in RPMI medium 1640 with 10% FCS. Splenocytes were cultured with 50 µg/ml of Escherichia coli lipopolysaccharide (LPS; Calbiochem, San Diego, CA) for 2, 4 and 9 days. Before each checkpoint, BrdU (Sigma Aldrich, St. Louis, MO) was added at a concentration of 30 ng/ml and incubation continued for 6 hours at 37°C/5% CO$_2$. MoAb anti-BrdU-FITC conjugated (Becton-Dickinson) staining and propidium iodide labeling were used to identify non-apoptotic cells undergoing DNA synthesis by flow cytometry.

**Immunoglobulin and antibody measurement**

Serum immunoglobulin (Ig) levels were measured by ELISA using polyvinyl chloride microtiter plates (Dynex Technologies, Chantilly, VA) and goat antimouse immunoglobulin antibodies labeled with alkaline phosphatase (AP) (Southern Biotechnology Associates, Birmingham, Al). A standard regression analysis curve was used to calculate relative Ig concentrations in individual samples based on OD measurements at 405nm in duplicate wells using p-nitrophenyl phosphate as AP substrate.
(Sigma Aldrich). Plastic wells were coated with goat anti-mouse immunoglobulins (1 µg/ml) overnight at 4°C and then blocked with 1% bovine serum albumin in PBS. Duplicates of a diluted serum sample were added and Ig isotypes were measured in serum samples by employing goat anti-mouse isotype specific antibodies conjugated with AP. Isotype specific anti-SRBC antibodies were measured by fixing SRBC onto the plastic plate \(^{21}\). Anti-PC (phosphocholine) specific antibodies were similarly measured in plastic wells coated with PC-BSA (a gift from Dr. John Kearney, UAB).

**Results**

*Tcl1 expression in normal lymphoid tissues*

In an analysis of the lymphoid tissues from wild-type mice, *Tcl1* transcripts could be detected by nested PCR in bone marrow, thymus, spleen, lymph nodes and peripheral blood lymphocytes, but not in lymphocytes from the Peyer’s patches or intestinal epithelium. To evaluate *Tcl1* expression as a function of lymphocyte differentiation, the levels of *Tcl1* transcripts were assessed in purified subpopulations of bone marrow, thymus and splenic lymphocytes. In the bone marrow, *Tcl1* transcripts were detected at low levels in the pro-B fraction of B220⁺CD43⁻IgM⁻ cells, and in slightly increased levels in pre-B cells (B220⁺CD43⁺IgM⁻) and immature B cells (B220⁺IgM⁺IgD⁻) (Fig. 1 A). Low levels of Tcl1 protein were also detectable in these cells by using a monoclonal antibody \(^{20}\).

When thymocyte subpopulations were examined, the levels of *Tcl1* transcripts were highest in double negative cells and in double positive thymocytes. No signal was detected in CD4 single positive thymocytes, and only a faint *Tcl1* band was visible in the
CD8 single positive thymocytes, perhaps reflecting the presence of the immature CD8 single positive cells in this population. To determine more precisely at which thymocyte progenitor stage Tcl1 expression appears, the CD4/CD8 double negative thymocytes were sorted on the basis of differential CD44 and CD25 expression. Tcl1 transcripts were expressed in easily detectable levels only in the late stage subpopulation of thymocyte progenitors (CD4^−CD8^−CD44^−CD25^−) that precedes the CD4^+CD8^+ double positive differentiation stage (Fig. 1). Tcl1 expression is thus preferentially expressed during the intermediate intrathymic differentiation stages when T cells are undergoing proliferation and clonal selection.

