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Mixed Phenotype Lymphomas in Thymectomized (SL/Kh×AKR/Ms)F1 Mice

Ling-Min Lu and Hiroshi Hiai[1]

Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501

Lymphomagenesis in mice is determined both by genetic and epigenetic mechanisms. The inbred strain SL/Kh mice selectively develop pre-B lymphomas and AKR/Ms, T-lymphomas. In crosses between SL/Kh and AKR/Ms, an AKR-derived dominant gene *Tlsm1* (*Thymic lymphoma susceptible mouse-1)* determines the type of lymphoma to be a T-lymphoma. As an approach to the role of *Tlsm1*, we studied the effect of thymectomy at 1 week of age in (SL/Kh×AKR/Ms)F1 hybrids. In intact F1 mice, the predominant type of lymphoma was of T-lineage, whereas in thymectomized mice, it was an unusual mixed-phenotype lymphoma. They were basically $CD5^+$ B-lymphomas with a rearranged immunoglobulin gene, but carried NK1 and Mac1 on the cell surface and large lysosomal granules in the cytoplasm. Histologically, the lymphoma consisted of large lymphoblastoid cells and infiltrated the spleen, lymph node and liver. Electron microscopy and histochemistry revealed numerous cytoplasmic granules containing acid phosphatase and lysozyme. These morphological features are suggestive of large granular lymphocytes. They expressed interleukin-4, perforin, and interferon-γ. On transplantation, these lymphoma cells grew equally well in intact and thymectomized F1 recipients.

Key words: Mouse lymphoma — Thymectomy — B-1 lymphoma — Large granular cells — SL/Kh mice

The pathogenetic process of lymphomagenesis is greatly affected by the genetic and epigenetic factors that determine the final form of lymphomas, the incidence and the length of the latent period. We have analyzed these factors in SL/Kh strain mice, an inbred strain highly susceptible to spontaneous pre-B lymphomas.[1)] In contrast to SL/Kh, AKR strain mice develop predominantly T-lymphomas. In the cross between AKR and SL/Kh, our previous study showed that an AKR-derived dominant gene *Tlsm1* on chromosome 7 determines the type of lymphomas to be T-lymphoma.[2)] In T-lymphomagenesis, the thymus has been assumed to provide either the target cells, the site of endogenous retrovirus expression, replication and interaction, or microenvironments supporting the growth and progression of early transformed T-cells.[1, 3–6)] Thymectomy of AKR mice remarkably lowers the lymphoma incidence, modifies the type of lymphomas and prolongs the latent period.[7)] In an attempt to characterize the role of the thymus in the action of *Tlsm1*, we thymectomized (Tx) (SL/Kh×AKR)F1 mice (SLAKF1) at 1 week of age. In contrast to Tx-AKR mice, Tx-SLAKF1 mice develop lymphomas with the phenotype of $CD5^+$ $sIgM^+$ B cells, showing NK-1 expression and large granular lymphocyte (LGL)-like morphology at a high incidence and at a relatively short latent period. Such unusual lymphomas have not previously been described in humans or animal models. We report here in the immunocytological properties of the lymphomas in Tx-SLAKF1 and discuss the possible role of the thymus.

MATERIALS AND METHODS

Mice AKR/Ms, originally obtained as a breeding pair from Dr. H. Shisa, Saitama Cancer Center Research Institute, has been maintained by brother-sister matings over 120 generations. SL/Kh is an inbred strain established in this laboratory[8)] and has been maintained over 80 generations. Their origin,[9)] virological profile[8, 10)] and extremely high susceptibility to spontaneous pre-B lymphomas[11)] were reported. SLAKF1 hybrids were produced by mating of SL/Kh females and AKR/Ms males.

Thymectomy F1 hybrids were thymectomized 3–5 days after birth under ether anesthesia. The upper thorax was incised and the thymus was removed by vacuum aspiration. To avoid residual thymus in Tx-mice, careful cleaning of upper mediastinum was done with a cotton wool stick after aspiration. All the mice were individually identified and carefully observed at least twice a week until 15 months of age. They were housed in a metal cage in an isolation rack with sterile ventilation. Mice were killed for further analysis when they showed clinical signs of lymphomas or became moribund. Any Tx-mice with residual thymus were excluded from this study.

