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Aberrant Activation of Interleukin-9 Receptor and Downstream Stat3/5 in Primary T-cell Lymphomas *In Vivo* in Susceptible B6 and Resistant C3H Mice

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Abstract. *Background: Interleukin (IL)-2 family cytokine-mediated signal transduction plays important roles not only in normal development but also in the malignant transformation of lymphoid cells. However, little is known about the status of receptor activation and downstream signal transduction in primary lymphomas in vivo. Materials and Methods: Primary T-cell lymphomas (TL) of mice were induced by X-ray irradiation. Expression and activation of IL-2 family cytokine receptors and downstream Janus kinase (Jak)-signal transducers and activators of transcription (Stat) pathway were determined. Results: IL-9Rα was exceptionally highly expressed and phosphorylated in primary TL. IL-9Rα proteins in TL were heterogeneous due to different glycosylation. Downstream Stat3 and 5, but not Stat1, were also phosphorylated. There was a clear strain difference between susceptible C57BL/6 and resistant C3H mice in Stat3 and 5 activation and expression of Cyclin D1. Conclusion: Aberrant expression, modification and activation of IL-9Rα and Stat proteins contribute to in vivo growth of TL in a manner linking to the genetic susceptibility to TL induction.*

Interleukin (IL)-2 family cytokines play important roles not only in normal development but also in the malignant transformation of lymphoid cells. These cytokines bind to heterodimeric or trimeric receptors comprising specific alpha, beta and gamma common (γc) chain, which is shared by the receptors for these cytokines. Upon receptor ligation, a change in receptor conformation results in auto and/or transphosphorylation of Janus kinases (Jak), which leads to phosphorylation of the receptor and of signal transducers and activators of transcription (Stat) 1, 3 and 5. Phosphorylated Stat molecules dimerize and migrate to the nucleus where they bind regulatory sequences for genes such as *c-Myc, c-Jun, Pim1* and *Cyclin D1* (1-3). Unusual expression of mRNA of receptors for IL-2 family has been frequently demonstrated in primary T acute lymphoblastic leukemia (T-ALL), cutaneous T-cell lymphoma and human T-cell leukemia virus (HTLV) 1-transformed lymphoid cells (4-6). However, little study has examined biochemical characteristics of the receptors in malignant cells *in vivo*.

The importance of genetic factors for predisposition to leukemia has been suggested by racial differences and the association of specific SNP of cytokines with non-Hodgkin's lymphoma (7, 8). A clear genetic susceptibility to radiation-induced T-cell lymphomas in mice, which has been used as a model of human T-cell lymphoma, is also present: C57BL/6 (B6), C57BL/10 (B10) and Balb/c mice are susceptible, whereas C3H/HeN (C3H) and STS/A are resistant (9, 10). Possible mechanisms behind this genetic susceptibility include the ability to repair DNA damage such as DNA-PKcs, susceptibility to oxidative stress and the tissue microenvironment where the lymphoma cells expand (11-13). It is interesting to examine if strain differences may exist in the activation of the cytokine-mediated signal cascade between these susceptible and resistant strains.

In an attempt to determine if activation of cytokine signal pathways contributes to TL induction and is associated with genetic susceptibility, we examined the expression and biochemical characteristics of γc-associated cytokine receptors and activation of downstream signals in radiation-induced TL in susceptible B6 and resistant C3H mice.

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Key Words: T-cell lymphoma, IL-9Rα, Stat, strain difference.