For analysis of Tcl1 expression by splenic B cells, marginal zone (MZ) B cells (B220^+CD21^highCD23^low), follicular B cells (B220^+CD21^intCD23^high), and newly formed B cells (B220^+CD21^lowCD23^low) were sorted. Tcl1 mRNA could be detected in all of these B cell subpopulations, although MZ B cells appeared to express Tcl1 in higher levels than follicular B cells (Fig. 1). Lymph node B cell subpopulations clearly expressed Tcl1 transcripts. Tcl1 transcripts were found only in trace levels in splenic CD4^+ and CD8^+ T cells, and not at all in lymph node T cells. Low levels of Tcl1 transcripts were detected in circulating B cells, but were not seen in other types of circulating lymphocytes. The overall pattern of Tcl1 expression in the mouse during T and B lineage differentiation thus closely resembles that observed in humans.

**Impaired T and B lymphopoiesis in Tcl1 deficient mice**

Although the Tcl1^−/− mice have a fertility defect that is manifested by reduced litter size, the Tcl1^−/− newborns appeared normal and continued to develop normally. Histological
examination of the lymphoid organs of the Tcl1−/− mice did not reveal obvious developmental abnormalities, nor did we observe significant differences in organ size and total body weight (data not shown). However, reduced numbers of lymphocytes were found in the bone marrow, thymus and spleen (Table 1). Both the percentages (wild type and heterozygous mice, 29.1 ± 5.2% vs. Tcl1+/−, 20.7 ± 5.3%; \( p = 0.004 \)) and numbers (wild type and heterozygous mice, 147.5 ± 13.3 x 10⁵ vs. Tcl1+/−, 99 ± 8.7 x 10⁵; \( p = 0.0007 \)) of B220+ B lineage cells were significantly decreased in the bone marrow of Tcl1−/− mice. The numbers of myeloid-lineage cells in bone marrow were comparable in Tcl1−/− and wild type control mice (Tcl1+/+ or Tcl1+/-), although slightly increased percentages of Mac-1+ myeloid cells were observed in the Tcl1−/− mice (Tcl1−/−, 72.0 ± 3.3% vs. wild type and heterozygous mice, 64.5 ± 6.9%). A 50% reduction in the numbers of cells in the pre-B and immature B cell subpopulations (\( p \) values of 0.0008 and 0.0009, respectively) was evident in the Tcl1−/− mice (Fig. 2). Mature B cell numbers were also lower in the bone marrow of Tcl1−/− mice, but the reduction was not significant (\( p > 0.05 \)). Impaired generation of B lineage cells in Tcl1−/− mice was thus manifested primarily at the pre-B and immature B cell stages in differentiation.

Analysis of thymocyte differentiation in the Tcl1−/− mice indicated a significant reduction in the numbers of immature CD4−/CD8− thymocytes and of intermediate CD4+/CD8+ thymocytes (Fig. 1). The CD4+ and CD8+ single positive subpopulations of mature thymocytes were also slightly lower than normal, but these reductions were not statistically significant. When the CD44 and CD25 cell surface antigens were used to subdivide the CD4−/CD8− thymocytes, the hypocellularity noted for this progenitor population was primarily attributable to reduced numbers of cells in the CD4−CD8−CD44−
CD25− subpopulation, the stage at which pre-TCR expression occurs (Fig. 2). The impaired thymopoiesis in Tcl1−/− mice thus is manifested principally during the pre-T and intermediate thymocyte stages in T cell differentiation.

A significant reduction was observed in the numbers of splenic follicular and marginal zone subpopulations of B cells and in the numbers of splenic CD4+ and CD8+ T cells in young adult Tcl1−/− mice (Fig. 2). Notably, these data indicate that Tcl1 deficiency does not lead to a complete differentiation block in either lymphoid lineage.