FACS analysis Surface phenotypes of lymphomas were analyzed by flow cytometry using a FACScan (Becton

[1] To whom correspondence should be addressed.
E-mail: hiai@path1.med.kyoto-u.ac.jp

Dickinson, Mountain View, CA) as described previously.[12] The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-coupled CD3, CD8, CD16, Mac-1 and IgM, PE-coupled NK1.1, B220, CD4 and CD5; Thy1.1 was stained indirectly with a second antibody, FITC-labeled anti mouse IgG_{2a}. To block Fc receptor, unlabeled CD16 antibody was used at a concentration of 0.1 mg/ml, at which staining of FITC-labeled anti-CD16 was totally abolished in a preliminary competition assay. These antibodies were purchased from PharMingen (San Diego, CA).

RT-PCR and Southern blotting Total RNA was extracted from tumor tissues. A 5 μg aliquot of RNA was denatured for 10 min at 65°C, quickly chilled on ice and incubated at 37°C for 1 h with a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). A 1 μl aliquot of cDNA from each sample was used for PCR amplification. The procedure and primers were as described by Sugie *et al*.,[13] with slight modifications for IL4, IFNγ and β-actin. The primer for perforin was as described by Rolink *et al*.[14] Amplification by PCR was done for 30 cycles for IL4, IFNγ and perforin, and 20 cycles for β-actin. The sizes of the PCR products were 270 bp for IL4, 396 bp for IFNγ and 610 bp for perforin. Each PCR product was electrophoresed in 1.2% agarose gel and transferred to "Hybond"-N^+ membranes (Pharmacia-Amersham UK, Bucks, UK). Prehybridization and hybridization were performed as described by Rolink *et al*.[14]

To examine immunoglobulin (Ig) gene rearrangement, *Eco*RI-digested DNAs from normal kidney and lymphomas in Tx-SLAKF1 mice were blotted and hybridized with ^{32}P-labeled J_{H4}.[11]

Pathology Tumor tissues were fixed in 10% formalin. Paraffin sections were stained with hematoxylin and eosin. Electron microscopy was conducted as described previously.[15] Enzyme histochemical staining of acid phosphatase was done in cytocentrifuge preparations. For lysozyme, paraffin sections were stained with rabbit anti-human lysozyme antiserum (DAKO A/S, Glostrup, Denmark) and biotinylated goat anti-rabbit Igs (DAKO).

Lymphoma transplantation Intact or Tx-SLAKF1 mice were injected i.p. with 1×10^6 lymphoma cells from lymph node under a sterile condition. Successful passage was confirmed by lymphoma development.

RESULTS

Lymphomas in intact and thymectomized SLAKF1 mice Incidence, latent period and types of the lymphomas that spontaneously developed in SLAKF1 mice are summarized in Table I. In SLAKF1 mice, the presence or absence of the thymus critically affected the type of disease, although the overall incidence of lymphomas remained as high as >90%. In intact SLAKF1 mice, 30 out of 36 lymphomas were T-lymphomas involving the thymus and bearing T-cell phenotypes, including CD3, CD4/CD8 and Thy1.1. In contrast, no T-lymphoma was observed in 41 Tx-SLAKF1 mice, but instead, 32 developed unusual mixed-phenotype lymphomas. The predominant sites of involvement were spleen, lymph nodes and

