

# Abnormal Thymic Development, Impaired Immune Function and $\gamma\delta$ T Cell Lymphomas in a TL Transgenic Mouse Strain

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## Summary

During derivation of transgenic mouse strains with various TL and TL/H-2 chimeric genes, one strain, Tg.Tla<sup>a</sup>-3-1, introduced with a TL gene (Tla<sup>a</sup>-3), was found to have an abnormal thymic T cell population and to develop a high incidence of T cell lymphomas. To investigate the etiology of the thymic abnormalities and of the lymphomas, the development of lymphoid organs in transgenic mice was studied. The thymus of these mice goes through three unusual successive events: perturbation of thymic development during embryogenesis, disappearance of thymocytes between day 14 and day 21 after birth, and subsequent proliferation of large blast-like cells. These events are associated with the abolishment of T cell receptor (TCR)  $\alpha\beta$  lineage of the T cell differentiation, leading to preponderance of cells belonging to the TCR  $\gamma\delta$  L3T4<sup>-</sup>Lyt-2<sup>-</sup> double negative (DN) lineage. Bone marrow transplantation and thymic graft experiments demonstrate that the abnormality resides in the bone marrow stem cells rather than in the thymic environment. The expression of TL antigen in the transgenic mice is greatly increased and TL is expressed in a wide range of T cells, including normally TL<sup>-</sup> DN cells and L3T4<sup>+</sup>Lyt-2<sup>-</sup> and L3T4<sup>-</sup>Lyt-2<sup>+</sup> single positive cells. These quantitative and qualitative abnormalities in TL expression most likely cause the abnormal T cell differentiation.

The  $\gamma\delta$  DN cells migrate into peripheral lymphoid organs and constitute nearly 50% of peripheral T cells. Immune function of the transgenic mice is severely impaired, as T cell function is defective in antibody production to sheep red blood cells, in mixed lymphocyte culture reaction to allogenic spleen cells and also in stimulation with concanavalin A. These results indicate that the  $\gamma\delta$  cells are incapable of participating in these reactions.

Molecular and serological analysis of T cell lymphomas reveal that they belong to the  $\gamma\delta$  lineage, suggesting that the  $\gamma\delta$  DN cells in this strain are susceptible to leukemic transformation. Based on cell surface phenotype and TCR expression of the DN thymocytes and T cell lymphomas, a map of the sequential steps involved in the differentiation of  $\gamma\delta$  DN cells is proposed. Tg.Tla<sup>a</sup>-3-1 mice should be useful in defining the role of TL in normal and abnormal T cell differentiation as well as in the development of T cell lymphomas, and further they should facilitate studies on the differentiation and function of  $\gamma\delta$  T cells.

Mouse TL antigens belong to the family of MHC class I antigens. However, the expression of TL is distinct from other MHC class I antigens, being restricted to normal thymocytes and T cell lymphomas. Not all strains of mice express TL in the normal thymus, but even in such TL<sup>-</sup> strains an anomalous TL expression is found on T cell lym-

phomas (1, 2). To understand the structural basis for TL expression, we and others have isolated and characterized TL genes (3-6), and also analyzed TL expression in vitro by transfecting TL and TL/H-2 chimeric genes into L cells (7). These studies showed that TL genes resemble other MHC class I genes in their overall structure but have unique 5' regulatory elements. To investigate this further, we have recently derived several transgenic mouse strains in C3H/He (TL<sup>-</sup>) background with two TL genes, Tla<sup>a</sup>-3 isolated from the A-strain mouse (Tla<sup>a</sup>, TL<sup>+</sup>), and T3<sup>b</sup> from TL<sup>+</sup> leukemia arising in a C57BL/6 (B6)<sup>1</sup> mouse (Tla<sup>b</sup>, TL<sup>-</sup>), and with

<sup>1</sup> Abbreviations used in this paper: B6, C57BL/6; DN, L3T4<sup>-</sup>Lyt-2<sup>-</sup> double negative; DP, L3T4<sup>+</sup>Lyt-2<sup>+</sup> double positive; HSA, heat-stable antigen; PFC, plaque-forming cell; Pgp-1, phagocytic glycoprotein 1; SP, L3T4<sup>+</sup>Lyt-2<sup>-</sup> or L3T4<sup>-</sup>Lyt-2<sup>+</sup> single positive.

two TL/H-2 chimeric genes (8). Analysis of the transgene expression showed that even TL<sup>-</sup> thymocytes of C3H/He provide conditions for the transcriptional activation of *Tla<sup>a</sup>-3*, and that *Tla<sup>a</sup>-3* contains necessary elements for correct tissue-specific expression in normal thymocytes, whereas *T3<sup>b</sup>* and the endogenous TL genes of C3H/He lack these elements. During the course of the study, we found that one of the transgenic mouse strains, Tg.Tla<sup>a</sup>-3-1, expresses large amounts of TL antigen in thymocytes, and has a small thymus consisting mainly of L3T4 (CD4)<sup>-</sup>Lyt-2 (CD8)<sup>-</sup> double negative (DN) cells. Furthermore, this strain develops a high incidence of lymphoid neoplasms.

The differentiation pathway of T cells bearing  $\alpha\beta$  TCR in the thymus has been extensively studied, and it has been conclusively demonstrated that the interaction between  $\alpha\beta$  TCR and MHC antigens plays a critical role in the development of thymic T cells (for review, see references 9–12). The maturation of  $\alpha\beta$  cells in the thymus has been staged using various cell surface markers (13, 14). The differentiation pathway of the other lineage,  $\gamma\delta$  cells, has also been vigorously investigated, and many aspects of  $\gamma\delta$  cells, including function, localization of different subsets, thymic selection of certain subsets and putative ligands, have been reported (for review, see references 15–18).  $\gamma\delta$  cells, particularly those localized in epithelial structures, are speculated to function in the primary defense of the body against infection and other insults (19). However, because of the limited number of  $\gamma\delta$  cells in lymphoid organs (20–22), analysis of their function and staging of their developmental pathway have been difficult.

In the present study, we have investigated the development of the immune system in Tg.Tla<sup>a</sup>-3-1 mice to elucidate the role of the transgene in abnormal thymic differentiation. We found that T cell differentiation in the transgenic mice is forced to deviate to the  $\gamma\delta$  lineage through at least three unusual events in the thymus and that consequently  $\gamma\delta$  cells become abundant rather than  $\alpha\beta$  cells. To delineate the function and the maturation steps involved in the differentiation of  $\gamma\delta$  cells, we characterize DN thymocytes,  $\gamma\delta$  T cell lymphomas and immune responses of Tg.Tla<sup>a</sup>-3-1 mice.

## Materials and Methods

**Mice.** Derivation of the TL transgenic mouse strain, Tg.Tla<sup>a</sup>-3-1, has been described elsewhere (8). Briefly, a DNA fragment containing *Tla<sup>a</sup>-3* isolated from an A-strain genomic library was injected into male pronuclei of fertilized eggs of C3H/He mice (TL<sup>-</sup>, *Tla<sup>b</sup>*) (8, 23). The founder mouse of the Tg.Tla<sup>a</sup>-3-1 strain contained seven copies of *Tla<sup>a</sup>-3* transgenes in a single chromosomal site. The offspring of the founder were interbred to produce homozygous stock, which then maintained by brother-sister mating. C3H/He mice were purchased from Japan SLC Inc. (Hamamatsu, Japan), and other mice were from our breeding colony or from the National Institute of Genetics (Mishima, Japan).

