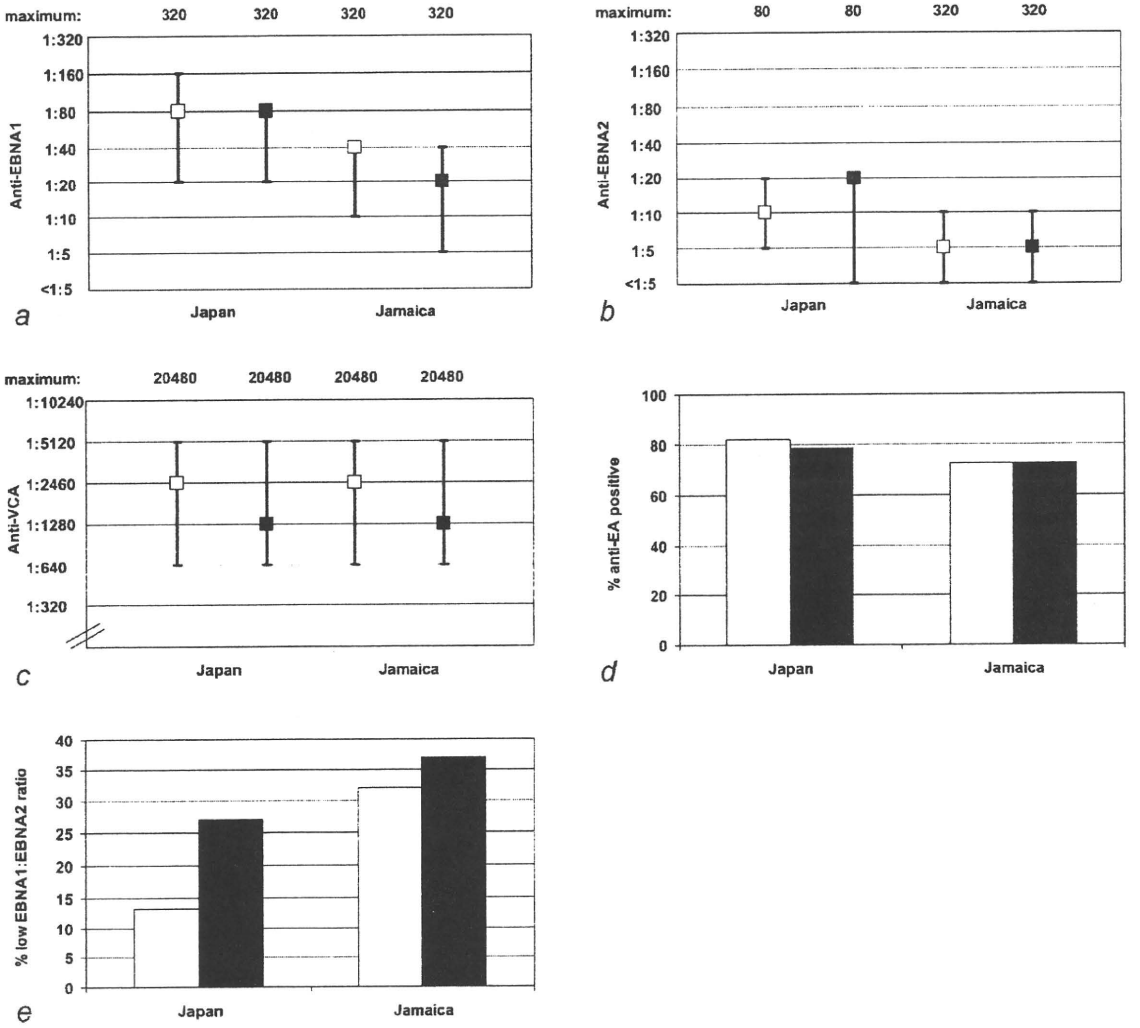

FIGURE 1 – Median titers, interquartile ranges and maximum observed titers of (*a*) anti-EBNA1, (*b*) anti-EBNA2 and (*c*) anti-VCA and prevalence of (*d*) anti-EA seropositivity and (*e*) low EBNA1:EBNA2 ratio, in HTLV-I-uninfected (□) and HTLV-I-infected (■) participants from Japan and Jamaica.

with anti-Tax positivity. We constructed a separate model for each immune marker, stratified by population. We also computed population-specific Spearman partial correlation coefficients (r) to estimate pair-wise correlations between each immune marker and the reciprocal of anti-HTLV-I titer and provirus load among HTLV-I carriers.

We used SAS® statistical software (SAS Institute, Cary, NC) for the statistical analyses. All statistical tests were 2-tailed. One EBV-negative individual (Japanese) and 12 persons with nonspecific anti-EBNA1 reactivity (8 Japanese, 4 Jamaican) were omitted from EBV antibody analyses.[22,23]

Results

Age, sex and blood collection year (*i.e.*, the matching factors) were distributed similarly by study population. The analysis included 45 (22%) persons aged 28–49, 81 (40%) aged 50–59, 53 (26%) aged 60–69 and 25 (12%) aged ≥70 years. Most matched sets (42/51; 82%) were female. We obtained 146 (72%) of the 204 plasma specimens from 1990 to 1993, and the remainder from 1987 to 1989.

Immune marker levels in persons uninfected with HTLV-I

Median EBV antibody titers and the prevalence of anti-EA seropositivity did not differ notably by population in HTLV-I noncarriers (Figs. 1*a*–1*d*). In contrast, a larger proportion of Jamaican than Japanese noncarriers had a low EBNA1:EBNA2 ratio (Fig. 1*e*). After adjustment for the matching factors, population differences in mean anti-EBNA1 titer and prevalence of a low EBNA1:EBNA2 ratio were statistically significant among noncarriers (Table I). HTLV-I noncarriers from Japan had lower median CRP levels (Fig. 2*a*) and higher median levels of sIL2R (Fig. 2*b*) and sCD30 (Fig. 2*c*) than those from Jamaica. There were no apparent differences in median neopterin (Fig. 2*d*) or total IgE levels (Fig. 2*e*) by population. After adjustment for the matching factors, the population differences in mean CRP, sIL2R and sCD30 levels were statistically significant (Table I).

Immune marker patterns associated with HTLV-I infection

Among Japanese participants, HTLV-I carriers had an increased prevalence of a low EBNA1:EBNA2 ratio compared to noncarriers (Fig. 1*e*). The remaining EBV antibody titers did not vary significantly by HTLV-I status among Japanese subjects (Figs.

TABLE I – PAIR-WISE COMPARISON OF GEOMETRIC MEAN LEVEL AND PREVALENCE OF IMMUNE MARKERS AMONG 102 PERSONS UNINFECTED WITH HTLV-I FROM JAPAN AND JAMAICA

Immune marker	Population		*p*-value
	Japan	Jamaica	
Anti-EBNA1, GMT[1]	71	27	0.005
Anti-EBNA2, GMT[1]	9	7	0.41
Anti-VCA, GMT[1]	2043	2120	0.87
Anti-EA seropositivity (%)[2]	82.0	72.6	0.18
Low EBNA1:EBNA2 ratio (%)[2,3]	13.3	31.9	0.02
CRP (μg/mL)[1,4]	0.43	1.1	0.0004
sIL2R (pg/mL)[1]	623	477	0.0008
sCD30 (U/mL)[1]	46	34	0.0001
Neopterin (nmol/L)[1]	5.6	5.6	0.94
Total IgE (ng/mL)[1,4]	154	146	0.82

EBNA1, Epstein-Barr virus nuclear antigen type 1: GMT, geometric mean titer; EBNA2, Epstein-Barr virus nuclear antigen type 2; VCA, viral capsid antigen; EA, early antigen; CRP, C-reactive protein; sIL2R, soluble IL-2 receptor-α; sCD30, soluble CD30; IgE, immunoglobulin E.

[1]Reciprocal of the geometric mean titer, or geometric mean level, from mixed-effects linear regression analyses adjusted for age (continuous, in years), sex and year of sample collection (continuous); *p*-values were obtained from the *t*-test and reflect covariate adjustment.–[2]*p*-values were obtained from the Mantel-Haenszel test, adjusted for age (< 60 *vs.* 60+ yr), sex and year of sample collection (1987–1989 *vs.* 1990–1993).–[3]A low EBNA1:EBNA2 ratio is a ratio of anti-EBNA1 to anti-EBNA2 titers ≤ 1.0.–[4]Values below assay detection level are assigned a value of one-half the assay detection limit and included in the estimation of the geometric mean.

1*a*–1*d*). With adjustment for the matching variables, Japanese HTLV-I carriers were 3 times as likely as Japanese noncarriers to have a low EBNA1:EBNA2 ratio ($p = 0.06$) (Table II). Japanese carriers also had higher median CRP levels (Fig. 2*a*) and a more than 2-fold increased odds of higher CRP levels ($p = 0.04$) (Table II) compared to Japanese noncarriers. Levels of sIL2R, sCD30, neopterin and total IgE did not vary markedly by HTLV-I status in Japanese subjects in crude comparisons (Figs. 2*b*–2*e*) or in multivariable regression analyses (Table II).

Among the Jamaican participants, neither the prevalence of a low EBNA1:EBNA2 ratio (Fig. 1*c*), nor the concentration of CRP (Fig. 2*a*) varied by HTLV-I serostatus. However, both were higher in Jamaican than in Japanese subjects regardless of HTLV-I status. Compared to Jamaican noncarriers of HTLV-I, Jamaican carriers had higher median sIL2R (Fig. 2*b*) and sCD30 levels (Fig. 2*c*) and 3- and 2-fold increases in the odds of higher sIL2R ($p = 0.01$) and sCD30 levels ($p = 0.09$), respectively (Table II). However, median sIL2R levels were similar in HTLV-I carriers from Japan and Jamaica (Fig. 2*b*). Levels of EBV antibodies, neopterin and total IgE did not differ greatly by HTLV-I status among Jamaican subjects in crude comparisons (Figs. 1*a*–1*d* and 2*d* and 2*e*, respectively) or in multivariable regression analyses (Table II).

Correlation of HTLV-I viral markers with plasma immune markers

Among Japanese carriers, anti-HTLV-I titer was significantly correlated only with anti-EA titer (Table III). In contrast, among Jamaican carriers, anti-HTLV-I titer was significantly correlated with anti-VCA, anti-EA, sIL2R, sCD30 and neopterin levels. Provirus load was not correlated with any immune marker in Japanese carriers but was correlated with anti-EBNA2, anti-EA, sIL2R, sCD30 and neopterin levels in Jamaican carriers (Table III). Anti-Tax seropositivity was more prevalent in Japanese carriers with lower anti-EBNA1 titers, higher anti-EBNA2 titers, a low EBNA1:EBNA2 ratio or detectable anti-EA, although the associations were not statistically significant (Table III). Among Jamaican carriers, anti-Tax was more commonly detected in those who had higher sIL2R, sCD30 or neopterin levels. CRP and total IgE levels were not associated with any viral marker in either population (data not shown).

Discussion

We undertook the present analysis to compare patterns of plasma markers of host immune status in Japanese and Jamaican carriers and non-carriers of HTLV-I using standardized laboratory assays. Some cytokines may be unstable, or present at levels below the lower detectable limits of commercial assays, in the plasma specimens available for our investigation.[30,31] Thus, we measured soluble protein correlates of cytokine levels that are well accepted as plasma immune markers, including several that we previously measured successfully.[32] We included EBV antibody profiles as markers of cellular immunity; studies have shown that the combination of lower anti-EBNA1 and higher anti-EBNA2 titers suggest poor control of EBV infection in the context of a weak CTL response.[22,29] We also measured concentrations of plasma markers of T-cell (sIL2R, sCD30), B-cell (total IgE), and inflammation or nonspecific (CRP, neopterin) immune activation.[7,25–28,33] Compared to cytokines, the surrogate markers are considered to be more stable, more frequently detectable and measurable with greater precision in peripheral blood specimens from asymptomatic individuals.[30,31]

To characterize underlying population differences in host immunity, we examined immune marker patterns in Japanese and Jamaican noncarriers of HTLV-I. In the Jamaican noncarriers we observed marker patterns that suggest less T-lymphocyte activation (*i.e.*, lower mean anti-EBNA1, sIL2R and sCD30 levels),[25,26] and diminished CTL control of EBV (*i.e.*, a greater prevalence of a low EBNA1:EBNA2 ratio),[22,29] compared to Japanese noncarriers. This lower level of T-cell activation may confer a lower risk of T-cell transformation and thus contribute to the relatively low risk of ATL in Jamaica. In contrast, higher mean CRP levels in Jamaican noncarriers compared to Japanese noncarriers are consistent with increased levels of inflammation or nonspecific immune activation in the Jamaicans.[27,28] The Jamaican subjects' mean CRP levels were lower than those typically associated with inflammation-related disease[34]; however, such subclinical increases in inflammation may contribute to the greater risk of HAM/TSP in Jamaica. The small sample sizes in the present analysis, as well as the use of surrogate rather than direct cytokine markers, warrant cautious interpretation. Nonetheless, the contrasting immune marker patterns in Japanese and Jamaican noncarriers of HTLV-I may reflect underlying population differences in host immune status. These differences may be due, in part, to genetics, nutritional status, social environment and/or co-morbidity such as parasitic burden.