Table I. *Sequences of primers and conditions of RT-PCR.*

Primer	Sense	Antisense	RT-PCR conditions	Cycles
G3pdh	TGAAGGTCGGTGTGA ACGGATTTGGC	CATGTAGGCCATGAGG TCCACCAC	72˚C 1.5 min/94˚C 30 s/60˚C 30 s	28
IL-2Rα	ATGGAGCCACGCTTG CTGATGTTG	CCATTGTGAGCACAA A TGTCTCCG	72˚C 1.5 min/94˚C 45 s/60˚C 45 s	30
IL-2Rβ	CAATGTCTCTTGCATG TGGAGCCAT	AAGACGTCTACGGGCC TCAAATTCCAA	72˚C 1.5 min/94˚C 45 s/60˚C 45 s	34
IL-4Rα	CTACTATACGGCGCGT GTGA	GGCACCTGTGCATCCT GAAT	72˚C 1.0 min/94˚C 30 s/58˚C 30 s	35
IL-7Rα	CAAAGTCCGATCCATT CCCCATAAC	GTTTTCTTATGATCGGG GAGACTAGG	72˚C 1.5 min/94˚C 45 s/60˚C 45 s	35
IL-9Rα	CACAAATGCACCTTCT GGGACA	TCACTCCAACGATACG GTCCTT	72˚C 30 s/94˚C 30 s/64˚C 30 s	35
IL-15Rα	CTACTGTTGCTCCGC TGAG	TGTCTCTGTGGTCATT TGCGGTAT	72˚C 1.5 min/94˚C 45 s/60˚C 45 s	35
γc	CCAGAGGTTCAGTGC TTTGTGT	ATCCACACTAGGCAG GGAGAAT	72˚C 1.5 min/94˚C 45 s/60˚C 45 s	33
Cyclin D1	GCGTACCCTGACACC AATCT	ACCAGCCTCTTCCTC CACTT	72˚C 30 s/94˚C 30 s/60˚C 30 s	35
Pim1	GCTCGGTCTACTCTG GCATA	GTAGCGATGGTAGCG AATCC	72˚C 30 s/94˚C 30 s/60˚C 30 s	33
c-Jun	CTGAGTGTGCGAGAG ACAGC	CGCTAGCACTCACGT TGGTA	72˚C 30 s/94˚C 30 s/60˚C 30 s	35
c-Myc	AGTGCATTGATCCCTC AGTGGTCTTTCCCTA	CAGCTCGTTCCTCCTC TGACGTTCCAAGACGTT	72˚C 30 s/94˚C 30 s/60˚C 30 s	30
Bcl-xl	TGGTGGTCGACTTTCT CTCC	AAGAGTGAGCCCAGCA GAAC	72˚C 30 s/94˚C 30 s/60˚C 30 s	30

Materials and Methods

Mice and tumor induction. Fifty C57BL/6N (B6) mice and one hundred C3H/HeN (C3H) female mice were obtained from Charles River, Inc. (Yokohama, Japan), and housed in a temperature- and light-controlled facility with food and water available *ad libitum*. Animal care and experimental protocols were approved by the National Institute of Radiological Sciences of Japan. Experiments were performed in strict accordance with the guidelines of the Institute. TL was induced by whole body X-irradiation split into 4 weekly doses of 1.6 Gy at 0.6 Gy/min (Pantak Ltd., East Heaven, CT, USA; 200 kVp, 20 mA with filters of 0.5 mm Cu and 0.5 mm Al), starting at 5 weeks of age. Mice were sacrificed when they became moribund. Sixty 8-week-old female mice for each strain were used as normal control.

Expression analysis of cytokine receptors by reverse transcriptase polymerase chain reaction (RT-PCR). Since the amount of mRNA from each TL was limited, a semi-quantitative RT-PCR assay was used as described elsewhere (14). The sequences of primers and annealing temperatures used for the analysis of *G3pdh, IL-2Rα, IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, γc, Bcl-xl, c-Jun, c-Myc, Pim1* and *Cyclin D1* are listed in Table I. PCR products were electrophoresed through a 2% Seakem agarose gel. The luminescence of gel bands was measured with a BioImage laser scanner (Genomic SolutionsTM, Ann Arbor, MI, USA), and the OD recorded for each gene was scaled to that of *G3pdh* to give the relative expression. RT-PCR for each gene was repeated twice.

Antibodies for Western blot and immunoprecipitation (IP). For Western blot, rabbit anti-mouse Stat1, phospho-Stat1, Stat3, phospho-Stat3, Stat5 and phospho-Stat5 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit anti-mouse IL-9Rα (M-20), goat anti-mouse β-actin (I-19) and donkey anti-goat IgG-horseradish peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Biosciences (Piscataway, NJ, USA). For immunoprecipitation (IP), mouse anti-phosphotyrosine (pY), clone 4G10, was purchased from Upstate, Inc. (Lake Placid, NY, USA). Antibody dilution was performed according to standard protocols.