**Histology.** Tissues were fixed with Bouin's solution and 5  $\mu$ m sections were stained with hematoxyline-eosin.

**Serological Analysis.** Two-color analysis of cell surface antigens was performed with a fluorescence-activated cell sorter (FACstar; Becton Dickinson and Co., Mountain View, CA) using mAbs to TL (HD168, reference 3), L3T4 (GK1.5, reference 24), Lyt-2 (53-7,

reference 25), TCR $\alpha\beta$  (H57-597, reference 26), TCR $\gamma\delta$  (3A10, reference 22), CD3 (145-2C11, reference 27), heat-stable antigen (HSA) (J11d, reference 28), phagocytic glycoprotein 1 (Pgp-1, Ly24) (NU5-50; provided by Dr. E. Nakayama, Nagasaki Univ., Nagasaki, Japan), IL-2 receptor  $\alpha$  chain (IL-2R $\alpha$ ) (7D4, reference 29), and Thy-1.1 and Thy-1.2 (purchased from Beckton Dickinson and Co.). Most antibodies were directly conjugated with fluorescein or biotin. The secondary reagents, PE streptavidin (Biomedex, Foster City, CA), FITC-labeled anti-hamster Ig (Caltag, South San Francisco, CA), anti-rat Ig (Tago Inc., Burlingame, CA) or anti-mouse Ig (DAKO, Glostrup, Denmark) were used as appropriate.

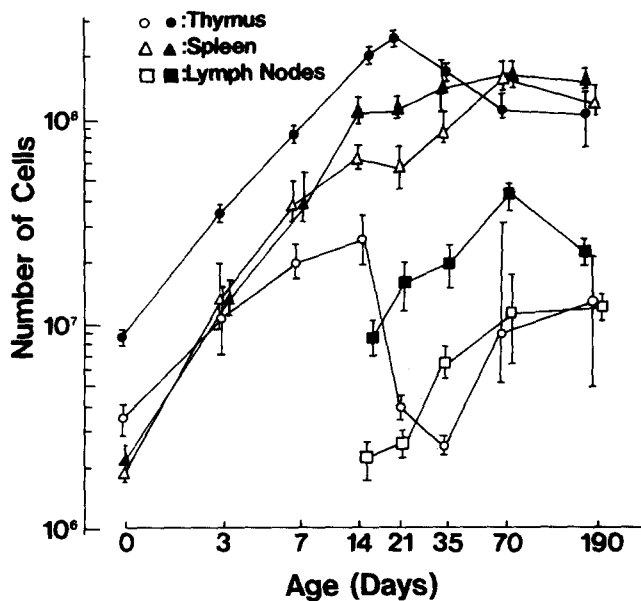
**Bone Marrow Transplantation and Thymic Graft.** Recipient mice were lethally irradiated (10 Gy, Hitachi MBR-1520R; Hitachi, Tokyo, Japan) and 1 d later injected intravenously with 10<sup>7</sup> bone marrow cells from donor mice. After a few weeks, the lymphoid system of the mice was totally reconstituted by cells of the donor origin as determined by the expression of cell surface antigens (Thy-1.1 or Thy-1.2, and TL<sup>+</sup> or TL<sup>-</sup>) of the donor origin. For the thymic graft, a lobe of thymus was inserted under the renal capsule of the host mice. The lymphoid cells in the grafted thymus were gradually replaced by cells of host origin, which was monitored by the analysis of cell surface antigens as in the bone marrow transplantation experiments.

**Immune Response.** (A) Plaque forming cell (PFC) assay: 10<sup>8</sup> SRBC were injected intravenously into the transgenic or control mice. 1 wk later, 10<sup>6</sup> spleen cells of the immunized mice were mixed with 2  $\times$  10<sup>8</sup> SRBC and incubated for 2 h at 37°C in agarose in the presence of guinea pig complement. The number of plaques producing IgM antibody to SRBC was counted (direct assay), and the number of plaques producing IgG and IgM antibodies was counted by addition of anti-mouse Ig (indirect assay). (B) MLC: 10<sup>6</sup> spleen cells of the transgenic or C3H/He mouse were mixed with irradiated (24 Gy, Hitachi MBR-1520R; Hitachi) spleen cells of BALB/c or B6 and incubated for 4 d at 37°C, followed by a 6-h incubation in the presence of 18.5 kBq [<sup>3</sup>H]thymidine. Cells were harvested on a filter by LABO MASH (Labo Science Inc., Tokyo, Japan) and precipitated with TCA. The amount of [<sup>3</sup>H]thymidine in the precipitates was counted with a scintillation counter (Beckman Instruments, Palo Alto, CA). (C) Con A stimulation. Total spleen cells or the T cell fraction of spleen purified by a T cell recovery column kit (Biotex, Alberta, Canada) were incubated in the presence of 2  $\mu$ g/ml Con A for 3 d, followed by a 6-h incubation with the addition of 18.5 kBq [<sup>3</sup>H]thymidine. The uptake of [<sup>3</sup>H]thymidine was measured as in the MLC experiments. (D) LPS stimulation. The experimental procedure was essentially the same as the Con A stimulation, except that 20  $\mu$ g/ml LPS (Sigma Chemical Co., St. Louis, MO) was added instead of Con A.

**DNA and RNA Blot Analysis.** Probes for Southern (30) and Northern (31) blot analyses were prepared either by nick-translation (32) or by the random priming method (33). The DNA rearrangement and RNA transcripts were analyzed with the following probes: TCR  $\alpha$  with p $\alpha$ 1 (34), TCR  $\beta$  with PL5 (provided by Dr. D. Y. Loh, Washington Univ., St. Louis, MO), TCR  $\gamma$  with p8/10-2 $\gamma$ 11 (35), TCR  $\delta$  with RAD11C (36), and TL with pFL1 (3). Conditions for hybridization and washing were described previously (3, 5).

## Results

**Abnormal Growth of the Thymus in Tg.Tla<sup>a</sup>-3-1 Transgenic Mice.** The growth of the thymus in Tg.Tla<sup>a</sup>-3-1 mice was



**Figure 1.** Change in cell number in thymus, spleen and lymph nodes during maturation. Open symbols are the results of transgenic mice and closed symbols are those of C3H/He mice. For counting the cells in lymph nodes, axillary, brachial, inguinal, and mesenteric nodes were combined. The mean cell number was calculated from data of at least three virgin female mice. The data for C3H/He mice are consistent with published results (37). Thymus and lymph nodes of Tg.Tla<sup>-3-1</sup> consistently contain fewer cells than those of age-matched C3H/He mice. The low cell number of newborn thymus, sudden disappearance of thymocytes between day 14 and day 21, and gradual increase in the number of thymocytes after day 35 are characteristic of the transgenic mice.

Changes in the weight of the lymphoid organs correlate well with those in the cell number. The weight of the thymus of transgenic mice starts from  $3.9 \pm 0.3$  mg at day 0, increase to  $21.1 \pm 2.7$  mg at day 14, then reduces to  $13.6 \pm 2.5$  mg at day 21 and recovers to  $21.9 \pm 6.2$  mg at day 190. In C3H/He mice, the weight of the thymus is  $4.8 \pm 0.3$  mg at day 0,  $57.8 \pm 6.9$  mg at day 14,  $64.1 \pm 2.3$  mg at day 21 and  $36.9 \pm 4.4$  mg at day 190. The weight of lymph nodes of transgenic mice is consistently smaller than the control;  $13.7 \pm 2.5$  mg versus  $24.8 \pm 2.5$  mg at day 14 and  $69.1 \pm 11.5$  mg versus  $92.2 \pm 16.2$  mg at day 190. Spleen weight or total body weight do not differ significantly between the transgenic and the control mice throughout the observation period.

assessed by changes in weight, cell number and histology, and was compared with that of the control C3H/He mice (Fig. 1 and Fig. 2). Three unusual events were found to occur during the development of the transgenic thymus. First, at birth, the thymus of the transgenic mouse is already smaller and contains fewer cells than the control. The thymus has a thin cortex layer and the boundary of cortex and medulla is not as distinct as in control mice. Second and most strikingly, lymphoid cells abruptly disappear from the transgenic thymus between day 14 and day 21; the thymus loses 50% of its weight and 85% of its lymphoid cell population. Consequently, the thymus of 21-d and also 35-d mice has only a few percent of the thymocytes present in the control thymus. The thymus at day 21 lacks small lymphocytes in the cortex, and the distinction between cortex and medulla becomes ambiguous. Third, uniformly large blast-like cells with high mi-

totic activity repopulate and occupy the thymus of 35-d or older mice. With increase in this cell population, both thymus weight and cell number show partial recovery.