We also observed marked population differences in HTLV-I-associated immune marker patterns. Diminished cellular immunity, as characterized by a low EBNA1:EBNA2 ratio,[22,29] was more frequent in Japanese carriers than noncarriers, consistent with previous reports.[13,16–19] In contrast, the prevalence of a low EBNA1:EBNA2 ratio did not differ by HTLV-I status in the Jamaican subjects, but was greater in both the carriers and noncarriers from Jamaica than in the Japanese carriers. Thus, the apparent population differences in HTLV-I-related EBV antibody patterns reflect the population differences we observed among the HTLV-I non-carriers.

Similarly, among Japanese subjects, HTLV-I carriers had higher CRP levels than noncarriers, but the median CRP levels were even higher in Jamaican subjects regardless of HTLV-I status. The HTLV-I-related increase in CRP levels in the Japanese subjects may reflect increased IL-6 expression,[35] possibly induced by Tax,[36] whereas the higher CRP levels in Jamaican noncarriers may have obscured any Tax-induced effects on CRP or IL-6 in the Jamaican carriers.

HTLV-I infection of T-cells results in upregulation of activation markers, including IL2R and CD30,[25,37] on infected cells. In addi-

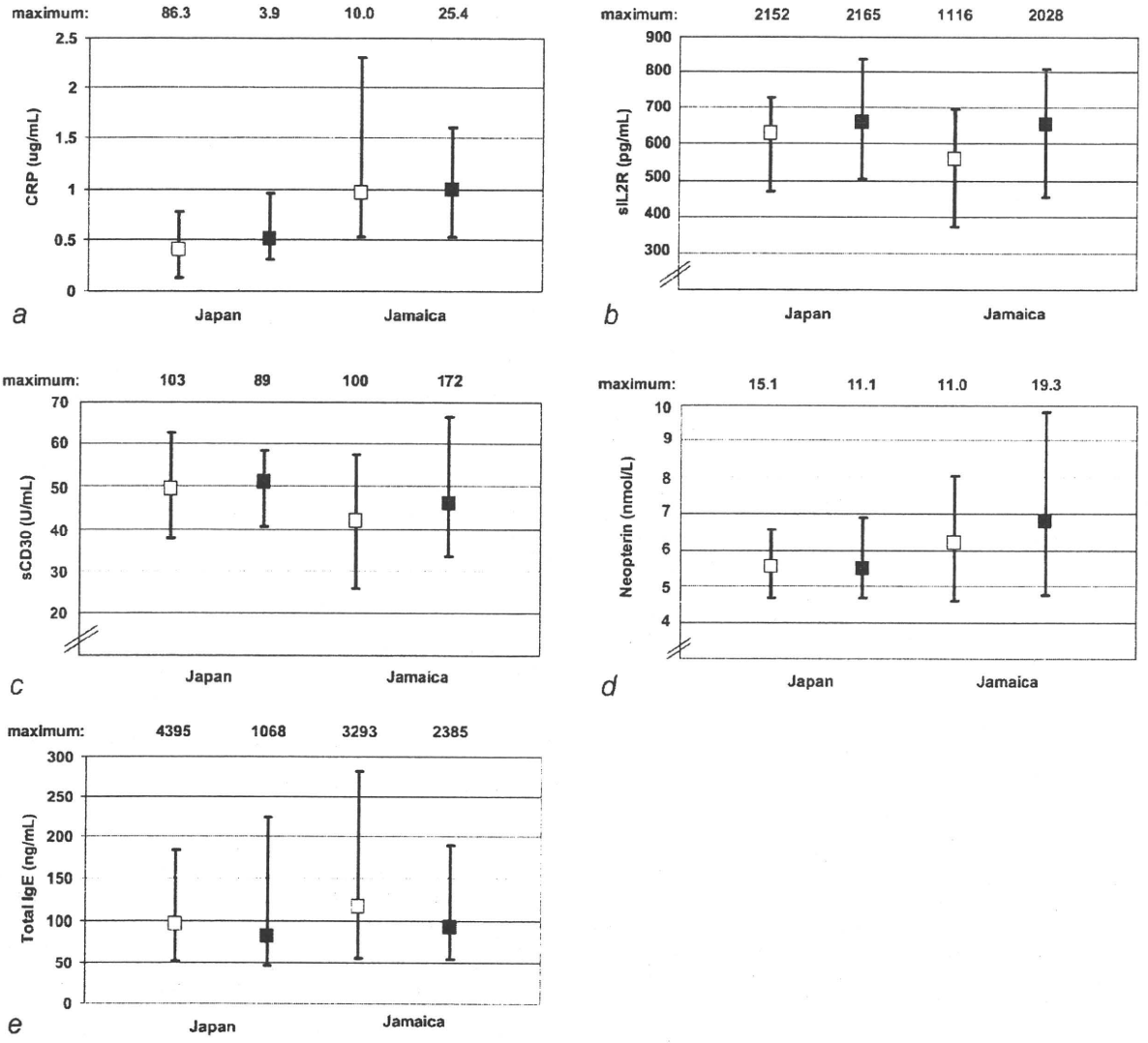

FIGURE 2 – Median levels, interquartile ranges and maximum observed plasma levels of (*a*) C-reactive protein, (*b*) soluble IL-2 receptor, (*c*) soluble CD30, (*d*) neopterin and (*e*) total IgE, in HTLV-I-uninfected (□) and HTLV-I-infected (■) participants from Japan and Jamaica.

tion, elevated plasma sIL2R[38,39] and sCD30 levels[40,41] have been reported in ATL patients and likely reflect tumor burden related to IL2R and CD30 expression by ATL cells.[25,26] HAM/TSP patients and asymptomatic HTLV-I carriers were also reported to have significantly higher sIL2R levels than noncarriers in a predominantly Caribbean population.[42] We previously reported similar sCD30 levels in asymptomatic Japanese carriers and noncarriers,[16] but the levels have not been well studied in Jamaican carriers. In the present analysis, we did not observe differences in sIL2R or sCD30 levels by HTLV-I status in Japanese subjects, whereas levels of both markers were higher in carriers than noncarriers from Jamaica, especially sIL2R. Those apparent HTLV-I-associated differences in sIL2R levels in the Jamaicans were due to lower levels in the Jamaican noncarriers rather than to unusually high levels in the Jamaican carriers. Therefore, the HTLV-I-related increases in these markers among the Jamaicans appear to reflect the lower background population levels of T-cell activation that we observed in the Jamaican noncarriers.

One Jamaican study has previously examined circulating neopterin levels in asymptomatic adults by HTLV-I serostatus[43] and found that the levels did not vary by HTLV-I status, consistent with the present findings. We also did not observe significant differences in total IgE levels by HTLV-I serostatus in either population, in contrast to prior reports of lower total IgE levels in HTLV-I carriers than noncarriers.[16,44,45]

In analyses restricted to carriers of HTLV-I, the immune markers generally showed more correlation with viral markers in Jamaican than in Japanese carriers. In the Japanese carriers, EBV antibody patterns consistent with diminished cellular immunity had suggestive associations with anti-Tax seropositivity. We conducted a cross-sectional analysis and therefore cannot determine the temporal relation of the viral and immune marker patterns. However, the observed correlations in Japanese carriers may reflect an increase in viral protein expression and division of HTLV-I-infected T-lymphocytes in persons with diminished type 1 immunity.[8] In Jamaican carriers, plasma markers of T-cell (sIL2R, sCD30)[25,26] and nonspecific (neopterin)[28] immune activation were correlated with HTLV-I provirus load and anti-HTLV-I titer and had suggestive associations with anti-Tax seropositivity. Those correlations are consistent with the prediction that active

TABLE II – ASSOCIATION OF HTLV-I SEROPOSITIVITY WITH IMMUNE BIOMARKER LEVELS BY STUDY POPULATION, WITH ADJUSTMENT FOR AGE, SEX AND YEAR OF SAMPLE COLLECTION

Immune marker category[1]	Japan				Jamaica			
	HTLV-I				HTLV-I			
	– (N = 51) %	+ (N = 51) %	OR[2]	(95% CI)	– (N = 51) %	+ (N = 51) %	OR[2]	(95% CI)
EBNA1:EBNA2 ratio								
>1.0	86.7[3]	72.9[4]	1.0	(Reference)	68.1[5]	63.0[5]	1.0	(Reference)
≤1.0	13.3[3]	27.1[4]	3.0	(0.9–9.9)	31.9[5]	37.0[5]	1.3	(0.5–3.0)
CRP								
≤Median	51.0	31.4	1.0	(Reference)	51.0	49.0	1.0	(Reference)
>Median	49.0	68.6	2.4	(1.1–5.5)	49.0	51.0	1.1	(0.5–2.4)
sIL2R								
≤Median	49.0	39.2	1.0	(Reference)	51.0	29.4	1.0	(Reference)
>Median	51.0	60.8	1.6	(0.7–3.8)	49.0	70.6	3.3	(1.3–8.6)
sCD30								
≤Median	51.0	45.1	1.0	(Reference)	51.0	35.3	1.0	(Reference)
>Median	49.0	54.9	1.3	(0.5–3.1)	49.0	64.7	2.1	(0.9–5.1)
Total IgE								
≤Median	51.0	52.9	1.0	(Reference)	51.0	62.7	1.0	(Reference)
>Median	49.0	47.1	0.8	(0.4–1.8)	49.0	37.3	0.6	(0.3–1.3)
Neopterin								
≤Median	51.0	52.9	1.0	(Reference)	51.0	45.1	1.0	(Reference)
>Median	49.0	47.1	1.0	(0.4–2.4)	49.0	54.9	1.3	(0.6–3.0)

HTLV-I, human T-lymphotropic virus type I; OR, odds ratio; CI, confidence interval; EBNA1, Epstein-Barr virus (EBV) nuclear antigen type 1; EBNA2, EBV nuclear antigen type 2; CRP, C-reactive protein; sIL2R, soluble IL-2 receptor-α (or CD25); sCD30, soluble CD30; IgE, immunoglobulin E.

[1]Above-median categories are defined according to the population-specific median levels observed among noncarriers of HTLV-I.–[2]From unconditional logistic regression models adjusted for age, sex and year of sample collection.–[3]45 Japanese HTLV-I-seronegative persons were analyzed; we omitted 1 EBV-seronegative subject and 5 subjects with nonspecific anti-EBNA reactivity.–[4]48 Japanese HTLV-I-seropositive individuals were analyzed; we omitted 3 subjects with nonspecific anti-EBNA reactivity.–[5]49 HTLV-I-seronegative and 49 HTLV-I-seropositive Jamaican persons were analyzed; we omitted 2 subjects with nonspecific anti-EBNA reactivity in each group.

TABLE III – ASSOCIATION OF HTLV-I VIRAL MARKERS WITH IMMUNE MARKER LEVELS AMONG JAPANESE AND JAMAICAN CARRIERS

HTLV-I viral marker/Immune marker	Geographic population			
	Japan		Jamaica	
	r[1]	(p-Value)	r[1]	(p-Value)
Anti-HTLV-I titer				
anti-EBNA2	0.07	(0.66)	0.28	(0.06)
anti-VCA	0.26	(0.08)	0.32	(0.03)
anti-EA	0.36	(0.01)	0.47	(0.001)
sIL2R	0.19	(0.22)	0.57	(< 0.0001)
sCD30	−0.07	(0.63)	0.47	(0.0009)
Neopterin	0.19	(0.21)	0.58	(< 0.0001)
HTLV-I provirus load				
anti-EBNA2	0.18	(0.24)	0.44	(0.002)
anti-VCA	0.07	(0.67)	0.07	(0.64)
anti-EA	0.22	(0.15)	0.31	(0.03)
sIL2R	0.22	(0.14)	0.58	(< 0.0001)
sCD30	0.08	(0.61)	0.49	(0.0006)
Neopterin	0.08	(0.61)	0.57	(< 0.0001)
Immune marker	**OR[2]**	**(95% CI)**	**OR[2]**	**(95% CI)**
Anti-Tax seropositivity				
anti-EBNA1 median	3.2	(0.8–12.4)	1.4	(0.4–4.6)
anti-EBNA2 > median	2.0	(0.6–6.7)	1.5	(0.4–5.2)
EBNA1:EBNA2 ratio ≤ 1.0	3.6	(0.8–15.1)	0.9	(0.3–3.3)
anti-EA-positive	2.3	(0.5–11.0)	0.7	(0.2–2.7)
sIL2R > median	1.3	(0.4–4.5)	2.7	(0.7–11.2)
sCD30 > median	1.7	(0.4–6.4)	2.8	(0.7–11.3)
Neopterin > median	1.8	(0.5–6.9)	3.4	(0.9–12.6)

HTLV-I, human T-lymphotropic virus type I; sIL2R, soluble IL-2 receptor; sCD30, soluble CD30; OR, odds ratio; CI, confidence interval; EBNA1, Epstein-Barr virus nuclear antigen type 1; EBNA2, Epstein-Barr virus nuclear antigen type 2; VCA, viral capsid antigen; EA, early antigen.