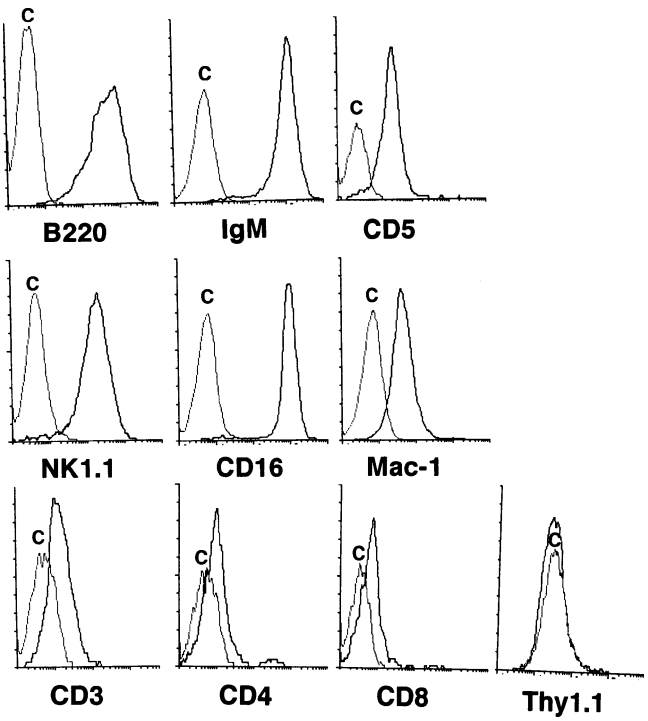

Fig. 1. Surface phenotype of a representative lymphoma in Tx-SLAKF1 mice. Letter C indicates an unstained control.

Table I. Lymphomas Developing in SLAKF1 Mice with or without Thymus

Thymus	No. of mice	Lymphoma (% incidence)	Latency (days)	Type of lymphomas			
				T	B1-$NK1^+$	B	Unclassified
Intact or sham-Tx	39	36 (92.3)	288±73	30	2[a)]	3	1
Tx	41	39 (95.1)	379±91	0	32	6	1

a) Developed in sham-Tx mice.

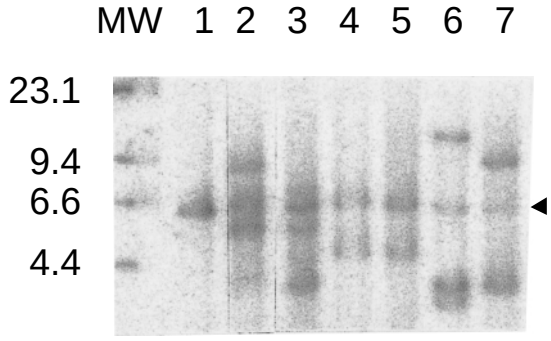

Fig. 2. Southern hybridization of immunoglobulin heavy chain genes. DNAs of normal SLAKF1 kidney (lane 1) and a T-lymphoma in an intact SLAKF1 (lane 2) and lymphomas in Tx-SLAKF1 (lanes 3–7) were digested with *Eco*RI and hybridized with ^{32}P-labeled J_{H4} probe. Digestion with *Nco*I yielded similar results (not shown). An arrowhead represents the germ line fragment. MW: molecular weight size markers (*Hin*dIII-digested λ phage DNA).

liver. Payer's patches were also frequently invaded. The lymphomas in Tx-SLAKF1 had a significantly longer latent period than those in intact mice (P<0.001). As can be seen in Table I, lymphomas with pre-B (BP1$^+$, B220$^+$, CD5$^-$, IgM$^-$) or mature B phenotypes (BP1$^-$, B220$^+$, CD5$^-$, IgM$^+$) developed in both groups at a low incidence. A few lymphomas were categorized as unclassified since they did not express definitive lineage markers.

Unusual phenotype of lymphomas in Tx-SLAKF1 mice

Fig. 1 shows the surface phenotype of a representative case of Tx-SLAKF1 lymphomas. Lymphoma cells expressed B220, CD5, IgM, CD16 and Mac1. For T-cell markers, faint stainings were observed with anti-CD3, CD4 and CD8, but not with anti-Thy1.1. The weak signal may represent contamination with normal T-cells to the extent of 5–10%, as judged from their smaller cell size. Furthermore, the lymphoma cells were highly positive for NK1.1 (Fig. 1). The cells harvested from such lymphomas were larger than most T-lymphoma cells as measured by forward scatter (data not shown). These phenotypes were