**Hypersensitivity of TCR-bearing thymocytes to CD3 ligation in Tcl1−/− mice**

Since earlier studies suggest roles for the human TCL1 gene product in cellular proliferation 7,8 and in anti-apoptotic signaling 10,16,17, we tested the effects of apoptotic and proliferative stimuli under in vivo and in vitro conditions. When the rate of spontaneous apoptosis was assessed by measuring cell viability in thymocytes cultured in media or for thymocytes stimulated with dexamethasone, significant differences were not observed for the wt and null mice (data not shown). We therefore examined the effect of TCR/CD3 ligation-induced apoptosis as a surrogate model for the negative clonal selection that occurs during thymocyte development 29,30. When an anti-CD3ε antibody was injected into Tcl1−/− mice, the CD4+/CD8+ subpopulation comprised only 20% of the thymocytes 48 h later, whereas 51% of the double-positive thymocytes survived this treatment in wild type mice. A 56.5-fold reduction in the numbers of CD4+/CD8+ thymocytes and a 5-fold reduction in single-positive CD4+ thymocyte subpopulation occurred in the Tcl1−/− mice treated with the anti-CD3ε antibody. These results contrasted with a 15.8-fold reduction in CD4+/CD8+ thymocytes and a 2.1-fold in the CD4+
thymocytes after corresponding treatment of control mice (Table 2). The results of these experiments indicate increased susceptibility of the CD4+/CD8+ thymocytes and, to a lesser extent, of the CD4+ thymocytes to CD3 ligation-induced apoptosis in Tcl1−/− mice.

Splenic B-cells derived from young mice were also assayed for apoptosis after stimulation with LPS at 2, 4 and 9 days. A slight increase (around 5%) in the number of apoptotic cells, measured by PI incorporation, was observed for Tcl1+/− splenocytes compared to Tcl1+/+ splenocytes. A modest, but significant, difference was instead observed in the percentage of B220+ splenocytes when cell proliferation was measured after LPS stimulation (Tcl1+/+ 13.75±6.2 vs Tcl1−/− 9.36±5.1; p=0.046; wt=10; N≥7 in each group). Since TCL1 is known to activate AKT, we examined the possibility that the phosphorylation level and kinase activity of Akt could be altered in splenocytes or thymocytes of null mice. We could not observe any difference in the rate of Akt phosphorylation and kinase activation (not shown), a result that may reflect an alternative mechanism of Akt activation in the absence of Tcl1.

**Immunoglobulin levels, antibody responses and expression of Tcl1b1-b5 in Tcl1+/− mice**

Serum IgG1 and IgG2b levels in Tcl1−/− mice were approximately 40% lower in Tcl1+/− mice than in control mice, whereas other immunoglobulin isotypes were not significantly affected (Table 3). To assess antibody responsiveness to a thymus-dependent antigen, mice were immunized intravenously with sheep red blood cells (SRBC) and bled one week later. The levels of SRBC specific IgG1 and IgG2b antibodies in Tcl1+/− mice were approximately 40% of those in the control mice (Fig. 4). Tcl1+/− and control mice
produced comparable levels of IgG2a, IgG3 (Fig. 4 A) and IgM anti-SRBC antibodies (not shown). Since isotype switching occurs primarily within the germinal centers, germinal center formation was evaluated after SRBC immunization by enumerating the PNA-reactive B cells in the spleen. Although clear histological differences in germinal center reactions were not evident, there were fewer PNA-staining cells. Four days after intraperitoneal immunization, the frequency of PNA-positive cells was significantly lower in Tcl1−/− mice than in wild type mice, (3.1 ± 0.13% vs 3.9 ± 0.34%, n=4; p=0.04).

Examination of the T-independent antibody response to the immunodominant phosphocholine epitope of S. pneumoniae indicated that the levels of IgM anti-PC specific antibodies produced in Tcl1−/− mice were approximately 45% of those observed in control mice one week after immunization (Fig. 4 B). The compromised T-independent antibody response and reduction in the marginal zone subpopulation suggested an impairment in the B1 subset of Tcl1−/− mice. In accordance with this inference, a 40% reduction of circulating B cells with the B1 phenotype, IgM\textsuperscript{high}, IgD\textsuperscript{low}, CD5\textsuperscript{+} B220\textsuperscript{+}, was observed in Tcl1−/− mice (2.77± 0.27 vs 1.50 ±0.36 , n=7; p=0.03) thereby indicating a role for Tcl1 in the development of this subset of B cells.