[1]Spearman partial correlations of $\log_{10}$-transformed HTLV-I viral markers with $\log_{10}$-transformed serologic immune marker levels, with mutual adjustment for the immune markers and for age, sex and year of sample collection.–[2]From logistic regression models adjusted for age, sex and year of sample collection.

host immune responses to HTLV-I in the context of persistent HTLV-I replication characterize the Jamaican carriers in this study population.[15]

A synthesis of the immune and viral marker data suggests several hypotheses regarding population differences in host immune status and control of HTLV-I. Among Japanese participants, noncarriers of HTLV-I appeared to have increased T-cell activation, but HTLV-I was not associated with a further increase in T-cell activation marker levels. Concurrently, the HTLV-I viral marker patterns in Japanese carriers did not indicate persistent HTLV-I replication or CTL responses to HTLV-I.[15] Thus, the Japanese carriers may not have effective virus-specific immune responses despite the evidence of T-cell activation. This suggestion is consistent with the present and previous observations of diminished type 1 immunity in Japanese carriers.[13,16–19] Furthermore, in the absence of evidence for persistent HTLV-I replication, the high provirus loads we observed in Japanese carriers are most likely a result of mitotic division of HTLV-I-infected T-lymphocytes.[15] The combination of ongoing but ineffective T-cell activation and mitotic proliferation of HTLV-I-infected T-lymphocytes could contribute to the increased risk of ATL that is characteristic of Japanese carriers. In contrast, in Jamaican carriers and noncarriers of HTLV-I we observed plasma marker levels that imply chronic inflammation. In addition, Jamaican carriers had plasma T-cell activation marker levels similar to those of Japanese subjects. HTLV-I viral marker levels in Jamaican carriers further indicated persistent HTLV-I replication and virus-specific CTL responses. The combination of persistent HTLV-I replication, CTL responses and inflammation is characteristic of a population at greater risk for HAM/TSP.[11,12,15] The lysis of HTLV-I-infected T-cells by a persistent CTL response may also contribute to the comparatively low risk of ATL observed in Jamaica.

The matched study design, direct comparisons of immune marker profiles across populations and standardized laboratory assays represent unique strengths of the present analysis. Nonetheless, limitations of this study should be noted. The immune marker findings may not be directly applicable to ATL or HAM/TSP patients, as all of the HTLV-I-infected participants were asymptomatic. Also, we could not adjust for factors that may modulate the host immune response to HTLV-I, including age at infection, route and dose of infection, nutritional status, social environment, co-morbidity and host genetics. The statistical power to detect significant small associations was limited due to the small sample size. However, we included as many participants as could be appropriately matched across populations; we are not aware of ongoing studies that could yield a larger sample size for a similar analysis.

In conclusion, we observed several intriguing new findings related to population differences in host immune status in carriers and noncarriers of HTLV-I. The findings corroborate the view that host immune dysregulation, perhaps even at a modest, subclinical level, contributes to the well established differences in natural history of HTLV-I infection in Japanese and Jamaican carriers. It remains unclear why HTLV-I infection results in contrasting types of immune dysregulation in the two populations. Possible mechanism(s) may be elucidated by longitudinal studies that include ATL and HAM/TSP patients and information on potential cofactors that was not available for the present analysis. The further development of informative, easily measured markers of immune status will also aid future studies. Those studies will provide important information to characterize the asymptomatic carriers of HTLV-I who are at an increased risk of ATL or HAM/TSP.

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References

1. Lairmore MD, Franchini G. Human T-cell leukemia virus types I and II. In: Knipe DM, Howley PM, eds. Fields virology, 5th edn. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2007. 2072–105.
2. International Agency for Research on Cancer (IARC). Human T-cell lymphotropic viruses. In: IARC monographs on the evaluation of carcinogenic risks to humans, Vol 67. Lyon: IARC, 1996. 261–390.
3. Mueller N, Birmann B, Parsonnet J, Schiffman M, Stuver S. Infectious agents. In: Schottenfeld D, Fraumeni JJ, eds. Cancer epidemiology and prevention, 3rd edn. New York: Oxford University Press, 2006. 507–48.
4. Mortreux F, Gabet AS, Wattel E. Molecular and cellular aspects of HTLV-1 associated leukemogenesis in vivo. Leukemia 2003;17:26–38.
5. Li HC, Walters M, Carrington M, Headley C, Amarasinghe A, Goedert J, Cranston B, Hanchard B, Hisada M. Serologic and molecular characteristics of atypical HTLV Western blot banding patterns in Jamaican blood donors [Abstract P56]. AIDS Res Hum Retroviruses 2005;21:479.
6. Barmak K, Harhaj E, Grant C, Alefantis T, Wigdahl B. Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration. Virology 2003;308:1–12.
7. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev 1996;9:532–62.
8. Akimoto M, Kozako T, Sawada T, Matsushita K, Ozaki A, Hamada H, Kawada H, Yoshimitsu M, Tokunaga M, Haraguchi K, Uozumi K, Arima N, et al. Anti-HTLV-I Tax antibody and Tax-specific cytotoxic T-lymphocyte are associated with a reduction in HTLV-I proviral load in asymptomatic carriers. J Med Virol 2007;79:977–86.
9. Mueller N, Okayama A, Stuver S, Tachibana N. Findings from the Miyazaki Cohort Study. J Acquir Immune Defic Syndr Hum Retrovirol 1996;13(Suppl 1):S2–S7.
10. Yokota T, Cho MJ, Tachibana N, McLane MF, Takatsuki K, Lee TH, Mueller N, Essex M. The prevalence of antibody to p42 of HTLV-I among ATLL patients in comparison with healthy carriers in Japan. Int J Cancer 1989;43:970–4.
11. Kubota R, Nagai M, Kawanishi T, Osame M, Jacobson S. Increased HTLV type 1 tax specific CD8 + cells in HTLV type 1-asociated myelopathy/tropical spastic paraparesis: correlation with HTLV type 1 proviral load. AIDS Res Hum Retroviruses 2000;16:1705–9.
12. Nagai M, Kubota R, Greten TF, Schneck JP, Leist TP, Jacobson S. Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11–19-specific memory and effector CD8+ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with HTLV-I provirus load. J Infect Dis 2001;183:197–205.
13. Imai J, Hinuma Y. Epstein-Barr virus-specific antibodies in patients with adult T-cell leukemia (ATL) and healthy ATL virus-carriers. Int J Cancer 1983;31:197–200.
14. Tendler CL, Greenberg SJ, Burton JD, Danielpour D, Kim SJ, Blattner WA, Manns A, Waldmann TA. Cytokine induction in HTLV-I associated myelopathy and adult T-cell leukemia: alternate molecular mechanisms underlying retroviral pathogenesis. J Cell Biochem 1991;46:302–11.
15. Hisada M, Stuver SO, Okayama A, Li HC, Sawada T, Hanchard B, Mueller NE. Persistent paradox of natural history of human T lymphotropic virus type I: parallel analyses of Japanese and Jamaican carriers. J Infect Dis 2004;190:1605–9.
16. Birmann BM, Mueller NE, Okayama A, Hsieh CC, Tsubouchi H, Harn D, Stuver SO. Patterns of serum type 1 and type 2 immune markers in healthy carriers of HTLV-I. J Med Virol 2006;78:847–52.
17. Tachibana N, Okayama A, Ishizaki J, Yokota T, Shishime E, Murai K, Shioiri S, Tsuda K, Essex M, Mueller N. Suppression of tuberculin skin reaction in healthy HTLV-I carriers from Japan. Int J Cancer 1988;42:829–31.
18. Murai K, Tachibana N, Shioiri S, Shishime E, Okayama A, Ishizaki J, Tsuda K, Mueller N. Suppression of delayed-type hypersensitivity to PPD and PHA in elderly HTLV-I carriers. J Acquir Immune Defic Syndr 1990;3:1006–9.

19. Kwon KW. Epstein-Barr virus-specific immunity in asymptomatic carriers of human T-cell leukemia virus type I. Hokkaido Igaku Zasshi 1995;70:315–28.
20. Murphy EL, Figueroa JP, Gibbs WN, Holding-Cobham M, Cranston B, Malley K, Bodner AK, Alexander SS, Blattner WA. Human T-lymphotropic virus type I (HTLV-I) seroprevalence in Jamaica. I. Demographic determinants. Am J Epidemiol 1991;133:1114–24.
21. Sawada T, Tohmatsu J, Obara T, Koide A, Kamihira S, Ichimaru M, Kashiwaqi S, Kajiyama W, Matsumura N, Kinoshita K, Yano M, Yamaguchi K, et al. High risk of mother-to-child transmission of HTLV-I in p40tax antibody-positive mothers. Jpn J Cancer Res 1989;80:506–8.
22. Lennette ET, Rymo L, Yadav M, Masucci G, Merk K, Timar L, Klein G. Disease-related differences in antibody patterns against EBV-encoded nuclear antigens EBNA 1, EBNA 2 and EBNA 6. Eur J Cancer 1993;29A:1584–9.
23. Nagy N, Maeda A, Bandobashi K, Kis LL, Nishikawa J, Trivedi P, Faggione A, Klein G, Klein E. SH2D1A expression in Burkitt lymphoma cells is restricted to EBV positive group I lines and is downregulated in parallel with immunoblastic transformation. Int J Cancer 2002;100:433–40.
24. Linde A, Andersson J, Lundgren G, Wahren B. Subclass reactivity to Epstein-Barr virus capsid antigen in primary and reactivated EBV infections. J Med Virol 1987;21:109–21.
25. Rubin LA, Nelson DL. The soluble interleukin-2 receptor: biology, function, and clinical application. Ann Intern Med 1990;113:619–27.
26. Younes A, Aggarwall BB. Clinical implications of the tumor necrosis factor family in benign and malignant hematologic disorders. Cancer 2003;98:458–67.
27. Fernandez-Real JM, Vayreda M, Richart C, Gutierrez C, Broch M, Vendrell J, Ricart W. Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. J Clin Endocrinol Metab 2001;86:1154–9.
28. Hoffmann G, Wirleitner B, Fuchs D. Potential role of immune system activation-associated production of neopterin derivatives in humans. Inflamm Res 2003;52:313–21.
29. Henle W, Henle G, Andersson J, Ernberg I, Klein G, Horwitz CA, Marklund G, Rymo L, Wellinder C, Straus SE. Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. Proc Natl Acad Sci USA 1987;84:570–4.
30. Aziz N, Nishanian P, Fahey JL. Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. Clin Diagn Lab Immunol 1998;5:755–61.
31. Whiteside TL. Cytokine measurements and interpretation of cytokine assays in human disease. J Clin Immunol 1994;14:327–39.
32. Birmann BM, Mueller N, Okayama A, Hsieh CC, Tachibana N, Tsubouchi H, Lennette ET, Harn D, Stuver S. Serologic assessment of type 1 and type 2 immunity in healthy Japanese adults. Cancer Epidemiol Biomarkers Prev 2004;13:1385–91.
33. Kishimoto T. The biology of interleukin-6. Blood 1989;74:1–10.
34. Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14.719 initially healthy American women. Circulation 2003;107:391–7.
35. Yamamura M, Yamada Y, Momita S, Kamihira S, Tomonaga M. Circulating interleukin-6 levels are elevated in adult T-cell leukaemia/lymphoma patients and correlate with adverse clinical features and survival. Br J Haematol 1998;100:129–34.
36. Yamashita I, Katamine S, Moriuchi R, Nakamura Y, Miyamoto T, Eguchi K, Nagataki S. Transactivation of the human interleukin-6 gene by human T-lymphotropic virus type I Tax protein. Blood 1994;84:1573–8.
37. Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, Gatter K, Falini B, Delsol G, Lemke H, Schwarting R, Lennert K. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 1985;66:848–58.
38. Araki K, Harada K, Nakamoto K, Shiroma M, Miyakuni T. Clinical significance of serum soluble IL-2R levels in patients with adult T cell leukaemia (ATL) and HTLV-I carriers. Clin Exp Immunol 2000;119:259–63.
39. Arisawa K, Katamine S, Kamihira S, Kurokawa K, Sawada T, Soda M, Doi H, Saito H, Shirahama S. A nested case-control study of risk factors for adult T-cell leukemia/lymphoma among human T-cell lymphotropic virus type-I carriers in Japan. Cancer Causes Control 2002;13:657–63.
40. Pfreundschuh M, Pohl C, Berenbeck C, Schroeder J, Jung W, Schmits R, Tschiersch A, Diehl V, Gause A. Detection of a soluble form of the CD30 antigen in sera of patients with lymphoma, adult T-cell leukemia and infectious mononucleosis. Int J Cancer 1990;45:869–74.
41. Dallenbach F, Josimovic-Alasevic O, Dürkop H, Schwarting R, Diamantstein T, Matsuoka M, Takatsuki K, Stein H. Soluble CD30 antigen in the sera of patients with adult T-cell lymphoma/leukemia (ATL): a marker for disease activity. In: Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEG, eds. Leukocyte typing IV. Oxford, UK: Oxford University, 1989. 426–8.
42. Tendler CL, Greenberg SJ, Blattner WA, Manns A, Murphy E, Fleisher T, Hanchard B, Morgan O, Burton JD, Nelson DL, Waldmann TA. Transactivation of interleukin 2 and its receptor induces immune activation in human T-cell lymphotropic virus type I-associated myelopathy: pathogenic implications and a rationale for immunotherapy. Proc Natl Acad Sci USA 1990;87:5218–22.
43. Giusti RM, Maloney EM, Hanchard B, Morgan OS, Steinberg SM, Wachter H, Williams E, Cranston B, Fuchs D, Manns A. Differential patterns of serum biomarkers of immune activation in human T-cell lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis, and adult T-cell leukemia/lymphoma. Cancer Epidemiol Biomarkers Prev 1996;5:699–704.
44. Hayashi J, Kishihara Y, Yoshimura E, Furusyo N, Yamaji K, Kawakami Y, Murakami H, Kashiwaqi S. Correlation between human T cell lymphotropic virus type-I and Strongyloides stercoralis infections and serum immunoglobulin E responses in residents of Okinawa, Japan. Am J Trop Med Hyg 1997;56:71–5.
45. Matsumoto T, Miike T, Mizoguchi K, Yamaguchi K, Takatsuki K, Hosoda M, Kawabe T, Yodoi J. Decreased serum levels of IgE and IgE-binding factors in individuals infected with HTLV-I. Clin Exp Immunol 1990;81:207–11.