**Abnormal Thymic T Cell Differentiation.** As shown in Fig. 3, the antigenic profile of thymocytes of Tg.Tla<sup>-3-1</sup> mice also undergoes drastic changes, in parallel with the growth of the thymus described above. The thymus at each age (before day 14, at day 21 and after day 35) shows a distinct and abnormal representation of the four T cell subpopulations belonging to the different maturation stages, the least mature L3T4<sup>-</sup>Lyt-2<sup>-</sup> DN, the immature L3T4<sup>+</sup>Lyt-2<sup>+</sup> double positive (DP) and the mature L3T4<sup>+</sup>Lyt-2<sup>-</sup> or L3T4<sup>-</sup>Lyt-2<sup>+</sup> single positive (SP) cells. Thy-1 expression by the thymic cell population indicates their T cell origin. Until day 14, the thymus has a T cell profile similar to the control, but it contains an expanded population of DN cells and a relatively small population of DP cells. The sudden disappearance of cells from the transgenic thymus between day 14 and day 21 involves 90% of DP thymocytes as well as 85% of DN thymocytes. As a result, the thymus of day 21 mice has all four subpopulations in equal proportion. At day 35, DN cells become the major population, with 90% of thymocytes being DN and the remaining 10% Lyt-2<sup>+</sup> SP cells, while DP and L3T4<sup>+</sup> SP cells become undetectable. This abnormal phenotype becomes stable after day 35, indicating that the differentiation pathway from DN to DP thymocytes has been lost, while the DN pathway remains active. As calculated from Fig. 1 and 3, the number of DP cells in transgenic thymus is  $<10^5$  after day 35, while the control thymus contains  $1.5 \times 10^8$  DP cells at day 35 and  $9 \times 10^7$  at day 70. In contrast, the number of DN thymocytes increases after day 21 and the thymus at day 70 contains  $8 \times 10^6$  DN thymocytes, twice as many as the age-matched control.

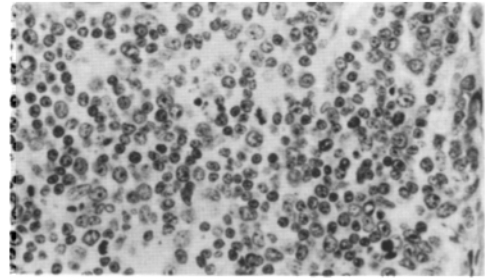
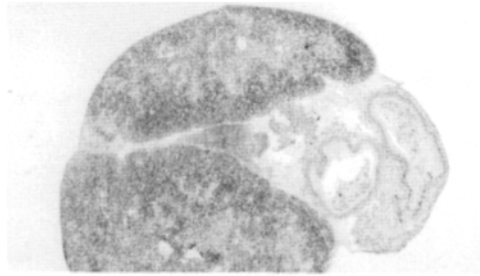
Studies of TCR expression show that DN cells in day 70 thymus express TCR  $\gamma\delta$  (Fig. 3, Row 4). However, this commitment of DN cells to TCR  $\gamma\delta$  becomes evident only at day 35, as the majority of DN cells are negative for TCR  $\gamma\delta$  expression before day 21. The differentiation pathway to TCR  $\alpha\beta$ <sup>+</sup> SP cells is disturbed in the transgenic thymus (Fig. 3, Row 3), since there are very few TCR  $\alpha\beta$ <sup>+</sup>L3T4<sup>+</sup> SP cells in 35-d or older mice. TCR  $\alpha\beta$  expression is detected on the relatively expanded SP thymocytes in day 21 mice and on Lyt-2<sup>+</sup> SP thymocytes in older mice. TCR  $\alpha\beta$  and  $\gamma\delta$  expression is always accompanied by CD3 expression (Fig. 3, Row 5), suggesting that both TCR  $\alpha\beta$  and  $\gamma\delta$  on the cell surface are functional.

A variable proportion of the DN thymocytes of young transgenic mice expresses IL-2 receptor  $\alpha$  chain (IL-2R $\alpha$ ), HSA, and Pgp-1. For example, of the DN thymocytes of day 21 mice, 69% are IL-2R $\alpha$ <sup>+</sup>, 30% HSA<sup>+</sup> and 16% Pgp-1<sup>+</sup>. The DN cells of the control thymus are consistently heterogeneous in terms of the expression of these antigens. However, TCR  $\gamma\delta$ <sup>+</sup> DN thymocytes of 70-d-old transgenic mice become homogeneously negative for these antigens (data not shown).

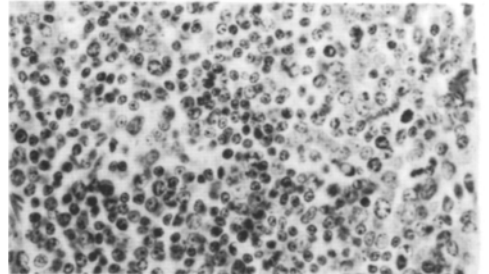
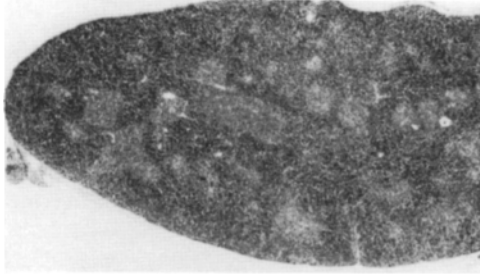
**T Cell Receptor (TCR)  $\gamma\delta$ <sup>+</sup> Double Negative (DN) T Cells in Peripheral Lymphoid Organs.** Lymph nodes of Tg.Tla<sup>-3-1</sup> are consistently smaller in size and contain fewer cells than

**Tg.Tla<sup>a</sup>-3-1**

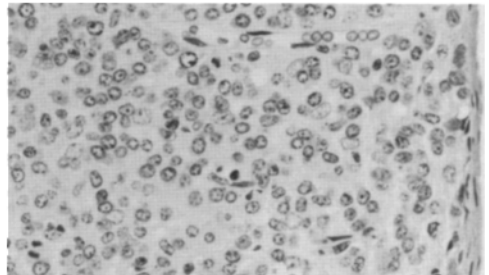
**Day 0**



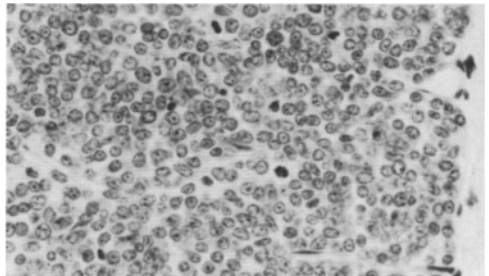
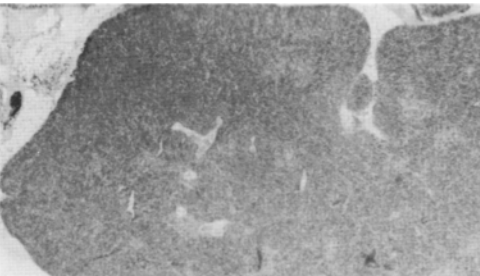
**Day 14**