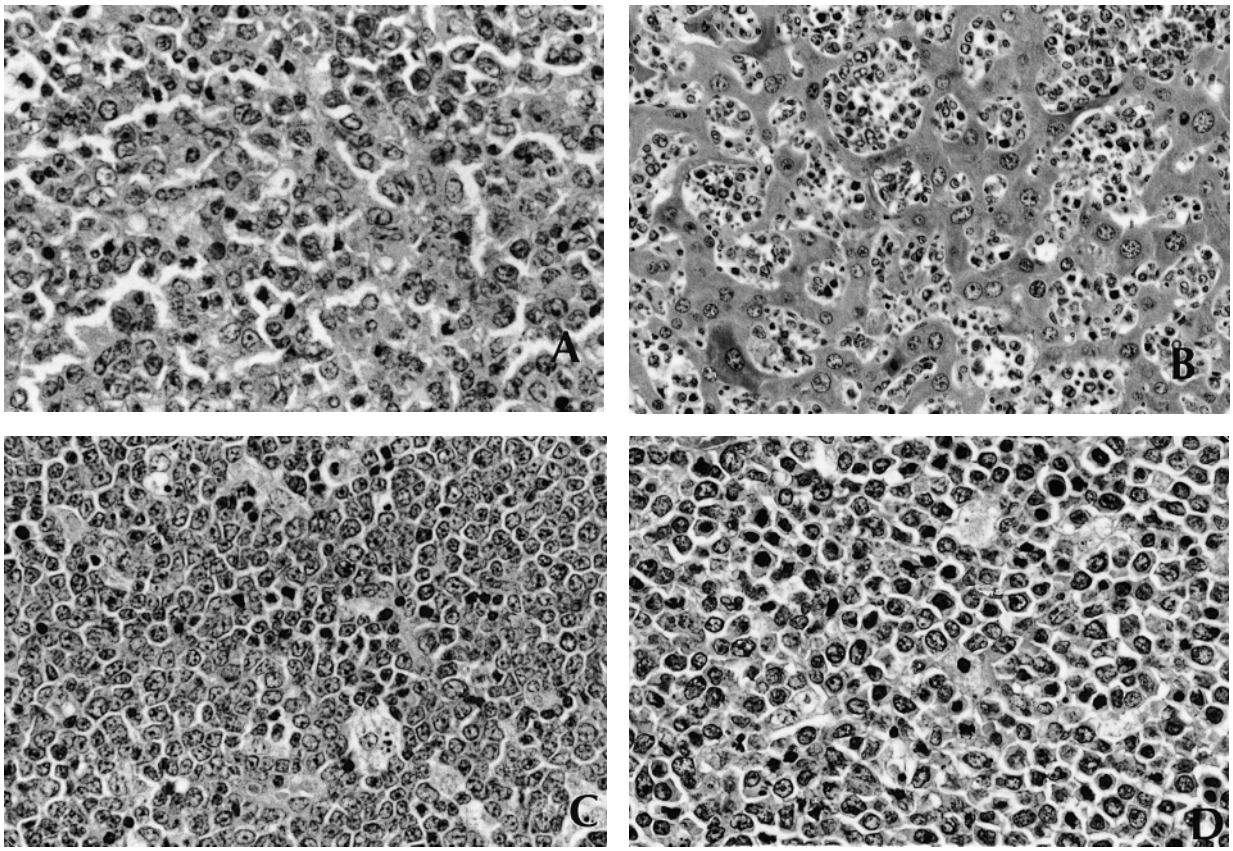

Fig. 3. (A) Histology of a representative Tx-SLAKF1 lymphoma growing in mesenteric lymph nodes. ×400. (B) The same lymphoma infiltrating the liver. ×200. (C) A T-lymphoma in an intact SLAKF1 (mesenteric lymph node). ×400. (D) A pre-B lymphoma in an intact SLAKF1 (mesenteric lymph node). ×400. Hematoxylin and eosin stain.