Identification of cancer stem cells in a Tax-transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma

*Jumpei Yamazaki,[1,2] *Takuo Mizukami,[1] Kazuya Takizawa,[1] Madoka Kuramitsu,[1] Haruka Momose,[1] Atsuko Masumi,[1] Yasushi Ami,[3] Hideki Hasegawa,[4] William W. Hall,[5] Hajime Tsujimoto,[2] Isao Hamaguchi,[1] and Kazunari Yamaguchi[1]

[1]Department of Safety Research on Blood and Biologics, National Institute of Infectious Diseases, Tokyo, Japan; [2]Veterinary Internal Medicine, University of Tokyo, Tokyo, Japan; Departments of [3]Animal Science and [4]Pathology, National Institute of Infectious Diseases, Tokyo, Japan; and [5]Centre for Research in Infectious Diseases, School of Medicine & Medical Science, University College Dublin, Dublin, Republic of Ireland

Adult T-cell leukemia/lymphoma (ATL) is a malignant lymphoproliferative disorder caused by HTLV-I infection. In ATL, chemotherapeutic responses are generally poor, which has suggested the existence of chemotherapy-resistant cancer stem cells (CSCs). To identify CSC candidates in ATL, we have focused on a Tax transgenic mouse (Tax-Tg) model, which reproduces ATL-like disease both in Tax-Tg animals and also after transfer of Tax-Tg splenic lymphomatous cells (SLCs) to nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Using a limiting dilution transplantation, it was estimated that one CSC existed per 10^4 SLCs (0.01%). In agreement with this, we have successfully identified candidate CSCs in a side population (0.06%), which overlapped with a minor population of $CD38^-/CD71^-/CD117^+$ cells (0.03%). Whereas lymphoma did not develop after transplantation of 10^2 SLCs, 10^2 CSCs could consistently regenerate the original lymphoma. In addition, lymphoma and CSCs could also be demonstrated in the bone marrow and $CD117^+$ CSCs were observed in both osteoblastic and vascular niches. In the CSCs, *Tax*, *Notch1*, and *Bmi1* expression was down-regulated, suggesting that the CSCs were derived from Pro-T cells or early hematopoietic progenitor cells. Taken together, our data demonstrate that CSCs certainly exist and have the potential to regenerate lymphoma in our mouse model. (Blood. 2009;114:2709-2720)

Introduction

Adult T-cell leukemia-lymphoma (ATL) is an aggressive and clonal lymphoproliferative disorder of mature T cells caused by infection with human T-lymphotropic virus type I (HTLV-1).[1] An estimated 10 million to 20 million people are infected with HTLV-1 globally, and infection is endemic in southwestern Japan, Africa, the Caribbean basin, and South America.[2] Although the majority of infected persons remain clinically asymptomatic, approximately 6.6% of males and 2.1% of females will develop ATL. In Japan, 1.2 million persons are infected with HTLV-1, and 800 to 1000 new ATL cases develop each year. ATL has been divided into 4 subtypes: chronic, smoldering, acute, and lymphoma-type.[3] Acute and lymphoma-type have an aggressive clinical course with lymphadenopathy, hepatosplenomegaly, visceral invasion by malignant cells, and the appearance of leukemic cells with multilobulated nuclei termed "flower cells" in peripheral blood. The most common cell phenotype in ATL is $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$, and $CD25^+$. Other phenotypes, which include $CD4^-/CD8^-$ double-negative (DN), $CD8^+$, and $CD4^+/CD8^+$ double-positive, occur more rarely.[4] FOXP3 (forkhead box P3) expression, which is predominantly expressed in $CD4^+/CD25^+$ regulatory T cells (Tregs), has also been detected in some ATL cases, suggesting that disease may originate in Treg cells.[5] In addition, HTLV-I can also infect human hematopoietic progenitor cells and immature thymocytes, which would also account for the range of phenotypes observed.[6]

Studies have shown that initiation of oncogenesis is triggered by the viral Tax protein. Tax interacts with the nuclear factor-κB (NF-κB)–Rel signaling complex and activates NF-κB, which results in the up-regulation of various cytokines and their receptor genes, and alterations in cell signaling and cell-cycle regulation.[7] Recent studies have clearly demonstrated the oncogenic properties of Tax in vivo. Specifically, transgenic animals with Tax expression restricted to developing thymocytes developed an ATL-like phenotype and leukemogenesis developed in both immature[8] and mature T cells.[9] The treatment of ATL is unsatisfactory. Various combination chemotherapy regimens have produced poor outcomes; however, intensive induction therapy (interferon-α and zidovudine) has produced significant complete remission rates in certain cases.[10,11] Unfortunately, most patients relapse, and this has been considered to be the result of the existence of cancer stem cells (CSCs) similar to what has been described both in other types of leukemia and solid tumors. Specifically, CSCs have been identified in malignancies of both hematopoietic origin[12,13] and in breast, brain, prostate, colon, and pancreatic carcinomas.[14] CSCs have the potential to self-renew, develop inherent drug-resistance, and can regenerate the original tumors when transplanted into severe combined immunocompromised nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.[15] As a consequence, CSCs are considered important targets for anticancer therapy.[16]

To identify and confirm the existence of CSCs in ATL, we have focused our initial studies on an HTLV-Tax transgenic mouse (Tax-Tg) model,[8] where transgene expression was controlled by the LCK promoter, which limits transgene expression to developing thymocytes. The clinical, pathologic, and immunologic features observed in this Tax-Tg model are similar to those observed in the

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*J.Y. and T.M. contributed equally to this study.

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aggressive forms of human ATL. Lymphoma and leukemia develop at 10 to 23 months, which is equivalent to human disease (20-60 years) and is characterized by the widespread distribution of lymphomatous cells in various organs and the presence of flower cells in peripheral blood. Lymphomatous cells displayed a $CD4^-CD8^-$ DN phenotype. In addition, lymphomas could be regenerated in NOD/SCID mice after transplantation of Tax-Tg splenic lymphomatous cells. In this study, we have successfully identified candidate CSCs using xenografting into NOD/SCID mouse and flow cytometric analysis. Specifically, we identified CSCs in a side population (SP; 0.06%), which overlapped with a minor population of $CD38^-/CD71^-/CD117^+$ cells (0.03%). Using the NOD/SCID transplantation assay, we found that 10^2 CSCs could regenerate the original lymphoma. This is first report describing a CSC candidate in an ATL model, and we think that similar studies will inform and may ultimately allow the identification of CSCs in human disease.

Methods

Animal models of ATL

Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Infectious Disease, Tokyo, Japan. For transplantation studies, we obtained NOD/SCID mice (NOD.CB17-Prkdc^{scid}/J) 6 to 12 weeks of age from The Jackson Laboratory, and these were housed under constant temperature and light.

Transplantation assays

ATL-like lymphoma and leukemia was first established in NOD/SCID mice by intraperitoneal injection of 10^5 frozen stock Tax transgenic (Tax-Tg) splenic lymphomatous cells (SLCs). After 40 days, ATL-like lymphoma developed in the NOD/SCID mouse spleen, and SLCs could regenerate the original ATL-like lymphoma when further injected. Using this NOD/SCID mouse transplantation system, SLCs could be serially passed as required. We used 4th-passage frozen stocked SLCs for the serial transplantation studies. We performed 3 consecutive serial transplantations by intraperitoneal injection of 10^5 SLCs (first transplantation [n = 12], second transplantation [n = 5], third transplantation [n = 7]). We also performed limiting dilution assays by intraperitoneal injection of 10^6 (n = 5), 10^5 (n = 4), 10^4 (n = 3), 10^3 (n = 5), and 10^2 (n = 7) of freshly isolated SLCs. To evaluate the lymphoma-forming ability of CSC candidates, we also transplanted 10^2 CSCs ($CD38^-/CD71^-/CD117^+$; n = 9), non-CSC fraction ($CD38^+/CD71^+/CD117^-$; n = 11), and SLCs (n = 11) into NOD/SCID mice.

Flow cytometry and SP analysis

To identify candidate CSCs in the SLCs, we performed SP cell analysis. SLCs were suspended in Hanks balanced salt solution medium (Invitrogen) containing 2% fetal bovine serum, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (Invitrogen), and incubated with Hoechst 33342 dye (2.5 μg/mL, Invitrogen, H-3570) with or without verapamil (Sigma-Aldrich) at 37°C for 60 minutes, according to the method outlined by Goodell et al.[17] After staining with or without Hoechst 33342, the cells were stained with phycoerythrin (PE) anti–mouse/rat Foxp3 (clone FJK-16s), PE anti–mouse CD3e (145-2C11), PE anti–mouse CD8 (53-6.7), PE anti–mouse CD127 (clone A7R34), PE anti–mouse CD38 (clone 90), fluorescein isothiocyanate (FITC) anti–mouse Sca-1 (clone D7), FITC anti–mouse CD2 (RM2-5), FITC anti–mouse CD4 (RM4-5), FITC anti–mouse CD123 (clone 5B11), FITC anti–mouse CD24 (clone 30-F1), FITC anti–mouse CD71 (clone R17217), and allophycocyanin (APC) anti–mouse CD25 (clone PC61.5), APC anti–mouse CD133 (clone 13A4), APC anti–mouse CD117(clone ACK2), APC anti–mouse CD25 (PC61.5), and purified anti–mouse CD44 (IM7) at 4°C for 30 minutes and then counterstained with 2 μg/mL propidium iodide (BD Biosciences). All antibodies were obtained from eBioscience. We performed flow-cytometric analysis and cell sorting using JSAN (Bay Bioscience) with 350-nm UV laser for SP analysis. Cytospin preparations of the sorted cells were prepared and subjected to Wright/Giemsa staining.

Histologic preparation and periodic acid-Schiff–hematoxylin staining

NOD/SCID mouse spleen, bone marrow (BM), liver, lung, lymph node, and epidermal tissues were harvested and fixed in Bouin solution (Sigma-Aldrich) or 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (pH 7.5) at 4°C for 24 hours. After fixation, samples were dehydrated in a graded ethanol series and cleared in xylene, and then embedded in paraffin; 4-μm semithin sections were prepared using a carbon steel blade (Feather Safety Razor Co) by microtome (Yamato Kouki). Tissue sections were mounted on super-frosted glass slides coated with methyl-amino-silane (Matsunami Glass). To identify periodic acid-Schiff (PAS)–positive ATL leukemia/lymphoma[18] in spleen, we performed PAS-hematoxylin staining as previously described.[19] Histologic images were aqquired using a NikonEclipse E1000 microscope equipped with 10×/0.30, 20×/0.50, 40×/0.75, and 100×/1.30 NA objective lenses. Images were captured with a Nikon DXM 1200F digital camera.

Immunohistochemistry

Anti–mouse CD3 antibody (ab5690; Abcam), anti–mouse CD44 (IM7; BioLegend), anti–mouse/human CD117 (C-19; Santa Cruz Biotechnology), and anti–mouse CD4 (RM4-5; eBioscience) were used as primary antibodies, and biotinylated goat anti–rat IgG-B (SC-2041, Santa Cruz Biotechnology) and biotinylated goat anti–rabbit IgG-B (SC-2040, Santa Cruz Biotechnology) were used as secondary antibodies. Staining was carried out as previously described.[20] Briefly, after blocking with 3% bovine serum albumin (BSA) in phosphate-buffered saline, sections (4-μm thick) were incubated with anti-CD3, -CD4, -CD44, and -CD117 antibody (each diluted 1:200) at 4°C overnight. Signals were detected using a Vectastain ABC Elite Kit (Vector Laboratories), and nuclei were stained with Gill III-hematoxylin.