**Day 21**

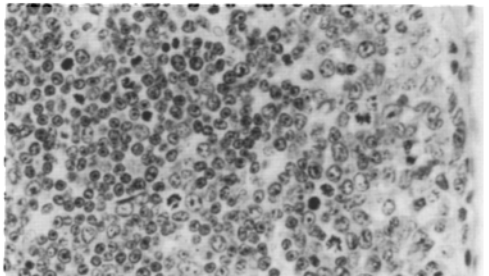
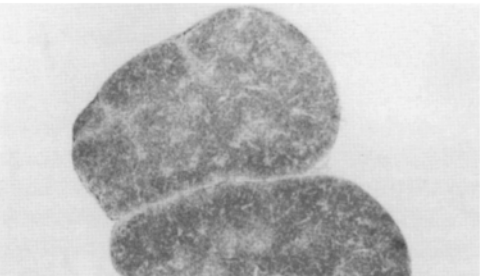


**Day 70**

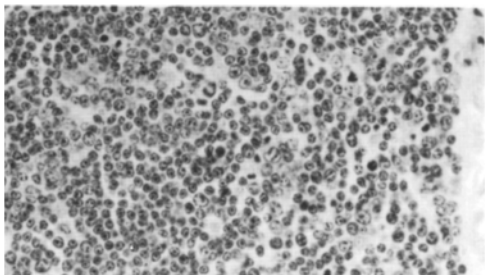
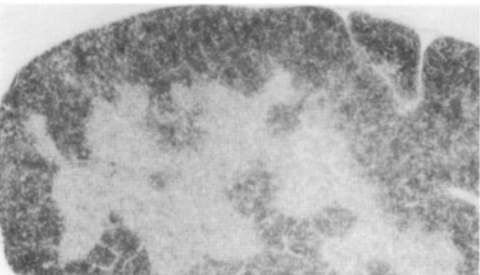