Western blot for cytokine receptors and downstream Stats. TL was lysed in cell lysis buffer with protease inhibitor (Protease Inhibitor Cocktail; Cell Signaling Technology). Western blot was performed according to standard procedures. Briefly, electrophoresis was performed on 20 μg of protein/sample on 10% SDS-polyacrylamide gel electrophoresis (PAGE). The separated gels were transferred to polyvinylidine difluoride (PVDF) Immobilon Transfer Membranes (Millipore, Billerica, MA, USA) for 1 hour at 4˚C. The membrane was incubated in blocking buffer containing 20 mM Tris, 150 mM NaCl, 0.05% Tween20 and 5% skim milk for 1 hour at room temperature. After incubating with antibodies, the membrane was washed, incubated in Electrochemiluminescence (ECL) plus detection reagent (Amersham Biosciences) and exposed to a Las3000 CCD camera (FUJI FILM, Tokyo, Japan). Imagine analysis

Table II. *Expressions of γc cytokine receptors in normal thymocytes and T-cell lymphoma cells.*

	B6			C3H		
	Normal (n=60)	TL (n=45)	Fold difference	Normal (n=60)	TL (n=17)	Fold difference
IL-2Rα	0.13±0.06	0.61±0.13[a]	4.67	0.06±0.02	0.43±0.13[a]	7.23
IL-2Rβ	0.45±0.20	0.61±0.15[b]	1.37	0.48±0.13	0.17±0.07	–0.35
IL-4Rα	1.24±0.09	0.83±0.13[a]	–0.67	1.77±0.51	0.53±0.14[a]	–0.30
IL-7Rα	1.54±0.63	1.04±0.15	–0.68	0.95±0.21	1.72±0.37	1.81
IL-9Rα	0.01±0.003	0.66±0.11[a,b]	65.90	0.02±0.01	0.44±0.10[a]	22.14
IL-15Rα	0.12±0.03	0.33±0.08[a,b]	2.69	0.23±0.12	0.05±0.02	–0.26
γc	0.65±0.05	1.56±0.23[a,b]	2.40	0.96±0.18	0.58±0.11	–0.61

Expression of γc cytokine-receptor mRNA, such as *IL-2Rα, IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα* and *γc* relative to the expression of *G3pdh* (mean±SE) in normal thymocytes and TL from B6 and C3H mice. Statistical analysis was performed by Student's *t*-test ([a]: TL *vs*. normal thymocytes and [b]: B6 *vs*. C3H with $p<0.05$).

Table III. *Expression of Stat5 target genes in normal thymocytes and T-cell lymphoma cells.*

	B6			C3H		
	Normal (n=60)	TL (n=45)	Fold difference	Normal (n=60)	TL (n=17)	Fold difference
Bcl-xl	1.18±0.15	0.15±0.02[a]	–0.13	1.12±0.15	0.14±0.02[a]	–0.13
c-Jun	0.99±0.49	0.81±0.19	–0.82	1.03±0.10	0.50±0.15	–0.49
c-Myc	0.14±0.04	0.15±0.02	1.07	0.06±0.02	0.07±0.02	1.17
Pim1	1.54±0.59	0.42±0.06[a]	–0.27	1.18±0.25	0.47±0.25[a]	–0.40
Cyclin D1	0.08±0.05	1.51±0.16[a,b]	18.9	0.07±0.06	0.60±0.19[a]	8.57

Expression of *Bcl-xl, c-Jun, c-Myc, Pim-1* and *Cyclin D1* mRNA relative to the expression of *G3pdh* (mean±SE) in normal thymocytes and TL from B6 and C3H mice. Statistical analysis was performed by Student's *t*-test ([a]TL *vs*. normal thymocytes; and [b]B6 *vs*. C3H with $p<0.05$).

was carried out by Image Gauge Software (FUJI FILM). Anti-β-actin antibody was used to confirm that an equal amount of protein was loaded onto each lane. Western blots were repeated twice.