Quantitative analysis of gene expression

Poly (A)+ RNAs were extracted from 5×10^3 candidate CSCs ($CD38^-/CD71^-/CD117^+$) and non-CSCs ($CD38^+/CD71^+/CD117^-$) using a MicroFast Track 2.0 Kit (Invitrogen), and cDNAs were prepared using SMART polymerase chain reaction (PCR) cDNA synthesis kits (Clontech) as previously described.[20] Real-time PCR reactions were performed using SYBR PreMix ExTaq (Takara Shuzo) and a Light Cycler (Roche Diagnostics). The primer pairs used in this study were *Notch1* (5′-CGTGGTCTTCAAGCGTGATG-3′ and 5′-AGCTCTTCCTCGTGGCCATA-3′), *CD44* (5′-AGCTGACGAGACCCGGAAT-3′ and 5′-CGTAGGCACTACACCCCAATC-3′), *Rex1* (5′-TGTGCTGCCTCCAAGTGTTG-3′ and 5′-ATCCGCAAACACCTGCTTTT-3′), and *N-cadherin* (5′-CACAGCCACAGCCGTCATC-3′ and 5′-GCAGTAAACTCTGGAGGATTGTCA-3′) and for *Tax*,[8] *CD117*, *Bmi1*, *SCL/tal-1*, and *β-actin* as previously described.[20] *β-Actin* was used as an internal control. Real-time PCR was carried out using 40 cycles at 94.0°C for 1 minute, 60°C for 25 seconds (2-step). Amplification of predicted fragments was confirmed by melt-curve analysis and gel electrophoresis. To determine the relative amounts of product, we used the comparative threshold cycle method, according to the manufacturer's instructions (Roche Diagnostics). Expression levels are reported relative to mouse *β-actin*.

Genomic DNA isolation and PCR assays for Tax transgene integration mapping

Genomic DNA was extracted from 5×10^6 splenic and BM mononuclear cells using the DNeasy Blood & Tissue Kit system (QIAGEN). Cells were isolated from NOD/SCID mice with or without transplantation of Tax-Tg-derived SLCs. All genomic DNAs were treated with RNase A (100 mg/mL).

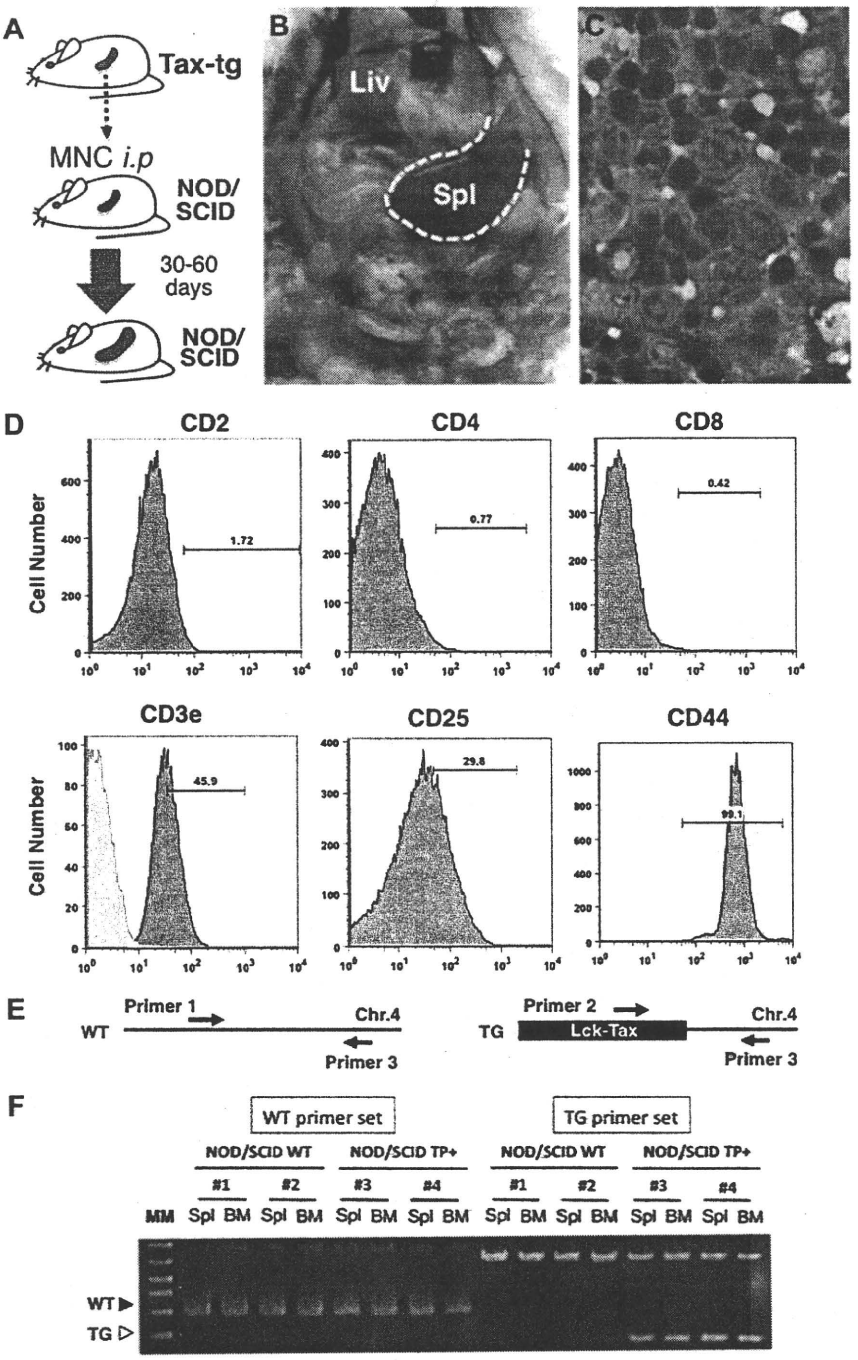

Figure 1. Transplantation of Tax-Tg mouse-derived splenic mononuclear cells to NOD/SCID mice. (A) Experimental design of the transplantation assay. (B) Remarkable splenomegaly was observed in the lymphoma-reconstituted NOD/SCID mouse. Spl indicates spleen; and Liv, liver. The dotted line shows the outline of the enlarged spleen. (C) Cytospin analysis of spleen cells isolated from reconstituted lymphoma in the NOD/SCID mouse. The spleen was filled with ATL-like lymphomatous cells. (D) Surface marker analysis of lymphomatous cells from spleen. These had the identical phenotype of lymphomatous cell reconstituted by Tax-Tg–derived splenic mononuclear cells: $CD2^-$, $CD4^-$, $CD8^-$, cytoplasmic $CD3^+$, and surface $CD25^+$ and $CD44^+$. (E) Schematic representation of the PCR assay to identify and confirm the Tax transgenic integration site. Lck-Tax transgenes were tandemly inserted on the chromosome 4 (Chr.4) in the original transgenic animals. (F) Genotyping of mononuclear cells in the spleen and BM. WT PCR product (300 bp) was detected in both the normal and CSC-transplanted NOD/SCID spleen and BM. However, Tax-Tg (TG) PCR products identifying the expected integration site of the transgene (200 bp) were detected only in the reconstituted ATL-like lymphomatous cells in spleen and BM. TP+ indicates transplantation.

PCR primers were constructed based on the integration site of Tax gene on chromosome 4 in the Tax-Tg mouse model.[8] The primers used to detect the Tax-Tg or wild-type (WT) NOD/SCID mouse derived genomic DNA were NOD/SCID mouse (5′-TGT TGC ATA CAG GAA GCC CA-3′ and 5′-GCG GTA CAG TGT GTG CTT TGA G-3′) and Tax-Tg (5′-GAC ACA GCA TAG GCT ACC TGG C-3′ and 5′-GCG GTA CAG TGT GTG CTT TGA G-3′; Figure 1E). PCR reactions were performed using ExTaq (Takara Shuzo) and a Thermal Cycler (Bio-Rad). The PCR was carried out using 95°C for 2 minutes, 40 cycles at 94.0°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The PCR products were analyzed by electrophoresis on 2% agarose gels.

Data analysis

Significant differences were calculated using the Student *t* test for gene expression analysis. Statistical analyses were performed using GraphPad Prism (Version 5, GraphPad Software) and Excel 2008 (Microsoft Japan).

Results

Lymphoma/leukemia regenerative activity in the Tax-Tg mouse and SLCs

Hasegawa et al reported that Tax-Tg SLCs could regenerate ATL-like lymphoma when transplanted into NOD/SCID mice and that Tax-Tg SLCs had the potential to regenerate the original lymphoma when further transplanted in NOD/SCID mice.[8] To assess the stem cell potential within the SLCs, we performed serial transplantation experiments by intraperitoneal injections of frozen stocked 4th-passed 10^5 SLCs into NOD/SCID mice (Figure 1A). SLCs could regenerate original lymphoma and leukemia in the first transplantation (12 of 12 animals) with the development of marked

splenomegaly (Figure 1B-C) and BM involvement resulting from infiltration of malignant cells. The phenotypes of the SLCs were similar to the original tumors in Tax-Tg animals. Surface staining for CD2, CD4, and CD8 was negative, but cytoplasmic CD3 and surface CD25 and CD44 were positive in the SLCs (Figure 1D). SLCs could regenerate original lymphoma and leukemia after second (5 of 5 animals) and third transplantation (6 of 7 animals) by day 40.

To confirm that the SLCs were derived from the original leukemic cells, we performed PCR analysis to confirm the Tax transgenic integration site. In the Tax-Tg model, the transgene was found to be integrated in the A9 region of chromosome 4. PCR analysis with amplification across the integration site demonstrated that this was identical in the lymphomatous cells arising in both spleen and BM (Figure 1E-F), confirming that they were derived from the original leukemic cells and that variant populations had not arisen nor were selected. These studies provided functional evidence for self-renewal and supported the existence of CSCs, which have leukemia/lymphoma regenerative potential, within the SLCs.

Identification of CSCs in SLCs by surface marker analysis

It has been suggested that CSCs are a small and minor population in both leukemias[12,13] and solid tumors.[14] To identify the candidate CSC populations in SLCs, we performed detailed cell surface marker analysis, to investigate phenotypic expression patterns previously observed in Tregs (CD25, CD127, FoxP3), hematopoietic stem cells (HSCs; CD117, CD34, CD38, Sca-1, c-kit), and markers previously identified in other CSCs (CD71, CD123, CD24, CD90, CD133). Surface marker analyses are shown in Figure 2A through D. Expression patterns were divided into 4 profiles: partial and low expression; CD127, CD117, CD123, FoxP3, CD133, CD90, and CD34 (Figure 2B); heterogeneous expression; CD71 and CD25 (Figure 2C); and major expression; CD38, CD24, and Sca-1 (Figure 2D).

Certain cases of human ATL are thought to originate from Tregs, which express CD4, $CD25^{int\text{-}hi}$, FOXP3,[21] and CD127.[22] Whereas Foxp3 expression was not detected (0.03%) in the SLCs, CD25 expression was heterogeneously detected (64.5%) and CD127 expression was detected at low levels (1.17%; Figure 2B-C). Recent studies have shown that leukemia stem cells can share HSC properties ($CD34^+/CD38^-$ cells), and it could also be shown that these could regenerate the original disease in NOD/SCID mice.[23] In the mouse, HSCs are enriched in $CD34^-$/c-kit^+/Sca-1^+/$Lineage^-$ cell population.[24] CD34 and CD117 (c-kit) expression in the SLCs was partially detected (0.63% and 0.56%). CD38 and Sca-1 highly expressed at 94.3% and 89.9%, respectively (Figure 2B,D). We also examined expression of CD123 (IL-3Ra), which is a well-established marker for leukemia stem cells,[12] CD133, which is a common CSC marker found in brain,[25] and colon cancer,[26] CD24, which has been identified in prostate CSCs,[27] and CD90, which is associated with CSCs in non-small-cell lung carcinoma.[28] In the SLCs, expression of CD71 and CD24 were 52.2% and 92.7%, respectively. In contrast, the expression of CD123, CD90, and CD133 was only detected at low levels, 0.13%, 0.89%, and 0.3%, respectively (Figure 2B-D).

Next, we performed multiple stainings for the identification of small populations within the SLCs (Table 1), and we successfully identified such a population, which was $CD38^-/CD71^-$. With a combination of CD117, we successfully confirmed the existence of a minor population (0.03%), which was $CD38^-/CD71^-/CD117^+$ (Figure 2E). In contrast, $CD38^-/CD71^-/CD133^+$ cells were not detected in SLCs (0%).

Identification of candidate CSCs for functional studies

Recent studies have shown that SPs are enriched for CSCs in various types of malignancies.[29] The SP phenotype is based on the ability of the cells to efficiently reflux the Hoechst 33342 fluorescent staining dye through the multidrug ABC transporter (ABCG2), and this property allows the isolation of the cells using flow cytometry. It is also considered that efflux efficiency in CSCs correlates with anticancer drug resistance and recurrence of disease after chemotherapy. To identify candidate CSCs, we investigated the SPs by evaluating efficient efflux of Hoechst 33342 dye in the SLCs. We successfully identified a small population (0.06%) corresponding to SP cells in the SLCs (Figure 3A); correspondingly, the SP fraction disappeared with treatment with the ABC transporter inhibitor verapamil. To further characterize the SP cells, we performed combination SP and surface marker analysis, and it could be shown that more than 50% of $CD38^-/CD71^-/CD117^+$ cells overlapped with the SP fraction in the SLCs (Figure 3B), suggesting that the CSC candidate(s) were associated with these populations.

The candidate CSC cells were also sorted for morphologic studies (Figure 3C). Isolated $CD38^-/CD71^-/CD117^+$ cells were blastoid cells and had scanty cytoplasm with no granules (Figure 3D). In contrast, $CD38^+/CD71^+/CD117^-$ cells were slightly larger and lymphocyte-like with dispersed chromatin and an irregularly shaped and prominent nucleus (Figure 3E).