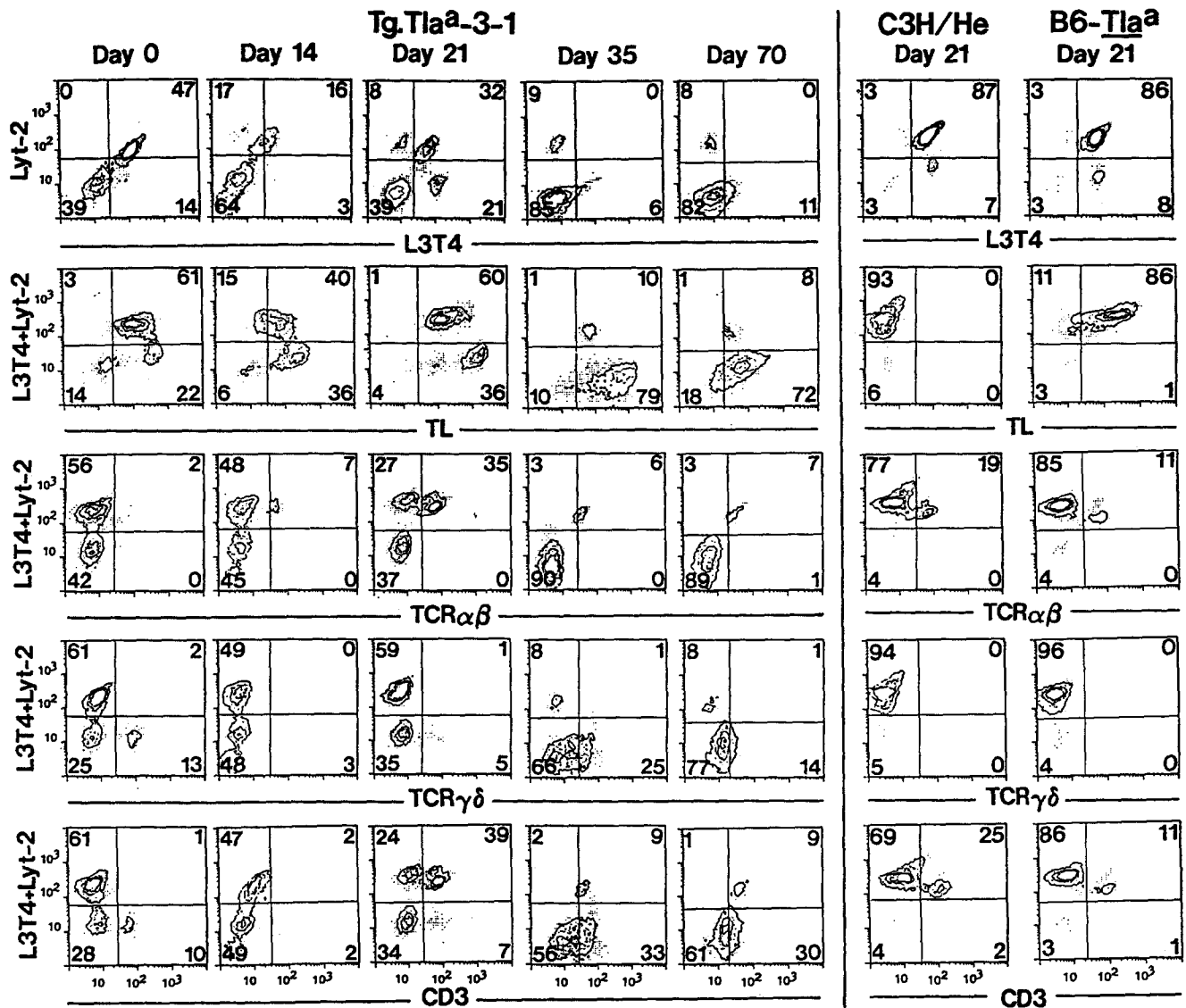
**C3H/He**

**Day 0**



**Day 70**



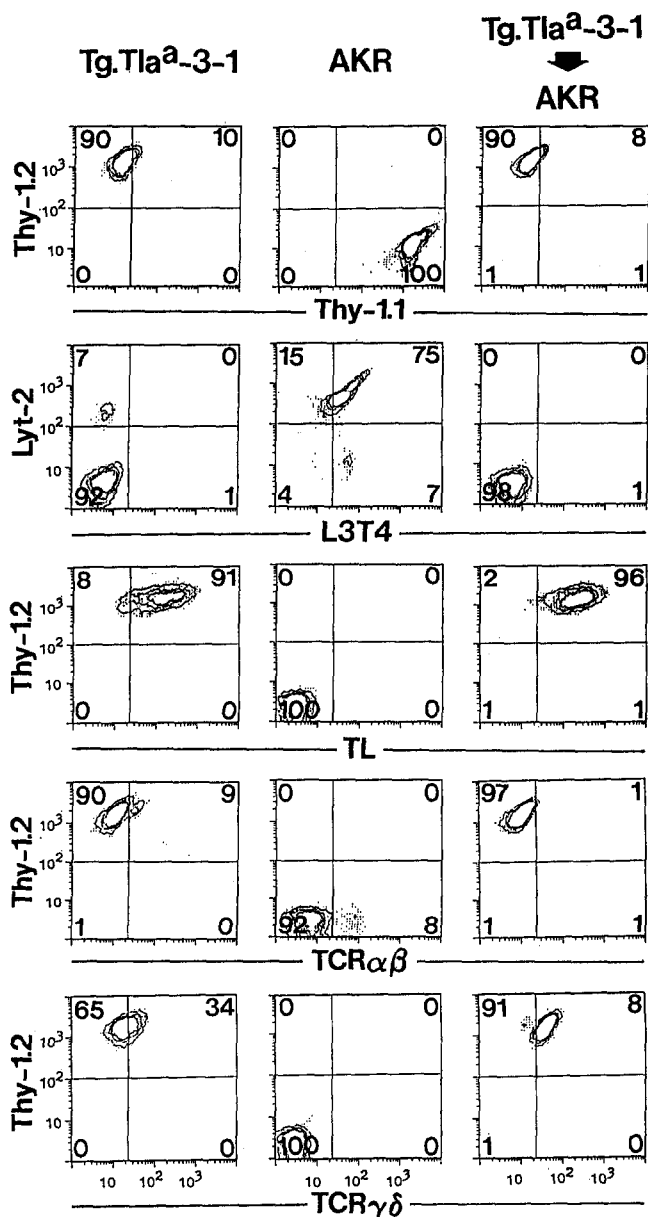


**Figure 3.** Surface antigenic profile of thymocytes. Thymocytes of Tg.Tla<sup>a</sup>-3-1, C3H/He and B6-Tla<sup>a</sup> were examined by two-color FACS analysis using various combinations of mAbs. To identify DN cells easily, a mixture of L3T4 and Lyt-2 antibodies was used in certain experiments; both DP and SP cells were positive with the mixture whereas DN cells were negative. The percentage of each population is shown in the four corners of each panel. The antigen profiles of day 3 and 7 thymocytes of transgenic mice were essentially identical to that of day 14 and are not shown in the figure. As the profile of C3H/He and B6-Tla<sup>a</sup> remained consistent with ages, only those of 21-d-old mice are shown in the figure. The T cell population in C3H/He and B6-Tla<sup>a</sup> thymus was comparable with published results (13, 14): 80–85% of thymocytes are DP cells, 3–9% L3T4<sup>+</sup> SP cells, 3–6% Lyt-2<sup>+</sup> SP cells, and 3–5% DN cells.

those of C3H/He mice (Fig. 1), with only one fifth to one half the numbers of T cells (data not shown). The spleen of transgenic mice does not differ significantly in weight or in cell number from age-matched controls, but contains about one half of the T cells present in control mice ( $14.1 \pm 1.3\%$

versus  $28.3 \pm 1.5\%$  Thy-1<sup>+</sup> spleen T cells, data not shown). Spleen and lymph nodes of Tg.Tla<sup>a</sup>-3-1 showed no obvious histological abnormality, but the white pulp in the lymph nodes and spleen of Tg.Tla<sup>a</sup>-3-1 is smaller than in the control (data not shown).

**Figure 2.** Histology of Tg.Tla<sup>a</sup>-3-1 thymus. The thymus of Tg.Tla<sup>a</sup>-3-1 maintains its basic structure up to day 14 (only the pictures of day 0 and day 14 thymus are shown). The distinction between cortex and medulla is clear, and typical thymocytes, small lymphocytes with basophilic nuclei and little cytoplasm, are relatively abundant in the cortex. However, the cortical area is smaller in the thymus of transgenic mice than in the control thymus. In day 21 thymus, the demarcation between cortex and medulla becomes unclear, and very few small lymphocytes are found in the cortex. Most remaining cells have vesicular nuclei and large cytoplasm. In the thymus of day 70, the distinction between cortex and medulla is barely visible, and the cortex is repopulated with large lymphocytes with less basophilic nuclei and large cytoplasm. Mitotic figures are often observed in these cells. In C3H/He thymus from day 0 to day 70, the demarcation of cortex and medulla is always clear, and the cortex is filled with typical thymocytes (the pictures of day 0 and day 70 are shown). Magnification is 25-fold in the left column and 260-fold in the right column.



**Figure 4.** Thymic population of bone marrow chimera between Tg.Tla<sup>a</sup>-3-1 and AKR mice. Lethally irradiated AKR mice were transplanted with bone marrow cells of 90-d-old Tg.Tla<sup>a</sup>-3-1 mice, and the thymocytes were analyzed 28 d later. Thymocytes from AKR and Tg.Tla<sup>a</sup>-3-1 of comparable age were tested as controls. The difference in Thy-1 allo-specificity between AKR (Thy-1.1) and Tg.Tla<sup>a</sup>-3-1 (Thy-1.2) enabled the origin of thymocytes to be determined. TL expression also distinguishes the host (AKR, TL<sup>-</sup>) and the donor (Tg.Tla<sup>a</sup>-3-1, TL<sup>+</sup>). Almost all thymocytes of the chimeric mice have the phenotype of Thy-1.1<sup>-</sup> Thy-1.2<sup>+</sup> TL<sup>+</sup>, indicating that they are derived from Tg.Tla<sup>a</sup>-3-1 bone marrow cells. The thymic population in chimeric mice is almost identical to that of transgenic mice, except for the absence of Lyt-2<sup>+</sup> SP cells in the chimeric thymus.

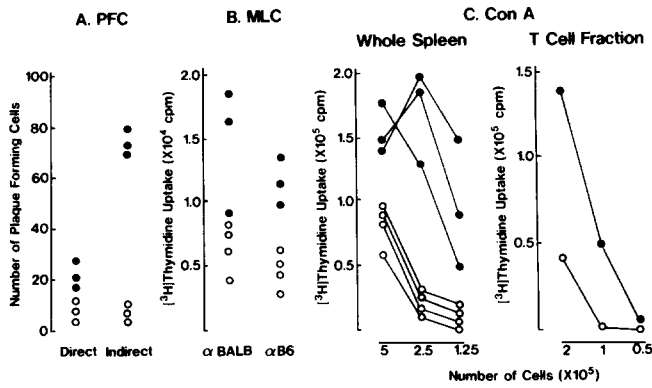
The antigenic profile of T cells in peripheral nodes also undergoes changes characteristic of Tg.Tla<sup>a</sup>-3-1 mice (data not shown). At day 21, the T cell population in the lymph nodes of transgenic mice is similar to that in control mice except the expression of TL; 95% of T cells are TCR αβ<sup>+</sup>

CD3<sup>+</sup> TL<sup>+</sup> SP cells and only 5% are TCR γδ<sup>+</sup> CD3<sup>+</sup> TL<sup>+</sup> DN cells. However, as transgenic mice age, the proportion of TCR γδ<sup>+</sup> CD3<sup>+</sup> TL<sup>+</sup> DN cells gradually increases, occupying 30% at day 70 and reaching nearly 50% at day 190. These results indicate that lymph nodes are first populated by TCR αβ<sup>+</sup> SP cells prior to day 21, and then by increasing numbers of TCR γδ<sup>+</sup> DN cells after day 21.

**Expression of the Transgene in Tg.Tla<sup>a</sup>-3-1 Thymus.** TL antigen, the product of the Tla<sup>a</sup>-3 transgene, is expressed in Tg.Tla<sup>a</sup>-3-1 thymus in a much larger quantity (about 10 times) than in the thymus of any inbred TL<sup>+</sup> strain, including the A-strain from which the transgene was isolated (see reference 8). Further, TL antigen is detected in almost all thymic T cells of Tg.Tla<sup>a</sup>-3-1, including DN and SP cells, although a fraction of DN cells in younger mice is TL<sup>-</sup> (Fig. 3, Row 2). The proportion of TL<sup>+</sup> DP cells decreases with aging, while TL<sup>+</sup> DN cells increase and become the major population of the thymus. In B6-Tla<sup>a</sup>, only DP thymocytes express TL antigens, while DN and SP thymocytes are TL<sup>-</sup>. TL antigen is expressed in almost all T cells in the lymph nodes of transgenic mice, including TCR γδ<sup>+</sup> DN and TCR αβ<sup>+</sup> SP cells. This contrasts with the situation in other TL<sup>+</sup> mouse strains, where peripheral T cells do not express TL antigen.