Glycosidase treatment of IL-9R protein. Protein lysates of TL were incubated at 37˚C overnight with or without *N*-glycosidase F (Roche, Mannheim, Germany) at 1-10U.

Analysis of IL-9Rα phosphorylation by IP. For IL-9Rα phosphorylation analysis, protein G sepharose (Pharmacia Biotech AB, Uppsala, Sweden) was added to anti-phosphotyrosine antibody and mixed. Proteins of 40 μg each were incubated with these mixtures for 1 hour. The immunoprecipitates were washed with lysis buffer and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted with anti-IL-9Rα antibody.

Immunohistochemistry staining. Cytospin slides were made by centrifuging TL cells with Cytospin3 cell preparation system (Pittsburgh, PA, USA), fixed with 100% cool methanol, air dried and saved at –80˚C. Slides were pre-treated with blocking buffer (10% goat serum in Block Ace, Dainippon Pharmaceutical Co., Osaka, Japan) and then incubated with diluted primary antibody (1:50) for 1 hour at room temperature. Slides were washed three times with Tris buffered saline with Tween 20 (TBST), incubated with a dilution of the peroxidase-conjugated goat anti-rabbit immunoglobulin (Histofine simple stain MAX PO (M) kit, Nichirei, Tokyo, Japan) for 30 minutes at room temperature. Signals were visualized with diaminobenzidine (DAB) staining (DAB Substrate kit; Vector Laboratories, Inc., Burlingame, CA, USA). Finally, specimens were counterstained with hematoxylin, dehydrated and mounted.

Statistical analyses. Statistical analysis for RT-PCR was performed by Student's *t*-test. For Western blot, Statistical analysis was performed by the Mann-Whitney test for activity level measurements.

Results

Incidence of thymic lymphoma. After split irradiation starting at 5 weeks of age, TL was induced at an incidence of 96% for B6 mice and only 24% for C3H mice ($p<0.01$). The average latent period was 108.4±25.0 days for B6 mice, while it was 172.6±68.5 days for C3H mice ($p<0.01$), indicating that the B6 strain is more susceptible to lymphomagenesis than the C3H strain.

Expression of cytokine receptors. We examined the relative expression of *IL-2Rα, IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα* and *γc* by *RT-PCR*. TL from B6 mice showed