Since other members of the TCL1 family gene have been described in humans and mice, we explored the possibility that these Tcl1 relatives could compensate in part for the effects of null Tcl1 alleles and thereby account for the modest immunodeficiency observed in the Tcl1−/− mice. When transcription of the Tcl1b1-5 genes was evaluated in bone marrow, thymus, spleen cells of Tcl1−/− mice by seminested RT-PCR, we were unable to amplify Tcl1b1, b2, b4 and b5 transcripts from any of the lymphoid organs.
whereas these transcripts were detectable in the analysis of a control egg library (data not shown), thereby suggesting that the \textit{Tcl1}^{-/-} phenotype is not affected by a compensatory expression of neighboring \textit{Tcl1b} genes.

**Discussion**

Since the recognition of TCL1 involvement in the pathogenesis of T cell leukemias, TCL1 has also been shown to be expressed in B cell tumor lines and during normal T and B lymphopoiesis in humans. The present analysis of \textit{Tcl1} expression in wild type mice and of the immune system alterations in \textit{Tcl1}^{-/-} mice indicates a significant role for \textit{Tcl1} in the generation of both T and B lymphocytes. In wild-type mice, \textit{Tcl1} expression was found to be upregulated during the early stages in lymphocyte differentiation when immature T and B lineage cells are undergoing proliferation and are highly sensitive to receptor mediated apoptosis \cite{38,39}. In \textit{Tcl1}^{-/-} mice, the numbers of thymocytes and bone marrow B lineage cells were significantly reduced. The differentiation stages most affected by the \textit{Tcl1} deficiency were the precursor subpopulations of T and B lineage cells that are undergoing positive and negative clonal selection via their antigen receptors \cite{40,41}.

The impairment in T and B lymphopoiesis that we observed in \textit{Tcl1}^{-/-} mice is also accompanied by functional deficits in immune responses. Following immunization with a T-dependent immunogen, the \textit{Tcl1}^{-/-} mice exhibited a reduction in numbers of germinal center B cells and impaired IgG1 and IgG2b antibody responses. Serum IgG1 and IgG2b levels were also significantly reduced in \textit{Tcl1}^{-/-} mice, whereas the levels of other immunoglobulin isotypes were not. This reduction in these switch isotypes was associated with reduced numbers of CD4$^+$ helper T cells and follicular B cells in the
spleen. \(Tcl1^{-/-}\) mice also displayed significantly impaired antibody responses to a T-independent antigen, the phosphocholine determinant of \(S.\ pneumoniae\). Reduced numbers of marginal zone B cells in the \(Tcl1^{-/-}\) mice further attest a \(Tcl1\) role in the response to polysaccharide antigens during bacterial infection. Together with the reduced numbers of B1 cells in these mice, these findings indicate a role for \(Tcl1\) in natural immunity. Conversely, overexpression of the human \(TCL1\) gene under the control of an Ig enhancer causes an expansion of B1 cells in transgenic mice that later undergo neoplastic transformation of their CD5\(^+\) B cells.

The impaired T and B cell generation observed in the \(Tcl1^{-/-}\) mice was relatively modest. This could reflect the relatively low levels at which Tcl1 is expressed in mouse lymphocytes relative to human lymphocytes. We also considered the possibility that the relatively modest phenotype seen in the \(Tcl1^{-/-}\) deficient mice might represent a functional compensation by Tcl1 gene relatives. However, expression of the Tclb1-b5 genes could not be detected in the lymphoid cells from either Tcl1 null or wild-type mice, thereby suggesting these genes exert their function in nonlymphoid cells. Tcl1b2 expression has been reported in lymphoid tissues, such as the spleen, but our analysis of isolated lymphocytes, the use of different primer pairs, and the study of different mouse strains could account for our failure to detect this transcript in lymphocytes of the \(Tcl1^{-/-}\) mice.