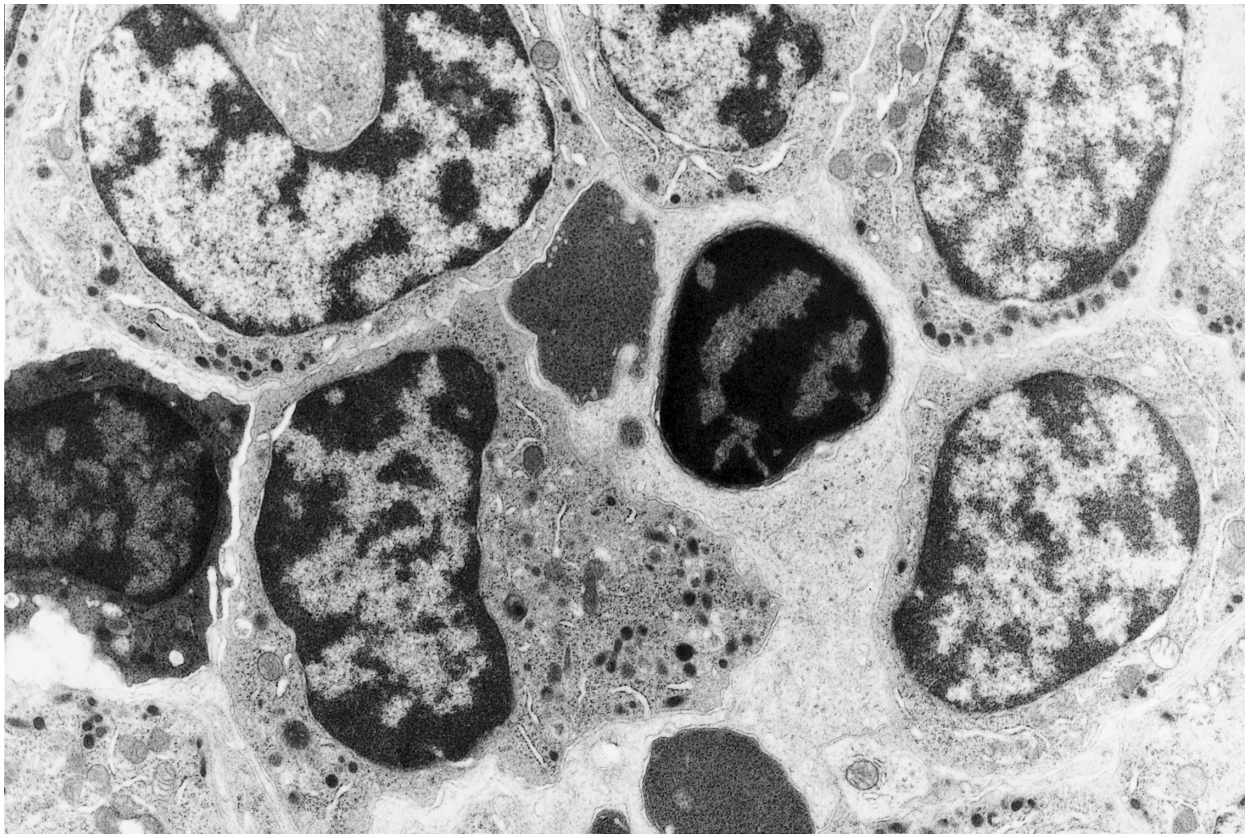

Fig. 4. Electron microscopy of a Tx-SLAKF1 lymphoma. Note characteristic cytoplasmic granules. ×5000.

consistent for all 32 lymphomas in Tx-SLAKF1. Subsequently DNAs of these lymphomas were examined for possible Ig heavy chain gene rearrangements. The *Eco*RI-digested DNAs of the lymphomas in Tx-SLAKF1 showed mono- or oligoclonally rearranged bands in Southern hybridization with Ig J_{H4} probe (Fig. 2).

Histologically they consisted of diffusely infiltrating large lymphoblastoid cells with abundant pale-stained cytoplasm and an indented nucleus carrying 2–4 prominent nucleoli (Fig. 3A). Infiltration in the liver was predominantly in sinusoids rather than in the periportal area (Fig. 3B). Histopathology distinguished them from T-lymphomas (Fig. 3C) or pre-B lymphomas (Fig. 3D), by their larger cell size and less evident starry sky appearance. In electron microscopy, the lymphoma cells had abundant cytoplasm, containing electron-dense granules (Fig. 4) resembling those seen in LGL. Most of the granules were acid phosphatase and lysozyme-positive (not shown). The lymphomas developing in Tx-SLAKF1 mice had a significantly longer latent period than those in intact F1 (P<0.001) (Table I). In contrast to T- and pre-B lymphomas, they grew rather slowly, so that the tumor-bearing mice survived for >2–3 months after the spleen became palpable.