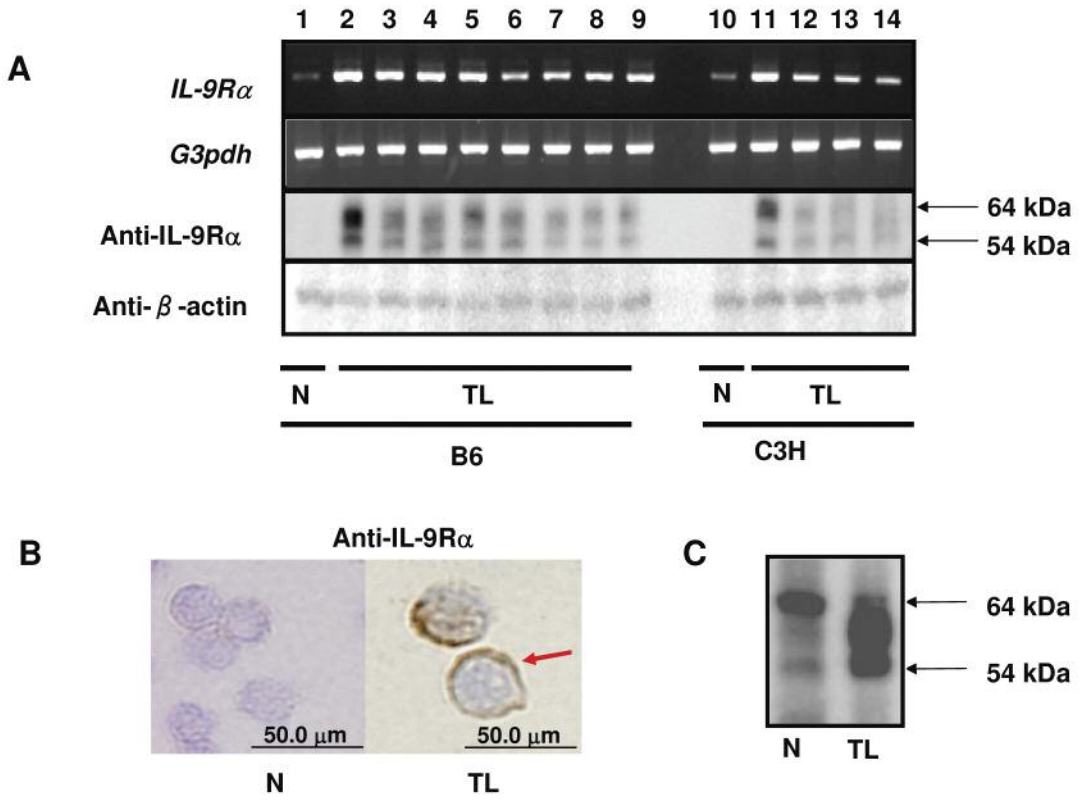

Figure 1. *A, Expression of IL-9Rα mRNA and IL-9Rα protein in normal thymocytes and TL cells. G3pdh and β-actin were used as internal controls, respectively. B, Immunohistochemistry for IL-9Rα in normal thymocytes (N) and TL cells (TL). Arrow indicates membrane-bound or cytoplasmic IL-9Rα. C, Comparison of the molecular size of IL-9Rα in normal thymocytes and TL. A total of 100 μg of protein (5-fold as large protein lysate of normal thymocytes as that of TL) was loaded to perform Western blot.*

higher expression of *IL-2Rα, IL-9Rα, IL-15Rα* and *γc* than normal thymocytes, while TL from C3H mice had higher expression of *IL-2Rα* and *IL-9Rα*. *IL-9Rα* expression increased by 66-fold in TL from B6 and by 22-fold in TL from C3H, compared to normal thymocytes (Table II). Expression of *IL-7Rα* and *IL-2Rβ* did not show significant differences between TL and normal thymocytes, while that of *IL-4Rα* decreased in both strains (Table II).

Characterization of IL-9Rα. Since IL-9Rα showed unusually high expression in TL, we focused on IL-9Rα. Western blot analysis indicated that IL-9Rα protein(s) were expressed in parallel with mRNA expression. They consisted of heterogeneous isoforms and were expressed abundantly in TL while these were negligible in control thymocytes (Figure 1A). Immunohistochemically, IL-9Rα protein(s) were located in the cytoplasm or plasma membrane (Figure 1B). To determine the size of IL-9Rα protein in normal thymocytes, we loaded 5-fold as much protein lysate of normal thymocytes as that of TL. We found that normal thymocytes expressed a large isoform, while TL cells expressed predominantly two smaller isoforms (Figure 1C). Although splice variants of IL-9Rα in human lymphoid cells and megakaryocytes have been demonstrated (15), we failed to observe any splice variants in mouse TL as determined by RT-PCR (data not shown). Human IL-9Rα is reported to be a glycoprotein that has two *N*-linked residues, Asn116 and Asn155, in extracellular domains, both of which are also present in mouse IL-9Rα (16). On treatment with glycosidase, the size of the two isoforms was reduced to the same single size of 54 kDa (Figure 2A), indicating that the size difference of IL9Rα was due to the glycosylation level (Figure 1C). We next examined the phosphorylation of IL-9Rα because *in vitro* studies reported that ligation with IL-9 results in phosphorylation of IL-9Rα (17). IP analysis indicated that three isoforms were phosphorylated, the non-glycosylated smallest isoform being the most strongly phosphorylated. Since this smallest band was hardly visible by conventional Western blot for whole cell lysate (Figure 1A), the result suggested partial deglycosylation during preparation for IP analysis (Figure 2B). The amount of IL-9Rα with