In vivo lymphoma reconstitution assay of candidate CSCs

We performed transplantation analysis to assess the functionality of the candidate CSCs using the NOD/SCID mouse model. In initial studies, we performed limiting dilution analysis to estimate the frequency of the CSCs in the SLCs. We transplanted 10^6, 10^5, 10^4, 10^3, and 10^2 SLCs into NOD/SCID mice. It has been previously demonstrated that 10^5 SLCs could regenerate original leukemia and lymphoma observed in the Tax-Tg mice,[8] and this was evident by marked splenomegaly and confirmed by cytology at 40 days after transplantation. Spleen weights were approximately 10- to 20-fold larger than non-treated NOD/SCID mouse spleen (data not shown). In addition to the development of lymphoma, ascites also developed in the NOD/SCID mice. Using these criteria, we evaluated the lymphoma-regenerative activity in the candidate CSC and non-CSC fractions (Table 2). Whereas 10^2 SLCs could not regenerate original lymphoma and leukemia (0%) in the NOD/SCID mouse, 10^4 SLCs could, as expected, regenerate the original lymphoma and leukemia in all animals (100%). Lymphoma and leukemia after transplantation of 10^3 SLCs developed in 20% of animals. These results suggested that one CSC existed in 10^4 SLCs (0.01%), and indeed this frequency estimate was consistent with our surface marker and SP analysis studies.

We then isolated CSCs from SLCs, and 10^2 CSCs were transplanted into NOD/SCID mouse. At 40 days after transplantation, lymphoma and leukemia formation was not observed in 6 of the CSC transplanted NOD/SCID mice examined. However, at 60 days after transplantation, ATL-like lymphoma was observed in all 9 of the 9 CSC-injected NOD/SCID mice. All developed typical splenomegaly (Figure 4A-B) and ascites (Figure 4C). Total spleen

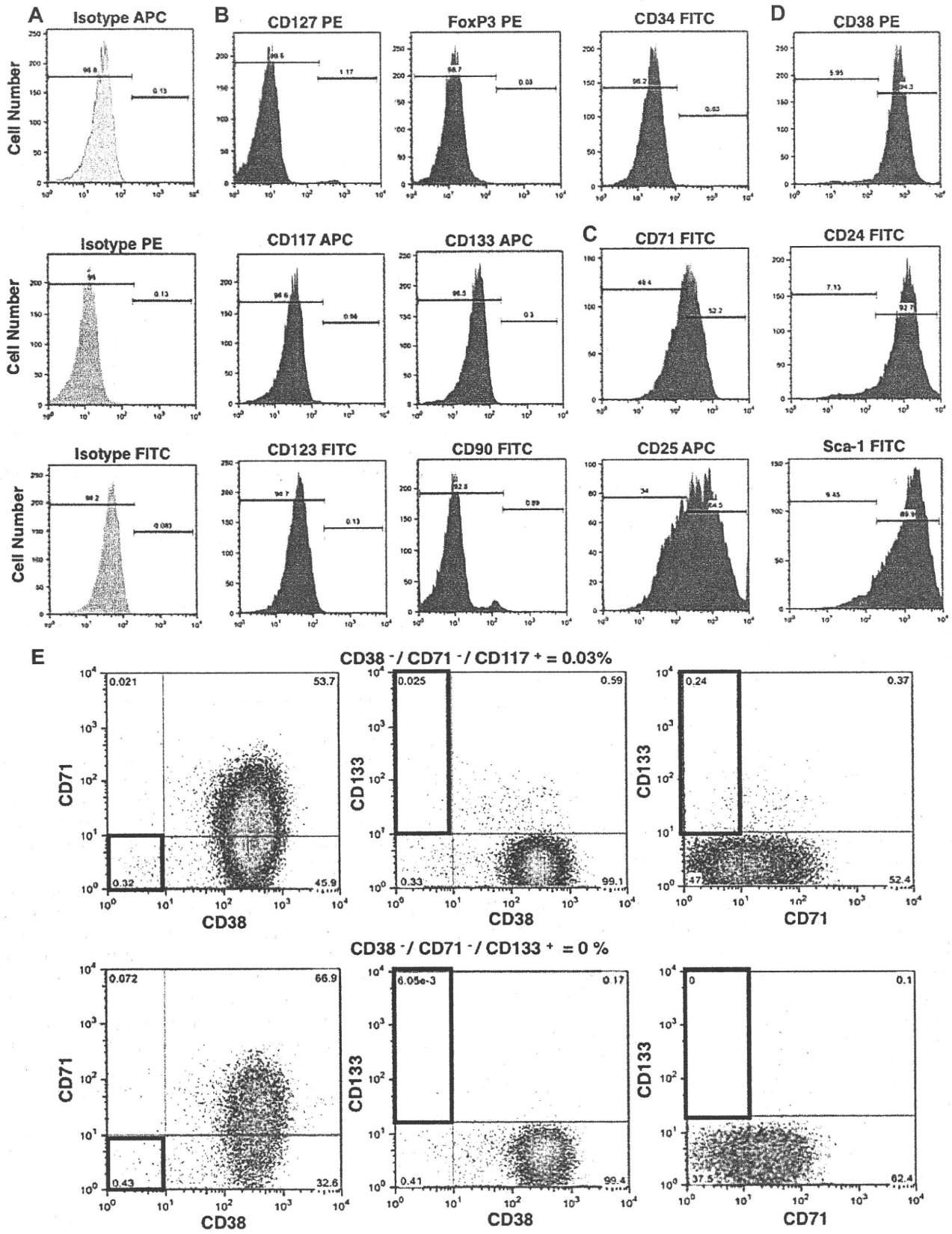

Figure 2. Flow cytometric analysis of NOD/SCID repopulating ATL-like lymphoma cells. Lymphoma and leukemia were generated in NOD/SCID mice by the transplantation of frozen stocked 4th-passage Tax-Tg SLCs. SLCs were isolated from spleen. (A) Histograms of isotype APC, PE, and FITC markers as controls. Expression profiles were divided into 4 patterns: partial and low, heterogeneous, and major types. (B) Partial and low expression: CD127, CD117, CD123, FoxP3, CD133, CD90, and CD34 are expressed at low levels in SLCs. (C) Heterogeneous expression: CD71 and CD25 are heterogeneously expressed in SLCs. (D) Major: CD38, CD24, and Sca-1 are highly expressed in SLCs. The percentage of individual subpopulations was determined according to isotype control in each assay. Dead cells were gated out by propidium iodide. (E) Triple-staining analysis with CD38, CD71, and CD117 or CD133 in the SLCs. $CD38^-/CD71^-/CD117^+$ cells were 0.03% and $CD38^-/CD71^-/CD133^+$ cells were 0% of total SLCs.

Table 1. Surface marker analysis of the NOD/SCID repopulating ATL-like lymphoma cells

Phenotype	Frequency (%)
$CD38^-/CD71^-/CD117^+$	0.03-0.05
$CD38^-/CD71^-/CD133^+$	0
$CD38^-/CD71^-$	0.3-0.5
$CD38^-/CD117^+$	0.10
$CD71^-/CD117^+$	0.15
$CD38^-/CD133^+$	< 0.01
$CD71^-/CD133^+$	< 0.01
Side population cells*	0.03-0.06
Aldehyde dehydrogenase activity*	0

Summary of surface marker combination analysis of ATL-like lymphoma cells in the NOD/SCID mouse. $CD38^-/CD71^-/CD117^+$ cells were overlapped with side population cells.

*Functional assay.

weight was significantly increased only in the CSC-transplanted-NOD/SCID mouse spleen (Figure 4D). No lymphoma was observed after transplantation of 10^2 SLCs (n = 11) and the non-CSC fraction ($CD38^+/CD71^+/CD117^-$; n = 11) at 60 days. Analysis of the splenic cells isolated from 10^2 CSC-transplanted NOD/SCID mouse showed similar profiles to the first transplantation experiment using 10^5 SLCs, in that they were $CD25^+/CD44^+$ (data not shown) and contained SP cells (0.06%; Figure 4E) and the CSC-associated ($CD38^-/CD71^-/CD117^+$) cell population (Figure 4F). These results clearly show that within this cell population are CSCs in that they have the SP phenotype and have the potential for self-renewal and the ability to regenerate the original lymphoma and leukemia.

Histologic analysis of spleen, BM, and other tissues

To confirm and characterize in more detail the lymphoma and leukemia in spleen after transplantation with the candidate CSCs,

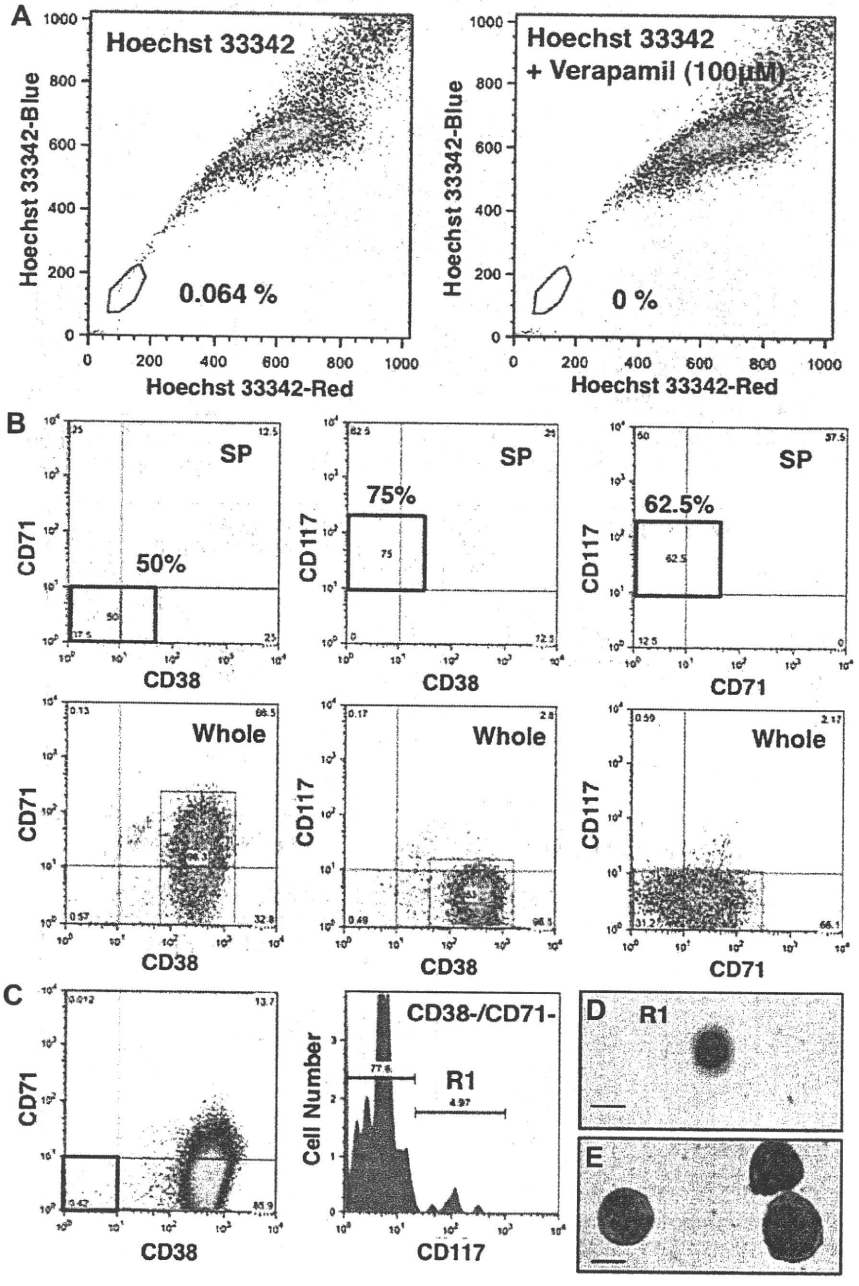

Figure 3. Functional analysis in the NOD/SCID repopulating ATL-like lymphoma cells. (A) SP cell analysis in the NOD/SCID repopulating ATL-like lymphoma cells. The SP regions are indicated by a trapezoid on each panel. (Left panel) Approximately 0.064% of SP cells were observed in the SLCs. (Right panel) SP cell analysis after treatment with verapamil (100 μM), where the SP fraction was lost. (B) Triple-staining analysis of CD38, CD71, and CD117 in the SP fraction. More than 50% of $CD38^-/CD71^-/CD117^+$ corresponded to the SP fraction. (C) FACS $CD38^-/CD71^-/CD117^+$ and $CD38^-/CD71^-/CD117^-$ cells. (D) Cytospin analysis of the CSC candidate ($CD38^-/CD71^-/CD117^+$) and (E) non-CSC candidate ($CD38^+/CD71^+/CD117^-$) populations.

Table 2. Limiting dilution analysis in assessing stem cell activity

No. of cells	SLCs	CSCs ($CD38^-/CD71^-/CD117^+$)	Non-CSCs ($CD38^+/CD71^+/CD117^-$)
Short-term incubation (40 days)			
10^6	5/5	NT	NT
10^5	3/4	NT	NT
10^4	3/3	NT	NT
10^3	1/5	NT	NT
10^2	0/7	0/6	NT
Long-term incubation (60 days)			
10^2	0/11	9/9	0/11

Assessment of stem cell activity in the ATL-like lymphoma cell using limiting dilution assay of transplantation cells. One CSC was thought to exist in 10^4 SLCs. At long-term incubation, only CSC fractions can repopulate and regenerate original ATL-like lymphoma in the NOD/SCID mouse.