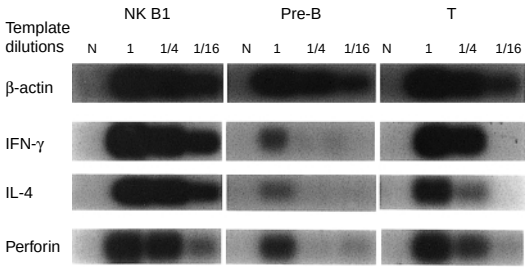

Fig. 5. Expression of IFNγ, IL4 and perforin by 3 types of lymphomas. The control was β-actin mRNA.

Production of cytokines is the distinctive function of NK and related cells. We further examined whether the lymphoma cells expressed IL4, IFNγ and perforin by means of RT-PCR followed by Southern hybridization using ^{32}P-labeled probes. A pre-B and a T-lymphoma from intact F1 mice were used as controls. As shown in Fig. 5,

the lymphomas in Tx-SLKAF1 mice expressed all 3 mRNAs at high levels. The levels of mRNAs of IL4, IFNγ and perforin were in the order of mixed phenotype lymphoma in Tx-SLAKF1 mice>T-lymphoma>pre-B lymphoma. Weak signals in pre-B lymphomas may well be derived from contaminating host T-cells, as production of IFNγ and perforin by pre-B cells has not been well established. These results indicate that the lymphomas in Tx-SLAKF1 mice have lineage markers for both B-1 and NK cells, and express several cytokines at high levels.

The lymphomas in Tx-SLKAF1 mice were transplantable by i.p. injection of 1×10^6 lymphoma cells from lymph nodes into F1 recipients. All of the recipients succumbed with lymphomas of the same phenotype about 30–70 days after grafting, irrespective of the presence or absence of thymus in the recipients.

DISCUSSION

The present study revealed that Tx-SLAKF1 mice developed lymphomas with unusual phenotype at a high incidence. These lymphoma cells had a rearranged Ig heavy chain gene and carried B220, IgM and CD5. Expression of T-cell markers was either negative, or very weak, possibly representing contaminating host cells. Expression of CD5, B220 and surface IgM by lymphoma cells is compatible with $CD5^+$ B (B1) lineage. Peled and Haran-Ghera[16] found that thymectomy of AKR mice at 1 month of age prevents T-lymphomas, but the Tx-AKR mice develop B-1 lymphomas much later in life at a lower incidence. Southern blot analysis of the IgH locus of B-1 lymphomas in Tx-AKR mice has revealed that rearrangement is mono- or oligoclonal.[17] Similar lymphomas have been described in old BALB/c mice or several autoimmunity-prone strains of mice.[18, 19] However, the lymphomas in Tx-SLAKF1 showed several unusual characteristics not reported in lymphomas in Tx-AKR or other B-1 lymphomas. They had cytoplasmic granules resembling LGL, and exhibited expression of NK1.1 as well as some cytokine mRNAs. Moreover, they had macrophage-like characteristics, such as Mac1 and CD16. The spectrum of cytokine messages was also unusual in these lymphomas. B-1 lymphoma cells can secrete IL4, but not IFNγ.[20] Perforin has been recognized as a cytotoxic component of CTL and NK cells. High expression of transcripts for IL4, IFNγ and perforin in Tx-SLAKF1 lymphomas is a further indication of their extraordinary character. Recently, several aberrant subsets showing dual lineage properties have been reported, e.g., NK^+ T-cells, T+B, T/macrophage and B-1/macrophage.[21–23] These lymphocytes with complicated lineage are mostly found in lymphomas or autoimmune diseases. The lymphomas in Tx-SLAKF1 mice are, therefore, probably not straightforward B-1 lymphomas. We propose that these lymphomas are $NK1^+$ B-1 cells or triphenotypic lymphomas. These lymphomas were occasionally associated with a variable number of normal T-cells, possibly induced by products of the lymphoma cells.[24] Whether or not the $NK1^+$ B-1 lymphoma has NK activity, for instance on YAC cells, is an interesting issue, but it has not yet been examined as several attempts to obtain an *in vitro* cell line have failed.