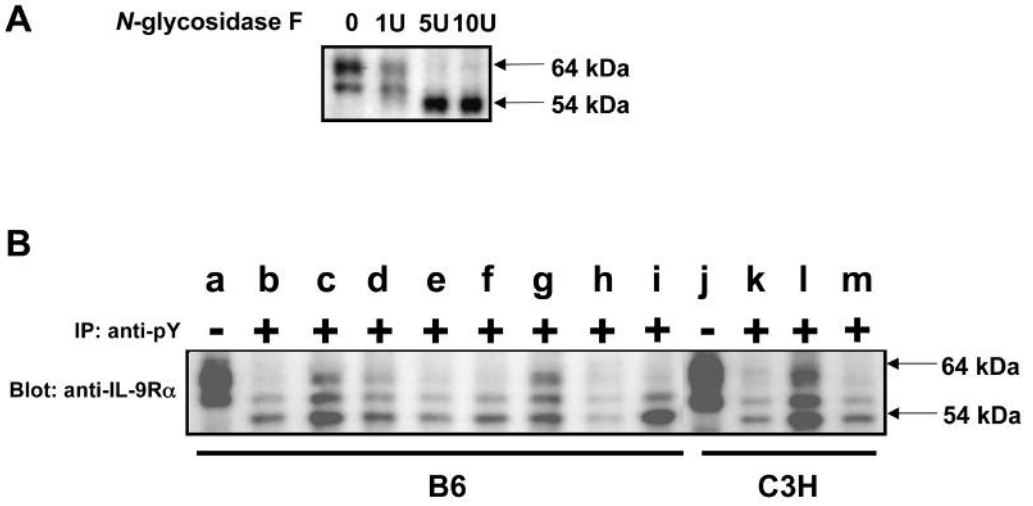

Figure 2. *A, Decrease in molecular size of IL-9Rα protein after treatment with N-glycosidase F at doses of 0, 1, 5 and 10 U. B, Status of IL-9Rα phosphorylation by IP analysis. Lanes a and j are the same TL sample as lanes b and k, respectively, but without IP.*