**T Cell Abnormality Resides in Bone Marrow Stem Cells.** To determine whether stem cells derived from the bone marrow or the thymic environment of Tg.Tla<sup>a</sup>-3-1 are responsible for the abnormal differentiation of T cells, bone marrow transplantation and thymus grafting were carried out. The thymus of AKR mice (Thy-1.1<sup>+</sup> TL<sup>-</sup>) reconstituted with bone marrow cells of transgenic mice showed the characteristics of the transgenic thymus; the majority of thymocytes were large blast-like cells and the cortex-medulla distinction was unclear (data not shown). The thymocytes of chimeric mice were entirely replaced by Thy-1.2<sup>+</sup> TL<sup>+</sup> cells of donor origin, and the major population was TCR γδ<sup>+</sup> DN cells (Fig. 4). Results from the reciprocal combination could not be obtained, because Tg.Tla<sup>a</sup>-3-1 recipients of AKR bone marrow cells did not survive. However, chimeric Tg.Tla<sup>a</sup>-3-1 mice receiving C3H/He bone marrow were produced and were found to have normal thymic morphology and a normal population of thymic T cells (data not shown). These results indicate that bone marrow stem cells rather than the thymic environment (including thymic epithelial cells) cause the abnormal thymic differentiation of Tg.Tla<sup>a</sup>-3-1 mice. This conclusion is confirmed by experiments involving reciprocal thymic graft. C3H/He thymus transplanted under the capsule of Tg.Tla<sup>a</sup>-3-1 kidney takes on the characteristics of the transgenic thymus. 5 wk after the grafting, the thymus becomes smaller with no clear cortex-medulla distinction and is constituted mainly of large lymphoid cells with a TL<sup>+</sup> TCR γδ<sup>+</sup> DN phenotype. In contrast, Tg.Tla<sup>a</sup>-3-1 thymus transplanted into C3H/He mice show normal T cell population and morphology (data not shown).

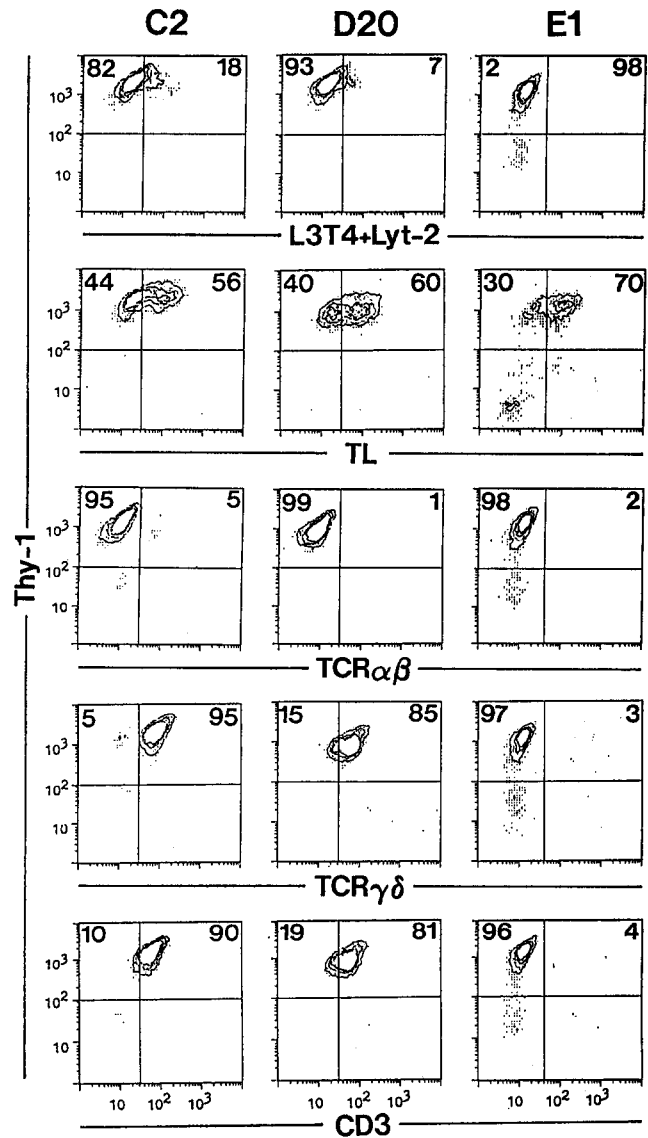
**Deficient T Cell Immune Function in Tg.Tla<sup>a</sup>-3-1 Mice.** Tg.Tla<sup>a</sup>-3-1 mice of day 70 or older were examined for their immune capacity in four different assays. In PFC assays in the response to SRBC, Tg.Tla<sup>a</sup>-3-1 mice yielded fewer IgM-



**Figure 5.** Immune responses of Tg.Tla<sup>a</sup>-3-1 mice. In all assays, 70-d or older mice were used. In all panels, open circles represent results with transgenic mice and closed circles with control C3H/He mice. (A) PFC response to SRBC. The mice were immunized with SRBC, and the number of PFCs in the spleen was enumerated by direct assay for cells producing IgM antibody and by indirect assay for cells producing IgG and IgM antibodies. Tg.Tla<sup>a</sup>-3-1 had consistently fewer PFCs in both assays. (B) Proliferative response to allogenic spleen cells in MLC. The transgenic and control spleen cells were cocultured with irradiated spleen cells of BALB/c or B6, both of which have different MHC class I and class II antigens from the transgenic and C3H/He mice. [<sup>3</sup>H]thymidine uptake by the spleen cells of the transgenic mice was less than the control MLC reactions with both BALB/c and B6. (C) Proliferative response to Con A. Unfractionated spleen cells or purified splenic T cell fraction were cultured in the presence of Con A. Unfractionated spleen cells and splenic T cell fraction of the transgenic mice were less reactive than the controls.

and IgG-producing cells than the control, as shown in Fig. 5 A. In vitro proliferative responses in MLC against allogenic BALB/c and B6 spleen cells showed that the transgenic mice were less reactive than the control (Fig. 5 B). Similarly, in the mitogenic response to Con A, transgenic spleen cells were less efficiently stimulated (Fig. 5 C). As the spleens of transgenic mice contain one half the T cells present in control spleens (see above), the same number of T cells isolated from Tg.Tla<sup>a</sup>-3-1 and C3H/He spleens were compared in their response to Con A. The results indicate that the T cells of transgenic mice are less reactive than those of the control. In contrast to these T cell dependent assays, the proliferative response of transgenic spleen cells to LPS was normal (data not shown). These results indicate that T cell function of Tg.Tla<sup>a</sup>-3-1 mice is impaired, whereas B cell function is not.

**Development of T Cell Lymphomas in Tg.Tla<sup>a</sup>-3-1.** Tg.Tla<sup>a</sup>-3-1 mice of 8–15 mo-old often developed enlargement of spleen and lymph nodes. Liver, lung and kidney were frequently involved, while overt enlargement of the thymus was rare. The infiltrating cells were larger than normal lymphocytes, with more vesicular nuclei and with relatively abundant cytoplasm. The gross and microscopic characteristics were similar in all cases, and these neoplasms could be classified as “lymphocytic leukemia” according to Dunn (38). The incidence was over 60% (32/51 mice) and the tumors were transplantable in C3H/He mice. C3H/He, used for the derivation of the transgenic strain, has a low incidence of lymphocytic leukemia (39).