The effect of thymectomy on mouse lymphomagenesis is model-dependent. Thymectomy of AKR mice effectively prevents T-lymphomagenesis, and instead, B-1 lymphomas develop at a low incidence at ~600 days of age.[7] In contrast, the pre-B lymphomas in SL/Kh are not affected by thymectomy except for slight prolongation of the latency period (Lu, unpublished observation). In SLAKF1, however, thymectomy dramatically changed the type of disease, e.g., from T- to $NK1^+$ B-1 lymphomas. To explain such differences, one should consider the genetic background of SL/Kh. In SL/Kh bone marrow, there is a polyclonal expansion of pre-B cells ~2 months in advance of monoclonal growth and such expansion is genetically determined by a quantitative trait locus *Bomb1* (*Bone marrow pre-B-1*).[25] Genetic abnormality in B-lineage, although its exact nature is unclear, may well predispose the animals to a high incidence of lymphomas, irrespective of the presence or absence of thymus. It is well known that the NK or NK T cell population increases in Tx-mice or nude mice.[21, 22, 26] Thymus might provide a negative signal to generation of NK-related cells. We are now studying the possibility that thymectomy may modify the differentiation of the extrathymic lymphoid precursors which include target cells leading to B-lineage lymphomas.

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REFERENCES

1) Hiai, H. Genetic predisposition to lymphomas in mice. *Pathol. Intern.*, **46**, 707–718 (1996).
2) Yamada, Y., Shisa, H., Matsushiro, H., Kamoto, K., Kobayashi, Y., Kawarai, A. and Hiai, H. T lymphomagenesis is determined by a dominant host gene *thymic lymphoma susceptible mouse-1* (*Tlsm-1*) in mouse models. *J. Exp. Med.*, **180**, 2155–2162 (1994).
3) Kaneshima, H., Itoh, M., Asai, J., Taguchi, O. and Hiai, H. Reorganization of thymic microenvironments during development and lymphomagenesis. *Jpn. J. Cancer Res.*, **78**, 799–806 (1987).
4) Law, L. W. and Miller, J. H. Observations on the effect of thymectomy on spontaneous leukemias in mice of the high leukemia strains RIL and C58. *J. Natl. Cancer Inst.*, **11**, 253–262 (1950).
5) Miller, J. F. A. P. Analysis of the thymus influence in leukaemogenesis. *Nature*, **191**, 248–249 (1961).
6) Metcalf, D. The effect of thymectomy on the lymphoid tissues of the mouse. *Br. J. Haematol.*, **6**, 324–333 (1960).
7) Haran-Ghera, N. Lymphomagenesis in AKR mice: B cell lymphomas as a model of tumor dormancy. *Adv. Cancer Res.*, **63**, 245–293 (1994).
8) Hiai, H., Kaneshima, H., Nakamura, H., Oguro, Y. B., Moriwaki, K. and Nishizuka, Y. Unusually early and high rate of spontaneous occurrence of nonthymic leukemias in SL/Kh mice, a subline of SL strain. *Jpn. J. Cancer Res.*, **73**, 704–712 (1982).
9) Abujiang, P., Yamada, Y., Haller, O., Kobayashi, H., Kamoto, T., Lu, L.-M., Ogawa, M., Ishimoto, A., Katoh, H., Kanehira, K., Ikegami, S., Fukumoto, M. and Hiai, H. The origin of SL family mice. *Lab. Anim. Sci.*, **46**, 410–417 (1996).
10) Yamada, Y., Matsushiro, H., Ogawa, M. S., Kamoto, K., Nakakuki, Y., Toyokuni, S., Fukumoto, M. and Hiai, H. Genetic predisposition to pre-B lymphomas in SL/Kh strain mice. *Cancer Res.*, **54**, 403–407 (1994).
11) Shimada, M. O., Yamada, Y., Nakakuki, Y., Okamoto, K., Fukumoto, M., Honjo, T. and Hiai, H. SL/Kh strain of mice: a model of spontaneous pre-B lymphomas. *Leuk. Res.*, **17**, 573–578 (1993).