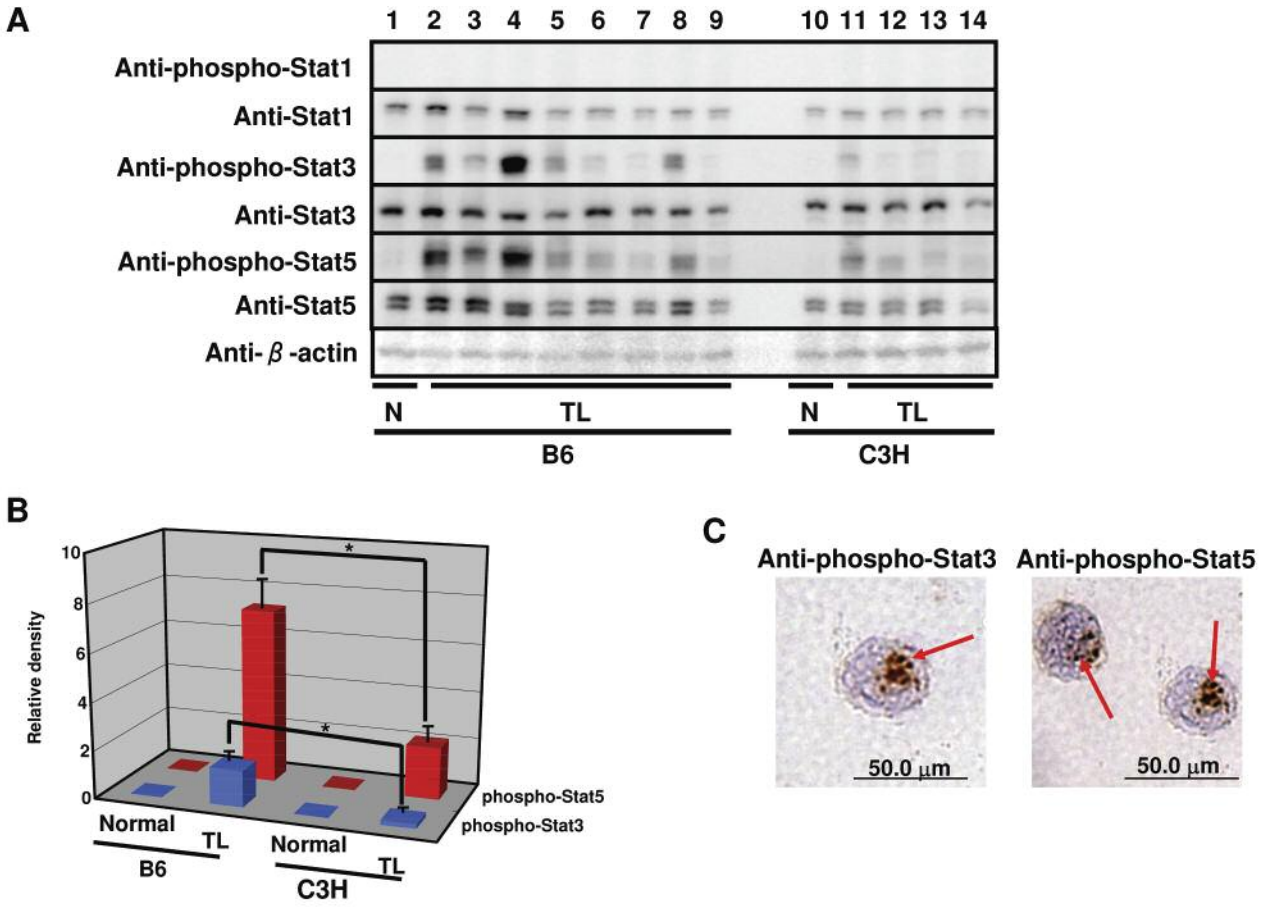

Figure 3. *A, Representative results of Stat1, Stat3 and Stat5 phosphorylation status in normal thymocytes (N) and TL cells from B6 and C3H mice by Western blot analysis. B, Comparison of phospho-Stat3 and phospho-Stat5 levels between normal thymocytes and TL cells in B6 (n=45) and C3H (n=15) mice. The graph represents the relative density of phospho-Stat3 and phospho-Stat5 to β-actin (mean ± SE). Strain difference was statistically significant by the Mann-Whitney test (*p<0.01). C, Immunohistochemical analysis of phospho-Stat3 and phospho-Stat5. Arrows indicate intranuclear phospho-Stat3 and phospho-Stat5.*

phosphorylation (lanes b and k in Figure 2B) was less than the total amount of IL-9Rα protein (lanes a *vs.* b and j *vs.* k in Figure 2B), and, therefore, some IL-9Rα protein might be activated *in vivo*.

Activation of the Stat cascade. Since IL-9Rα activates the Stat signal pathway (18, 19), we then examined the expression and phosphorylation of Stat1, Stat3 and Stat5. Stat1, Stat3 and Stat5 protein(s) were constitutively expressed in both TL and normal thymocytes at a similar level regardless of mouse strain. While normal thymocytes did not show any phosphorylation of Stat3 or Stat5, phosphorylation was evident in TL (Figure 3A). Phosphorylation was more evident in TL of B6 than C3H, suggesting a strain difference (Figure 3B). Immunohistochemical examination clearly showed focal accumulation of phospho-Stat3 and phospho-Stat5 in nuclei (Figure 3C). We failed to detect, however, any Stat1 phosphorylation either in TL or in normal cells from both strains (Figure 3A).

Expression of Stat5 target genes. Next we elucidated the expression of target genes of Stat5. The genes included *Bcl-xl, c-Jun, c-Myc, Pim1* and *Cyclin D1*. The fold increase of gene expression in TL compared to normal thymocytes was large for *Cyclin D1*, whereas that for *Bcl-xl* and *Pim1* was small (Table III). The expressions of *c-Jun* and *c-Myc* were almost comparable in normal thymocytes. A strain difference was evident; TL from B6 expressed greater *Cyclin D1* than that from C3H.

A lack of expression of IL-9 from TL. We attempted to amplify the IL-9 mRNA in TL in order to determine if an autocrine loop of IL-9 and IL-9Rα was established. No transcripts were observed in TL from either B6 or C3H. Thus, activation of IL-9Rα could not be ascribed to autocine released IL-9.