The impaired generation of early T and B lineage cells in \(Tcl1^{-/-}\) mice may reflect an increased susceptibility to receptor-induced apoptosis. In support of this hypothesis, we observed an increased vulnerability of the CD4\(^+\)/CD8\(^+\) thymocytes and, to a lesser extent, of the CD4\(^+\) and CD8\(^+\) thymocytes to receptor mediated apoptosis when \(Tcl1^{-/-}\) mice
were treated with an anti-CD3ε antibody. This finding is concordant with a recent report indicating that Tcl1 overexpression in a human cell line markedly enhances cell survival following treatment with the apoptosis inducing agents, anti-CD3, anti-Fas, and dexamethasone. It is not clear why the single positive thymocytes that do not express Tcl1 at easily detectable levels were hypersensitive to CD3 ligation in Tcl1-/- mice. It is possible that undetectable levels of Tcl1 expression in a subpopulation of the thymic CD4 cells may have a role in their survival.

Human TCL1 can bind to the pleckstrin domain of AKT with resultant enhancement of the AKT kinase activity and nuclear translocation. Mutational and structural TCL1 analyses indicate its interaction with AKT facilitates the formation of AKT-TCL1 oligomers. In the presence of TCL1, AKT Ser-473 phosphorylation may result from the transphosphorylation of other AKT molecules in the oligomeric complexes. Enhanced phosphorylation of both threonine residues (308/309/305 in Akt1/2/3, respectively) and serine residues (473/474/472) ensures maximal kinase activity. Tcl1 may thus facilitate a structural amplification loop in the PI3-kinase Akt pathway. However, following apoptotic or proliferative stimuli we did not observe changes in Akt or downstream targets that would have been predicted in this mouse model. This might reflect the very low expression levels of Tcl1 in the mouse relative to humans, or there could be an alternative mechanism for Tcl1-Akt interaction to compensate for the deficiency of Tcl1 in mice. Noteworthy in this regard, there are seven mouse orthologs and three human TCL1 family genes. Moreover, previous studies and our own work does not clarify whether or not murine Tcl1 binds to Akt. Although the crystal structures for human and murine Tcl1 are similar, the sequence conservation is only 50% and replacement of single
key residues in a way that would allow overall structural integrity could still disrupt TCL1/AKT-binding interactions.

On the other hand, the phenotypical changes here described are consistent with those observed in other murine models of Akt transgenic or null mice. Akt has been functionally linked to B and T cell development in studies that have demonstrated the activation of Akt in response to both BCR- and TCR-receptor mediated signaling. Additional insight into this developmental role has been obtained in studies of animal models wherein Akt is overexpressed or underexpressed. Akt1−/− mice display increased thymocyte apoptosis and hypersensitivity to apoptosis induction by gamma irradiation and dexamathasone treatment. Conversely, immune system alteration in Akt transgenics is manifested by B and T cell hypercellularity, enhanced immunoglobulin levels, especially IgG2a and IgA, and increased resistance to FAS mediated apoptosis induced by CD3 stimulation. Transgenic mice that overexpress either Tcl1 or its MTCP1 homologue also have exaggerated T and B cell proliferation and they develop leukemias. By contrast, Tcl1 deficient mice are shown in the present study to manifest precisely the opposite effects of those seen in Akt transgenic mice. It may therefore be of interest to cross these animal models in future studies to explore the coordinate roles of the Tcl1 and Akt genes in BCR/TCR mediated signaling and clonal selection.
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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
References