**Figure 6.** Surface antigenic profile of Tg.Tla<sup>a</sup>-3-1 lymphocytic leukemias. Two-color analysis of cell surface antigens of tumor cells was carried out as described in the legend to Fig. 3. The results with three tumors are illustrated in this figure. All three are Thy-1<sup>+</sup> TL<sup>+</sup> L3T4<sup>-</sup> Lyt-2<sup>-</sup> TCR αβ<sup>-</sup>; two (C2 and D20) are TCR γδ<sup>+</sup> CD3<sup>+</sup>, while one (E1) is TCR<sup>-</sup> CD3<sup>-</sup>. Most other lymphomas are similar to C2 and D20 (see Table 1).

The cell surface phenotype of these lymphoma cells was examined (Fig. 6 and Table 1). Cells from 21 lymphomas were Thy-1<sup>+</sup> TL<sup>+</sup> L3T4<sup>-</sup> Lyt-2<sup>-</sup> TCR αβ<sup>-</sup>. Most cells expressed TCR γδ and CD3, while a few were negative for TCR γδ and CD3. The expression of IL-2Rα and HSA was generally not detected. The tumors were heterogeneous with regard to the expression of Pgp-1.

The structure and expression of TCR genes were also examined and the results are summarized in Table 1. All except one lymphoma (A8) had rearranged TCR γ and δ genes and transcribed γ and δ mRNA of the functional sizes: A8 had germline TCR γ genes and no γ transcripts of the functional size. No tumor expressed both TCR α and β functional tran-



**Table 1.** Characteristics of *Thy-1<sup>+</sup> TL<sup>+</sup> L3T4<sup>-</sup> Lyt-2<sup>-</sup>* Lymphomas of *Tg.T1a<sup>+</sup>-3-1*

Tumor line	TCR gene structure*			TCR transcripts (functional)†				Cell surface phenotype‡				
	$\gamma$	$\delta$	$\beta$	$\gamma$	$\delta$	$\alpha$	$\beta$	TCR $\gamma\delta$	TCR $\alpha\beta$	CD3	HSA	Pgp-1
A8	G*	R*	G	-	+	-	-	-	-	-	+	+
E1	R	R	G	+	+	-	-	-	-	-	-	+
C2	R	R	G	+	+	-	-	+	-	+	-	+
B9	R	R	G	+	+	-	-	+	-	+	-	+
B1	R	R	G	+	+	-	-	+	-	+	-	-
D20	R	R	G	+	+	-	-	+	-	+	-	-
G2	R	R	G	+	+	-	-	ND <sup>  </sup>	ND	ND	ND	ND
C5	R	R	G	+	+	+	-	ND	ND	+	ND	ND
A1	R	R	R	+	+	-	-	+	-	+	-	+
G262	R	R	R	+	+	-	-	+	-	+	-	-
G3	R	R	R	+	+	-	-	+	-	+	-	-
A4	R	R	R	+	+	-	-	ND	ND	+	ND	ND
HA48	R	R	R	+	+	-	-	ND	ND	+	ND	ND
A2	R	R	R	+	+	-	-	ND	ND	+	ND	ND
A5	R	R	R	+	+	-	-	ND	ND	+	ND	ND
A3	R	R	R	+	+	-	-	ND	ND	+	ND	ND
D11	R	R	R	+	+	-	+	+	-	+	-	+
D2	R	R	R	+	+	-	+	ND	ND	+	ND	ND
H5	R	R	R	+	+	-	+	ND	ND	+	ND	ND
H4	R	R	R	+	+	-	+	ND	ND	+	ND	ND
G4	R	R	R	+	+	-	+	ND	ND	ND	ND	ND

A summary of the results of TCR gene structure in Southern blot analysis with EcoR I, Hind III, BamH I, Xba I and Pvu II digested DNA. The tumors, which showed a germline configuration with one enzyme, often showed rearranged bands with other enzymes. (G) TCR gene in germline. (R) TCR gene in clonal rearrangement. Probes used for the analysis are described in Materials and Methods.

† A summary of the results of TCR transcripts detected in Northern blot analysis. Two different sizes of mRNAs are identified for each TCR gene. For all TCRs, the larger mRNAs (1.5 kb for  $\gamma$ , 2.0 kb for  $\delta$ , 1.7 kb for  $\alpha$ , and 1.3 kb for  $\beta$ ) are the functional transcripts, while smaller mRNAs (1.2 kb for  $\gamma$ , 1.5 kb for  $\delta$ , 1.3 kb for  $\alpha$ , and 1.0 kb for  $\beta$ ) are non-functional aberrant transcripts (34, 36). Only functional transcripts are scored. Probes are described in Materials and Methods.

‡ see Fig. 6.

<sup>||</sup> Not done.

scripts simultaneously, although some expressed either TCR  $\alpha$  or  $\beta$  transcripts of functional sizes. These results correlate well with the results of flow cytometry as described above, although a discrepancy was found with one tumor (E1), which expressed functional  $\gamma$  and  $\delta$  mRNA, but was negative for TCR  $\gamma\delta$  expression. These results of molecular and serological analyses indicate that the majority of "lymphocytic leukemias" of *Tg.T1a<sup>+</sup>-3-1* mice originate from DN cells belonging to the TCR  $\gamma\delta$  lineage, with a few lymphomas arising from the DN cells before lineage commitment.

## Discussion

The present study of a TL transgenic mouse strain characterized by high levels of TL antigen expression in thymocytes and abnormal thymic development, reveals that thymic

T cell differentiation in this strain is deviated almost exclusively toward the  $\gamma\delta$  lineage. Bone marrow transplantation and thymic graft experiments clearly demonstrate that the abnormality resides in the stem cells of bone marrow and not in the thymic environment including the thymic epithelial cells. The most striking event is a sudden disappearance of small lymphocytes from the thymus between day 14 and day 21. This event is distinct from the disappearance of thymocytes induced by stress, irradiation or administration of cortisone. The thymus of *Tg.T1a<sup>+</sup>-3-1* mice shows neither a "moth-eaten" structure nor the accumulation of macrophages, eosinophils or necrotic cells in the cortex, which are associated with the disappearance of thymocytes by these other causes (40). The loss of thymocytes in transgenic mice between day 14 and day 21 is dependent on the genetic background, since a similar phenomenon occurs but much delayed in (C3H/



He  $\times$  Tg.Tla<sup>a</sup>-3-1), (B6  $\times$  Tg.Tla<sup>a</sup>-3-1) and (B6-Tla<sup>a</sup>  $\times$  Tg.Tla<sup>a</sup>-3-1) F<sub>1</sub> mice. The presence of Lyt-2<sup>+</sup> SP cells in older thymus seems not to be essential for the event, because no Lyt-2<sup>+</sup> SP cells are present in the thymus of the bone marrow chimera mice as shown in Fig. 4. The gradual increase of large blast-like  $\gamma\delta$  DN thymocytes in older mice is another feature of the transgenic thymus. After day 21, DN thymocytes become the major population and their differentiation appears to be restricted to  $\gamma\delta$  DN cells. Two possible mechanisms may account for the disappearance of thymocytes and proliferation of  $\gamma\delta$  DN thymocytes. One possibility involves a defect in proliferation and differentiating capacity of stem cells involved in the development of T cells, with an abnormal deviation toward the  $\gamma\delta$  lineage. The other possibility is the rapid removal of cells belonging to the  $\alpha\beta$  lineage including all DP and some DN cells, while leaving those in the  $\gamma\delta$  lineage intact. Although no visible cell death was observed in day 14 or day 21 thymus, further study of this possibility is required, e.g., molecular analysis to detect DNA fragmentation, a sign of apoptosis, in vitro organ culture of the thymus, and determination of TCR repertoires of the  $\gamma\delta$  DN thymocytes. The embryonic thymus of transgenic mice is not grossly abnormal, and the growth of the thymus after birth is close to normal until day 14. However, an event occurring during embryonic life may trigger the abnormal T cell differentiation, and a detailed analysis of thymic development during embryogenesis is now under way.