Mutation of the IL-9Rα gene. Since constitutive activating mutations in *c-kit* have been reported in leukemia (20), gain-of-function mutation of *IL-9Rα* was analyzed. There was no mutation in *IL-9Rα* cDNA in any TL (data not shown). No SNP was identified between B6 and C3H.

Discussion

The present study demonstrated that IL-9Rα in TL was much more highly expressed and phosphorylated compared to normal thymocytes in both susceptible B6 and resistant C3H mice. IL-9Rα proteins in TL comprised heterogenous molecules with different glycosylation which were smaller than those in normal thymocytes. There was a clear strain difference in activation status of IL-9Rα and downstream Stat3 and Stat5 in TL, which may be linked to genetic susceptibility to TL development. Neither mutation of *IL-9Rα* nor autocrine production of IL-9 was demonstrated in TL in the two mouse strains.

We showed here the difference in glycosylation between TL and normal thymocytes. *N*-linked glycosylation is a posttranslational modification of protein which affects protein folding and trafficking, thereby changing the integrity or binding of cytokine to its receptor. It is well known that tumorigenesis is frequently associated with altered structure and expression of oligosaccharaides on cell surface glycoprotein (21). However, only few data are available on quantitative and qualitative changes in glycosylation of specific proteins during tumorigenesis. It has been demonstrated that aberrant glycoslylation of CML granulocytes alters the binding of GM-CSF to their receptor(s), but not of normal granulocytes (22). A functional role for the modulation by glycosylation of glucose transporter GLUT1 in the tumorigenic behavior of HeLa cells has also been suggested (23). We showed that IL-9Rα protein expression increased in TL and that it existed as heterogeneous molecules due to a difference in *N*-glycosylation. It was previously reported that IL-9 does not induce the proliferation of freshly isolated normal human T-cells or mouse thymocytes at prelymphoma stages despite a significant expression of IL-9R mRNA (14, 24). The difference in glycosylation may explain for this discrepancy. The role of glycosylation of IL-9Rα in the binding ability of IL-9 and subsequent activation of downstream signal cascades remains to be elucidated.

Activation of Stat3 and Stat5 is a general mechanism for the development and growth of T-cell malignancies. Proliferation of adult T-cell leukemia caused by HTLV-1 is reported to be associated with the activation of Stat3 and Stat5 (25). Weber-Nordt *et al*. reported constitutive Stat5 activation in 63% patients in human T-ALL (26). Frequent activation of Stat3 and Stat5 in primary mouse TL was shown in the present study. Activation of Stat1, however, has rarely been reported. Its activation is associated with growth arrest and acts as proapoptotic factor, suggesting its inactivation promotes tumor cell survival (27). Enforced expression of Stat1 mediates the induction of differentiation genes such as *granzyme A, Ly6A/E* and *L-selectin* in T lymphomas (28). Therefore, the cells take advantage of activation of Stat3 and Stat5 and inactivation of Stat1 for proliferation and antiapoptosis.

Genetic factors influence the predisposition to leukemia. An increased risk of a subset of myeloma is associated with race (7). Polymorphisms of *TNF* and *IL-10* could affect susceptibility to non-Hodgkin lymphoma (8). Although we failed to detect SNP in the *IL-9Rα* gene, we found strain differences in its expression in TL. Some reports have described the strain dependency of cytokine production such as IL-9 in splenocytes after ConA stimulation and TGFβ1 in

lung after irradiation (29, 30). The present study also demonstrated clear strain differences in the activation of Stat 3 and Stat5. TL of susceptible B6 mice exhibited more frequent activation of Stat 3 and Stat5 than that of resistant C3H mice did. It was reported that thymic stromal cells of C3H mice lose the capability to sustain thymocyte differentiation after irradiation, while those in TL-prone AKR mice are supportive (31). Since irradiation up-regulates several cytokines in regenerating thymus (30), it would be interesting to determine the expression of these cytokines upstream of Jak/Stat signals after irradiation in thymus of B6 and C3H mice.

In conclusion, the present results demonstrate that aberrant expression, modification and activation of IL9Rα and Stat3/5 contribute to *in vivo* growth of TL in a strain dependent manner, linking genetic susceptibility to TL induction.

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