NT indicates no transplantation.

we performed histologic and immunohistochemical analyses. It has been reported that ATL leukemic cells have abundant PAS-strong positive cytoplasmic inclusions.[18] We could show that PAS-hematoxylin strong positive staining was only observed in lymphoma and leukemia in spleens after transplantation with 10^5 SLCs and 10^2 CSCs (Figure 5A-B). PAS-strong positive cells were not identified in the spleen after transplantation with 10^2 non-CSCs and SLCs (Figure 5C). To confirm the existence of malignant cells, we performed immunohistochemistry for CD3, CD4, CD44, and CD117. $CD3^+$ leukemic cells were observed only in the splenic lymphoma after transplantation of 10^2 CSCs and 10^5 SLCs. No $CD4^+$ cells were observed in the CSCs and non-CSC–transplanted

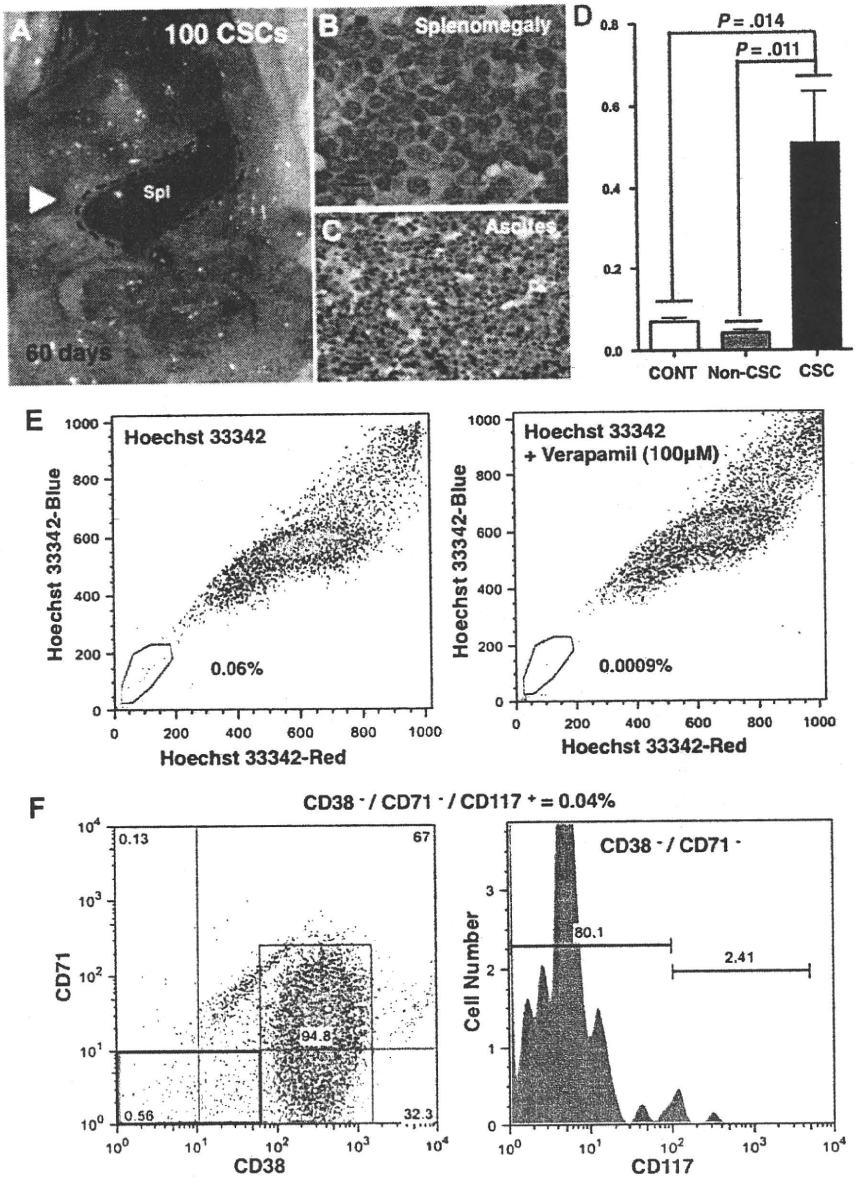

Figure 4. Regenerative potential of ATL-like lymphoma in 10^2 CSCs. ATL-like lymphoma-regenerative activity was assessed by the transplantation of 10^2 CSCs, the non-CSC fraction, and SLCs. (A) ATL-like lymphoma was regenerated by the transplantation of 10^2 CSCs into the NOD/SCID mouse at 60 days. (B) Marked splenomegaly was observed in the NOD/SCID recipient mice. (C) Ascites was also observed in the NOD/SCID recipient mice. (D) Total spleen weight was significantly increased only in the CSC-transplanted NOD/SCID mouse. (E) SP analysis of ATL-like lymphoma cells generated by the transplantation of 10^2 CSCs. (Left panel) The SP fraction (total = 0.066%) was present after CSC transplantation. (Right panel) The SP fraction was lost in the dot plot with treatment by 100 μM verapamil. (F) Surface marker analysis in the lymphoma cells regenerated by the 10^2 CSC transplantation. The CSC candidate cells ($CD38^-/CD71^-/CD117^+$) were also regenerated after transplantation of 10^2 CSCs.

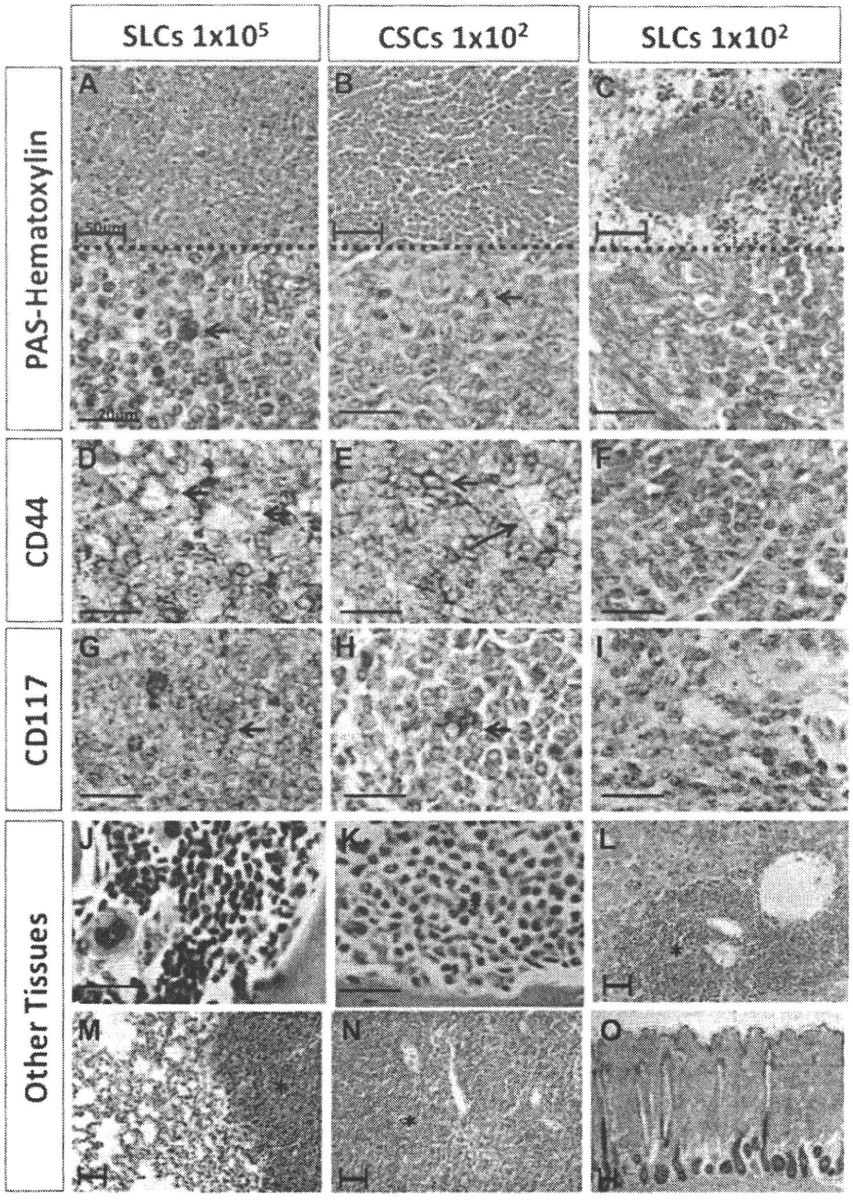

Figure 5. Histologic and immunohistochemical analyses of spleen after transplantation. At 60 days, lymphoma was regenerated after the transplantation of 10^5 SLCs and 10^2 CSCs. No lymphoma was observed after the transplantation of 10^2 non-CSCs. (A) PAS-hematoxylin staining in the 10^5 SLCs recipient spleen. (Top panel) Low magnification. (Bottom panel) High magnification. Strong PAS$^+$-stained cells were observed (←). (B) PAS-hematoxylin staining in the 1×10^2 CSCs recipient spleen. (C) PAS-hematoxylin staining in the 1×10^2 non-CSCs recipient spleen. No strong PAS$^+$ staining cells were evident. (D-I) Immunohistochemistry of CD44 and CD117, shown in the 10^5 SLC recipient spleen (D,G), in the 10^2 CSC recipient spleen (E,H), and in the 1×10^2 SLC recipient spleen (F,I). CD44 and CD117 expression is detected in the lymphoma in the spleen after 1×10^5 SLC and 1×10^2 CSC transplantation. (J) Hematoxylin and eosin (H&E) staining of the normal NOD/SCID mouse BM. Various types of blood cells, including megakaryocytes and erythroid cells, were evident. (K) Hematoxylin and eosin staining of lymphoma reconstituted in the NOD/SCID mouse BM. The BM tissue was uniformly filled with ATL-like lymphomatous cells. (L) Infiltration of lymphomatous cells was also observed in the liver. (M) Infiltration of lymphomatous cells in lung. (N) Infiltration of lymphomatous cells in lymph nodes. (O) Infiltration of lymphomatous cells was not observed in the epidermal tissues. *Lymphomatous cells. (Closed scale bar, 50 μm; open scale bar, 20 μm.)

spleen (data not shown). Interestingly, CD44 strongly positive cells were identified only in the lymphomatous spleen (Figure 5D-E) and not in the nonlymphomatous spleen (Figure 5F). Moreover, CD117$^+$ cells serving as a surrogate CSC marker were identified only in the lymphomatous spleen (Figure 5G-H) and not in the nonlymphomatous spleen (Figure 5I).

To confirm the infiltration of lymphomatous cells in other tissues, we next performed histologic analysis on BM, liver, lung, lymph node, and epidermal tissues. In the BM, as expected, various types of blood cells, including megakaryocytes and erythroid cells, were observed in WT NOD/SCID BM (Figure 5J). However, ATL-like lymphomatous cells uniformly filled in the BM of transplanted animals (Figure 5K). In addition to the BM, lymphomatous cells were also observed in the liver (Figure 5L), lung (Figure 5M), and lymph node (Figure 5N). Infiltration of lymphomatous cells was not observed in the epidermal tissues (Figure 5O).

Identification of lymphomatous cells in BM

We performed cell surface analysis of the BM mononuclear cells isolated from NOD/SCID mouse, after transplantation with 10^5 SLCs. CD38$^-$/CD71$^-$/CD117$^+$ cells (0.05%) could also be detected in BM (Figure 6A). Although, in the normal NOD/SCID mouse BM, CD3$^+$ cells were rare (Figure 6B), in contrast, almost all cells in the transplanted NOD/SCID mice were CD3$^+$ (Figure 6C).

In normal hematopoiesis, HSCs are located in specific microenvironments (niches) that also play a role in maintaining stem cell function. The osteoblastic niche in the trabecular bone and vascular niche in the medullary region are important for both the maintenance of hematopoietic[20] and leukemic stem cell function. To identify the niches of the CSCs in the BM, we performed CD117 staining as a surrogate CSC marker. Notably, CD117$^+$ cells were located both in the osteoblastic niche region (Figure 6D) in the trabecular bone and vascular niche in the medullary region (Figure 6E).