What is the role of the transgene in these abnormal events, and what is the function of TL in normal differentiation? It is certain that TL expression in the thymus of the transgenic mice is not a cause of these events, since conventional TL<sup>+</sup> strains and the other transgenic strain, Tg.Tla<sup>a</sup>-3-2, do not show the abnormality. We can not exclude the possibility that the abnormality is a result of insertional activation or mutagenesis of genes unrelated to TL. However, this genetic alteration has to be under the control of the inserted TL transgene, since the abnormal phenotype is inherited as a dominant trait and is restricted to tissues that express the transgene. These requirements make unrelated insertional mechanisms unlikely. However, to eliminate this possibility, over 20 new transgenic mice with various TL and TL chimeric genes have been derived and are now being established. The possibility we feel best explains the results obtained thus far is that quantitative or qualitative abnormalities in TL expression in the transgenic mice above a certain threshold cause abnormal T cell differentiation in the transgenic mice. The facts are that (a) these mice express over 10 times the level of TL antigen found in conventional TL<sup>+</sup> or Tg.Tla<sup>a</sup>-3-2 mice, (b) TL is expressed on DN thymocytes which are negative in conventional TL<sup>+</sup> mice, and (c) the abnormality is restricted to tissues that express TL. F<sub>1</sub> mice of (C3H/He  $\times$  Tg.Tla<sup>a</sup>-3-1), (B6  $\times$  Tg.Tla<sup>a</sup>-3-1) and (B6-Tla<sup>a</sup>  $\times$  Tg.Tla<sup>a</sup>-3-1) crosses show the characteristic abnormalities of Tg.Tla<sup>a</sup>-3-1 mice, but the onset of the abnormalities is delayed and not so pronounced as in parental homozygous Tg.Tla<sup>a</sup>-3-1 mice (data not shown). These results indicate dominant inheritance of the abnormality with a gene dosage effect, suggesting that an amplified level of TL expression is causally

related to abnormal thymic differentiation. If this assumption is correct, one can assume that TL plays a critical role in T cell development in the thymus and that thymocytes of TL<sup>-</sup> mice must express other MHC class I antigens that are functional equivalents to TL. It has been reported that some MHC class I genes mapping to the Tla region function as ligands for  $\gamma\delta$  TCRs (15, 16, 41, 42). However, unlike TL genes, these other class I genes are expressed in a variety of tissues. Recently, the expression of TL in intestinal epithelium has been described and its relation to  $\gamma\delta$  cells has been discussed (43, 44). It remains to be seen whether TL serves as ligands for certain  $\gamma\delta$  TCRs, whether TL is involved in the selection process of  $\gamma\delta$  cells, and whether altered expression of TL disturbs the normal differentiation of  $\gamma\delta$  cells.

Much effort has been made to characterize  $\gamma\delta$  cells and a vast amount of information has been accumulated (15-18). However, analysis of  $\gamma\delta$  cells in the peripheral lymphoid organs has been difficult because of their minor representation (20-22). With the advantage of the relative abundance of  $\gamma\delta$  cells and  $\gamma\delta$  T cell lymphomas in Tg.Tla<sup>a</sup>-3-1, we have attempted to stage the maturation of  $\gamma\delta$  cells. From analysis of surface antigens, the following pathway for  $\gamma\delta$  DN cells is proposed: the least mature stage 1, IL-2R $\alpha$ <sup>+</sup>HSA<sup>+</sup>Pgp-1<sup>+</sup>TCR<sup>-</sup>; stage 2, IL-2R $\alpha$ <sup>-</sup>HSA<sup>+</sup>Pgp-1<sup>+</sup>TCR<sup>-</sup>; stage 3, IL-2R $\alpha$ <sup>-</sup>HSA<sup>-</sup>Pgp-1<sup>+</sup>TCR<sup>-</sup> $\gamma\delta$ <sup>+</sup>; and the most mature stage 4, IL-2R $\alpha$ <sup>-</sup>HSA<sup>-</sup>Pgp-1<sup>-</sup>TCR<sup>-</sup> $\gamma\delta$ <sup>+</sup>. The appearance of surface TCR  $\gamma\delta$  accompanying CD3 expression occurs between stage 2 and 3. This proposed pathway is obviously not complete and may not be applicable to all subsets of  $\gamma\delta$  cells, but this sequence of changes in the surface phenotype is similar to that proposed for TCR  $\alpha\beta$  lineage (13, 14). Determination of the V region usage (45) in the  $\gamma\delta$  thymocytes and T cell lymphomas should reveal which subsets of  $\gamma\delta$  cells belong to this pathway. Immunization using DN cells and T cell lymphomas from Tg.Tla<sup>a</sup>-3-1 mice may produce reagents specific to the  $\gamma\delta$  lineage and may identify counterparts of Lyt-2 and L3T4 on  $\gamma\delta$  DN cells.

The immune response of Tg.Tla<sup>a</sup>-3-1 mice is severely impaired, with T cells being inefficient in helper function for antibody production, in allogeneic MLC and in Con A stimulation. Since nearly one half of the peripheral T cells in mature Tg.Tla<sup>a</sup>-3-1 mice are  $\gamma\delta$  DN cells, the results strongly suggest that  $\gamma\delta$  cells cannot participate in or are less active in these T cell dependent reactions. Further, the results indicate that the function of  $\alpha\beta$  cells in these transgenic mice is also affected, suggesting that only incompetent  $\alpha\beta$  cells are produced because of the abnormal thymic development. Alternatively, it is conceivable that the function of  $\alpha\beta$  cells is suppressed by  $\gamma\delta$  cells in these transgenic mice. The function of  $\gamma\delta$  cells is largely unknown and their reactivity to allo-antigens or to mitogens is still unclear. Accordingly, the use of isolated population of  $\gamma\delta$  cells from Tg.Tla<sup>a</sup>-3-1 mice in the analysis of other immune functions such as reactivity to mycobacterial antigens and generation of lymphokines, should help in dissecting the functions of  $\gamma\delta$  cells and their relation to the functions of  $\alpha\beta$  cells.

Development of  $\gamma\delta$  T cell lymphomas is another important feature of Tg.Tla<sup>a</sup>-3-1 mice. A relatively long latent

period and clonality of these lymphomas clearly indicates that tumor development requires other genetic events in addition to the transgene, in a manner similar to lymphomagenesis in *c-myc* transgenic mice (46, 47). Why are Tg.Tla<sup>a</sup>-3-1 mice susceptible to lymphomas and why do all lymphomas originate from the  $\gamma\delta$  lineage? An obvious reason for the  $\gamma\delta$  phenotype is that the preponderance of thymocytes in older transgenic mice are  $\gamma\delta$  cells, while  $\alpha\beta$  cells are a minor population. There is no evidence to indicate that  $\gamma\delta$  cells are more susceptible to lymphomagenesis than  $\alpha\beta$  cells, as there have been only a few reports on  $\gamma\delta$  T cell lymphomas in mice or in human (48, 49). A higher mitotic rate of thymocytes in trans-

genic mice than in nontransgenic mice indicates that transgenic thymocytes are rapidly dividing, suggesting that one of the events necessary for the ultimate development of lymphomas may have already occurred in these cells. To understand the genetic basis for the sensitivity of  $\gamma\delta$  cells to lymphomagenesis and how this relates to abnormal T cell differentiation, crosses between Tg.Tla<sup>a</sup>-3-1 mice and high or low lymphoma strains have been made. These mice should provide useful information on the role of TL in the development of the  $\gamma\delta$  T cell lymphomas and also advance our understanding of the significance of TL expression in radiation-induced lymphoma and prelymphoma cells (50-52).

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