Figure Legends

Figure 1. Expression of Tcl1 mRNA in flow-sorted lymphocyte subpopulations from bone marrow, thymus, spleen, peripheral blood and lymph nodes of wild-type mice.
For each lymphocyte subpopulation 5 x 10^5 cells were used for total RNA isolation. Pro-B cells were defined as B220^-CD43^+IgM^-, pre-B cells as B220^-CD43^-IgM^-, immature B as B220^+IgM^+IgD^-, mature B as B220^+IgM^-IgD^+. The DP thymocytes were defined as CD4/8 double positive. The CD4/8 double negative (DN) thymocytes were further subdivided based on differential CD25 and CD44 expression: DN-A, CD4^-CD8^-CD44^+CD25^-; DN-B, CD4^-CD8^-CD44^-CD25^-; DN-C, CD4^-CD8^-CD44^-CD25^+; DN-D, CD4^-CD8^-CD44^+CD25^-; DN-D, CD4^-CD8^-CD44^-CD25^+. Splenic B cells were divided into three subpopulations based on expression of the B220, CD21, and CD23 cell surface markers: MZ, marginal zone B cells (B220^hiCD21^hiCD23^int), FO, follicular B cells (B220^hiCD21^intCD23^hi); NF, newly formed B cells (B220^hiCD21^loCD23^lo). Peripheral blood mononuclear cells are designated PBMC.

Figure 2. Hematopoietic and lymphoid cell subpopulations in Tcl1^-/- deficient and wild-type (Tcl1^+/+ and Tcl1^-/-) mice. Analysis of bone marrow B lineage cells in Tcl1-deficient and wild-type mice was conducted by three-color flow cytometric analysis of cells from both femoral and tibial bones (mean ± 1 standard error). Splenic marginal zone (MZ) cells were defined as CD19^-CD21^hiCD23^int, follicular cells (FO) as CD19^-CD21^intCD23^hi, and newly formed (NF) cells as CD19^-CD21^loCD23^lo. Both B220 and CD19 antibodies stained splenic B cells similarly when used in combination with CD21 and CD23 antibodies. Each group included 7-10 mice 6–12 weeks of age. Results
expressed as mean ± 1 standard error. Asterisks indicate statistically significant differences (p<0.01, as assessed by Student’s t test) between Tcl1-deficient and wild-type mice.

**Figure 3.** Apoptotic (a) and proliferation (b) rate in cultured Tcl1−/− and Tcl1+/+ splenocytes stimulated with LPS. See result section for detailed description.

**Figure 4.** Evaluation of immune responses to T-dependent and T-independent antigens. (A) Analysis of anti-sheep red blood cells (SRBC) antibody response. Tcl1−/− mice and Tcl+/+/Tcl+/− control mice immunized intravenously with SRBC were bled at days 7 and 15, and the serum samples analyzed for SRBC specific IgM and IgG subclasses by ELISA (relative units/ml). Day 7 data only is shown here since antibody levels were similar at both time points. *Differences between Tcl1-deficient and wild type mice were statistically significant (p<0.02) by Student’s t test analysis.

(B) Analysis of anti-phosphocholine (PC) antibody responsiveness. Seven 11-16 week-old mice in the experimental and control groups were immunized intraperitoneally with 1x10⁸ S. pneumoniae and serum samples obtained one week later were analyzed for PC specific IgM antibodies by ELISA (relative units/ml). *Differences between Tcl1-deficient and wild type mice were statistically significant (p<0.02) by Student’s t test analysis.
Fig. 1

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>Thymocytes</th>
<th>DN Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-B</td>
<td>prev-B</td>
<td>Imm. B</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spleen</th>
<th>PBMC</th>
<th>Lymph Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T</td>
<td>CD8+ T</td>
<td>B220+</td>
</tr>
</tbody>
</table>

- $TcII$
- β-actin

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Table 1. Cellularity of Hematopoietic and Lymphoid Tissues in TcIl−/− Deficient and Non-deficient Mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>Number of nucleated cells (x 10⁶)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TcIl+/+ and TcIl+/-</td>
<td>TcIl−/−</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>51.1 ± 4.0c</td>
<td>38.4 ± 1.4*</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>110.4 ± 6.3</td>
<td>82.5 ± 5.1*</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>116.8 ± 6.9</td>
<td>84.1 ± 7.1*</td>
<td></td>
</tr>
</tbody>
</table>

*aCells were harvested from both femoral and tibial bones of 7-9 mice per group at 6–9 weeks of age.