12) Lu, L.-M., Ogawa, M., Kamoto, T., Yamada, Y., Abujiang, P. and Hiai, H. Expression of LECAM-1 and LFA-1 on pre-B lymphoma cells but not on preneoplastic pre-B cells in SL/Kh mice. *Leuk. Res.*, **21**, 337–342 (1997).
13) Sugie, T., Kubota, H., Sato, M., Nakamura, E., Imamura, M. and Minato, N. NK1.1$^+$ T CD4$^-$CD8$^-\alpha\beta$ T cells in the peritoneal cavity—specific T cell receptor-mediated cytotoxicity and selective IFN-production against B cell leukemia and myeloma cells. *J. Immunol.*, **157**, 3925–3935 (1996).
14) Rolink, A., Boekel, E. E., Melchers, F., Fearon, D. T., Krop, I. and Andersson, J. A subpopulation of B220$^+$ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.*, **183**, 187–194 (1996).
15) Iida, F., Matsushima, Y., Hiai, H., Uga, S. and Honda, Y. Rupture of lens cataract: a novel hereditary recessive cataract model in the mouse. *Exp. Eye Res.*, **64**, 107–113 (1997).
16) Peled, A. and Haran-Ghera, N. High incidence of B cell lymphomas derived from thymectomized AKR mice expressing TL.4 antigen. *J. Exp. Med.*, **162**, 1081–1086 (1985).
17) Rosner, A., Peled, A., Haran-Ghera, N. and Canaani, E. Analysis of Ly-1$^+$ B cell populations and IgH rearrangements in "normal" spleens and in lymphomas of AKR/J and AKR *Fv-1^b* mice. *Cancer Res.*, **53**, 2147–2153 (1993).
18) Slavin, S. and Strober, S. Spontaneous murine B-cell leukemia. *Nature*, **372**, 624–626 (1978).
19) Okada, T., Takiura, F., Tokushige, K., Nozawa, S., Kiyosawa, T., Nakauchi, H., Hirose, S. and Shirai, T. Major histocompatibility complex controls clonal proliferation of CD5$^+$ B cells in H-2 congenic New Zealand mice: a model for B cell chronic lymphocytic leukemia and autoimmune disease. *Eur. J. Immunol.*, **21**, 2743–2748 (1991).
20) O'Garra, A., Stapleton, G., Dhar, V., Pearce, M., Schumacher, J., Rugo, H., Barbis, D., Stall, A., Capp, J., Moore, K., Vieira, P., Mosmann, T., Whitmore, A., Arnold, L., Haughton, G. and Howard, M. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int. Immunol.*, **2**, 821–831 (1990).
21) Sykes, M. Unusual T cell populations in adult murine bone marrow: prevalence of CD3$^+$ CD4$^-$CD8$^-$ and $\alpha\beta$ TCR$^+$ NK1.1$^+$ cells. *J. Immunol.*, **145**, 3209–3215 (1990).
22) Watanabe, H., Miyaji, H. C., Seki, S. and Abo, T. C-kit$^+$ stem cells and thymocyte precursors in the livers of adult mice. *J. Exp. Med.*, **184**, 687–693 (1996).
23) Berrello, M. A. and Phipps, R. P. The B/macrophage cell: an elusive link between CD5$^+$ B lymphocytes and macrophages. *Immunol. Today*, **17**, 471–475 (1996).
24) Willoughby, P. B., Jennette, J. C. and Haughton, G. Analysis of a murine B cell lymphoma, CH44, with an associated non-neoplastic T cell population. *Am. J. Pathol.*, **133**, 507–515 (1988).
25) Lu, L.-M., Shimada, R., Higashi, S., Zeng, Z.-Z. and Hiai, H. Bone marrow pre-B-1 (*Bomb1*): a quantitative trait locus inducing bone marrow pre-B cell expansion in lymphoma-prone SL/Kh mice. *Cancer Res.*, **59**, 2593–2595 (1999).
26) Sato, K., Ohtsuka, K., Hasegawa, K., Yamakiwa, S., Watanabe, H., Asakura, H. and Abo, T. Evidence for extrathymic generation of intermediate T cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone marrow transplantation. *J. Exp. Med.*, **182**, 759–767 (1995).