*bTwelve mice per group, ages 7–11 weeks.

*cResults expressed as mean ± 1 standard error.

*p<0.01
FIG. 2

A

B

C

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Table 2. Comparison of Anti-CD3 Effects on Thymocytes in *Tcl1*−/− Deficient versus Non-deficient Mice

<table>
<thead>
<tr>
<th>Thymocyte Population</th>
<th><em>Tcl1</em> +/+ and +/−</th>
<th><em>Tcl1</em>−/−</th>
<th>Treated animals received 25 µg of anti-CD3 antibody intraperitoneally 2 days before analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Ant-CD3a Fold decrease</td>
<td>Control Anti-CD3a Fold decrease</td>
<td>Control Anti-CD3a Fold decrease</td>
<td>Control Anti-CD3a Fold decrease</td>
</tr>
<tr>
<td>CD4−CD8−</td>
<td>9.0 ± 1.7</td>
<td>3.6</td>
<td>24.1 ± 2.1</td>
</tr>
<tr>
<td>CD4+CD8+</td>
<td>64.4 ± 15.5</td>
<td>15.8</td>
<td>679 ± 104.1</td>
</tr>
<tr>
<td>CD4+CD8−</td>
<td>29.4 ± 2.5</td>
<td>2.1</td>
<td>50.7 ± 6.5</td>
</tr>
<tr>
<td>CD4−CD8+</td>
<td>12.6 ± 1.0</td>
<td>2.1</td>
<td>23.7 ± 3.1</td>
</tr>
<tr>
<td>Total</td>
<td>115.4 ± 19.5</td>
<td>9.6</td>
<td>801 ± 101</td>
</tr>
</tbody>
</table>

*Results for six 8 – 12 week old mice in each group are expressed as mean cell number (x 10^5) ± 1 standard error.*
FIG. 3

A

% Apoptotic B Cells

-5
0
5
10
15
20
25
30
35
40
45

2 days
4 days
9 days

Tcl1-/-

Tcl1+/+

B

% BrdU+ splenocytes

20
18
16
14
12
10
8
6
4
2
0

Tcl1+/+

Tcl1-/-
FIG. 4

A  Anti SRBC Antibody

B  Anti PC Antibody

Day 0  Day 7
### Table 3

Table 3. Serum Immunoglobulin Levels in the *Tc11*<sup>-/-</sup> Deficient and Non-deficient Mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Ig isotype</th>
<th>Micrograms/ml</th>
<th>(Tc11&lt;sup&gt;+/+&lt;/sup&gt; and Tc11&lt;sup&gt;+-&lt;/sup&gt;)</th>
<th>Tc11&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>482.6 ± 33.6</td>
<td>262.8 ± 37.8</td>
<td>0.00027</td>
<td></td>
</tr>
<tr>
<td>IgG2a</td>
<td>270.2 ± 51.2</td>
<td>321.9 ± 37.8</td>
<td>0.439</td>
<td></td>
</tr>
<tr>
<td>IgG2b</td>
<td>945.9 ± 94.2</td>
<td>556.6 ± 81.8</td>
<td>0.0063</td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>243.1 ± 24.8</td>
<td>172.4 ± 23.1</td>
<td>0.046</td>
<td></td>
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<tr>
<td>IgM</td>
<td>280.6 ± 32.0</td>
<td>204.0 ± 21.1</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>160.5 ± 30.9</td>
<td>135.6 ± 23.9</td>
<td>0.517</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Twelve to 14 mice per group, ages 8 - 13 week.
<sup>b</sup>Results expressed as mean ± 1 standard error.
Impaired T and B Cell Development in Tcl1 Deficient Mice

Sang-Moo Kang, Maria Grazia Narducci, Cristina Lazzeri, Adriana M Mongiovi, Elisabetta Caprini, Antonella Bresin, Fabio Martelli, Jay Rothstein, Carlo M Croce, Max D Cooper and Giandomenico Russo