Molecular characterization and Tax expression in CSCs

To identify specific molecular markers in CSCs, we performed real-time PCR analysis on isolated CSC and non-CSC fractions (Figure 7). Recently, several molecules have been indentified as

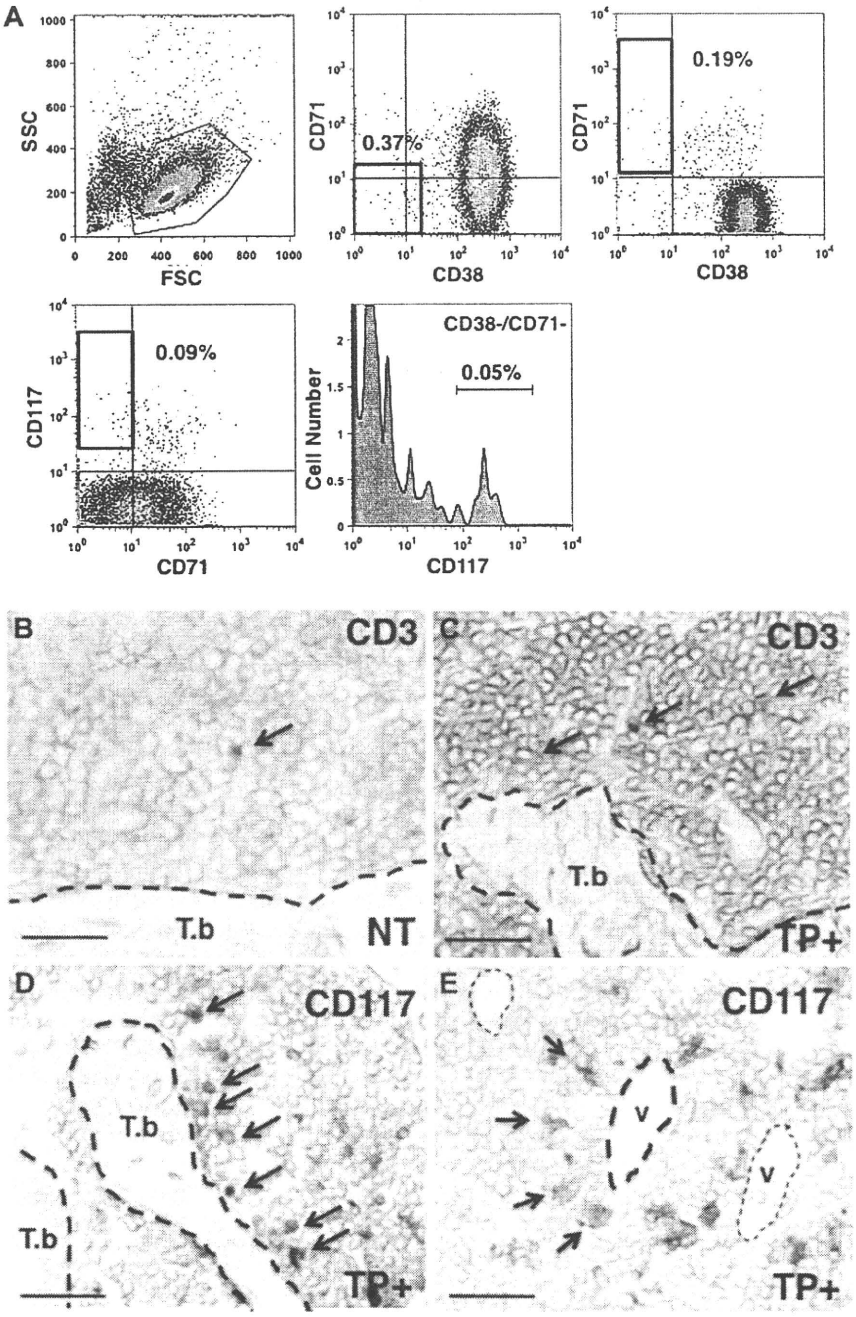

Figure 6. Flow cytometric analysis of lymphomatous cells in the NOD/SCID BM. NOD/SCID repopulating lymphomatous cells were isolated from BM. (A) Triple staining analysis with CD38, CD71, and CD117 in the BM SLCs. As was observed in spleen, the CSC candidates were also observed in the BM. (B) Histologic analysis of ATL-like lymphomatous cells in the NOD/SCID BM. In the normal NOD/SCID BM, $CD3^+$ cells were a rare population (←). (C) In the reconstituted NOD/SCID mouse BM, $CD3^+$ cells were readily identified (arrow). (D) $CD117^+$ cells as a surrogate CSC marker could be detected in the osteoblastic niche of the trabecular bone (arrow). (E) In the medullary region, $CD117^+$ cells (CSCs) were also detected in the vascular niche (←). T.b. indicates endosteal region in the trabecular bone; V, vascular zone; TP+, transplantation; and NT, no transplantation. Scale bar represents 20 μm.

being associated with tumor progression. Several embryonic stem cell markers have been shown to be associated with osteosarcoma[30] and bladder cancer,[31] and several HSC markers were found to be associated with leukemia development.[32] We examined *Notch1*, *CD44*, *Oct-4*, *Nanog*, *Rex1*, *Bmi1*, *SCL/tal-1*, *Flt3*, *N-cadherin*, and viral *Tax* expression in the CSCs. A total of 5×10^3 CSCs and non-CSCs were isolated by fluorescence-activated cell sorter (FACS) for RNA isolation and cDNA synthesis. These amplified cDNA were initially validated by *CD117* expression (Figure 6A). The expression level of *CD117* in the CSCs was higher than in the non-CSC fraction. In contrast, expression of *HTLV-1 Tax* mRNA was extremely low compared with the non-CSC fraction. Although expression of *CD44* in the CSC was higher than in the non-CSC fraction, *Notch1* and *Bmi-1* expression was lower than in the non-CSCs (Figure 6B). No differences were observed in *Rex1*, *Flt3*, *SCL/tal-1*, *N-cadherin*, *Oct-4*, and *Nanog* expression (data not shown).

Discussion

In this study, we have successfully identified a candidate CSC population in a mouse model of ATL, which has been shown to exhibit many of the clinical, pathologic, and immunologic features of human disease. It has been clearly established that CSCs are a specific and minor cell population, which have the potential for self-renewal, differentiation, aggressive proliferation, and chemotherapy resistance and can successfully regenerate the original malignancy when transplanted into immunocompromised mice.[14] In hematologic malignancies, several CSC candidates have been identified in acute myeloid leukemia,[12] chronic myeloid leukemia,[13] and acute lymphoblastic leukemia (ALL). Recently, Cox et al have characterized different CSC populations in ALL, which

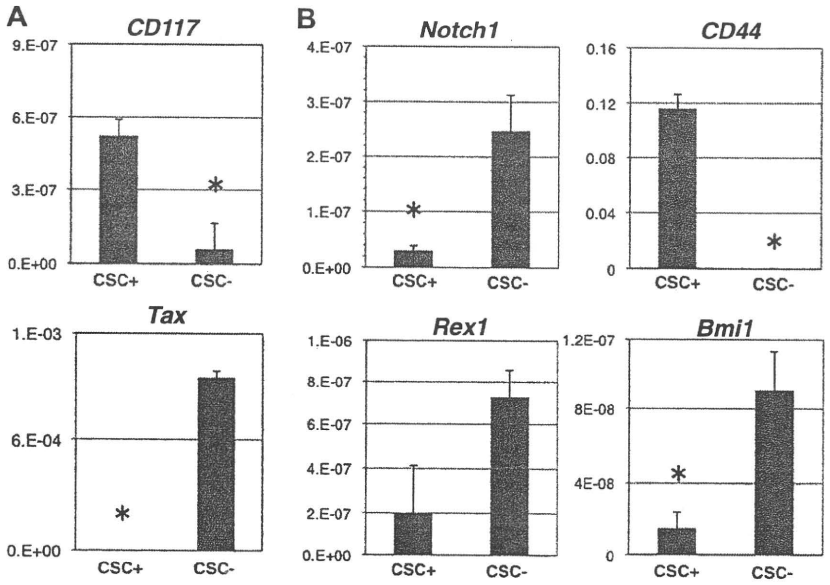

Figure 7. Molecular characterization of the CSCs and non-CSC fraction. FACS-sorted 5000 CSCs and non-CSCs were used to purify RNA and synthesize cDNA. Gene expression level was determined relative to *β-actin*. (A) *CD117* expression was used to evaluate the efficacy of cell sorting. (Top panel) *CD117* expression was higher in the CSCs than in the non-CSCs. (Bottom panel) Tax gene expression was not detected in CSCs. (B) *Notch1* and *Bmi1* expression was down-regulated in CSCs. *CD44* expression was up-regulated in CSCs. No difference was observed in the expression of the other genes, *Rex1*, *Flt3*, *SCL/tal-1*, *N-cadherin*, *Oct-4*, and *Nanog* in the CSCs and non-CSC fraction. $^*P < .05$ (significant).

include the phenotype $CD34^+/CD10^-$ or $CD34^+/CD19^-$ subpopulations in B-ALL and $CD34^+/CD4^-$ or $CD34^+/CD7^-$ subpopulations in childhood T-ALL.[33,34] Although it has been suggested that leukemic transformation occurs in a committed T- and B-cell lineage,[35] both of these candidates express the hematopoietic progenitor marker CD34, suggesting that T- and B-cell leukemia may have arisen in an early hematopoietic precursor.

ATL is a lymphoproliferative disorder caused by infection with HTLV-1.[1] Although various chemotherapeutic regimens have provided significant initial complete remission rates, most treated patients relapse, and these observations have suggested the existence of drug-resistance CSCs in aggressive disease. In this study, we have, for the first time, successfully identified and enriched a candidate CSC population in an ATL model mouse using SP analysis in conjunction with cell surface marker identification by flow cytometry. The candidate CSCs identified by their SP phenotype, which were found to overlap with a $CD38^-/CD71^-/CD117^+$ population, could regenerate the original lymphoma/leukemia with the expected phenotype when transplanted in SCID/NOD mice.

Recently, Kayo et al reported the existence of SP cells in several cultured ATL cell lines, which were defined by efficient efflux of Hoechst 33342 dye.[36] However, in these studies, both SP cells and non-SP cells could reconstitute both SP and main population cells, suggesting that SP cells in the cell lines had no specific CSC-like function in vitro.[36] The reasons for this are unclear but may well reflect changes in the cell populations as a result of culture. It has also been reported that CD90 may be a useful marker to identify CSCs in ATL cell lines. CD90 (Thy-1) is a hematopoietic progenitor marker, which is not expressed in mature T cells. In our experiments, CD90 expression was detected in approximately 1.5% of SLCs; however, expression was not associated with the CSCs. These disparate results may again reflect the differences between our transgenic and SCID/NOD animal models and ATL cell lines, which may have changed during culture.

CSCs, especially in the hematologic malignancies, share many properties with HSCs, and indeed almost all CSCs are thought to be derived from HSCs.[32] Although It has been shown that human ATL is a leukemia that, in most cases, is derived from $CD4^+$ mature T cells,[3] others exhibit a phenotype of cells at early stages in T-cell development. In our Tax transgenic mouse model, it has been shown that the ATL-like lymphoma cells were derived from an immature pre-T cell (DN2), which expresses cytoplasmic CD3, and surface CD25 and CD44.[8] In the present study, we could clearly demonstrate that the $CD38^-/CD71^-/CD117^+/CD44^+$ fraction have lymphoma and leukemia-initiating potential and proliferation activity, and have markers expressed in hematopoietic progenitor cells (CLP; common lymphoid progenitor) or Pro-T cells (DN1; double negative). CD117 (also known as c-kit) is useful marker for identifying long-term and short-term repopulating HSCs. Recently, it has shown that CD117 and its ligand stem cell factor also have important roles in early T-cell development,[37] and Notch1 with IL-7 induced differentiation of early progenitors with lymphoid and myeloid properties (EPLM) into T-cell lineage is dependent on CD117 signaling.[38] In addition to CD44, CD117 expression and the SCF/CD117 signaling pathway are also required to initiate the development of several lymphocyte populations.[39] *Notch1* is expressed in long-term HSCs and regulates the self-renewal activity of long-term HSCs in the osteoblastic niche via *Nocth1* receptor ligand *Jagged1*.[40] However, in the T-cell commitment process, whether activation of *Notch1* signaling occurs before or after EPLMs enter the thymus is still controversial.[41] In our experiments, *Notch1* expression in CSCs was down-regulated compared with the non-CSC fraction, suggesting that CSCs were derived from the EPLM or hematopoietic progenitor cells and that activation of Notch1 may be required to induce T-cell lymphoma and leukemia. It has been shown that a mutation (t(7;9) translocation) and constitutive activation of *Notch1* are frequently observed in T-ALL.[42] Whether activation of NOTCH1 signaling and in our animal model or indeed in ATL patients is related to HTLV-1 infection and Tax expression is still unclear and remains to be investigated. However, our data suggest and support the view that, at least in our mouse model, the T-cell lymphoma and leukemia-initiating stem cell is derived from an HTLV-1–infected EPLM or early hematopoietic progenitor cell and suggest that CD44 and CD117, if present, in human ATL CSCs could be possible therapeutic targets for treatment of aggressive disease.

It has been suggested that HTLV-1–derived Tax gene expression plays a key role in the development of ATL and particularly in the early stages of cell proliferation and transformation. Paradoxically, Tax gene expression is either undetectable or only at very low levels in fresh uncultured human ATL cells. In our Tax-transgenic

mouse model, Tax gene expression was also expressed at very low levels only being detected by sensitive reverse-transcription RT-PCR assays. In the present study, we found a significant further down-regulation of Tax gene in the CSCs compared with cells in the non-CSC fraction. These data support our hypothesis that CSCs are derived from EPLM or early pro-T cells (so-called DN1), as the *Lck* promoter will drive partially from DN2 and completely from the DN3 stages.[43] Recent studies have also shown that HTLV-1 can infect HSCs,[6] and lentiviral-mediated expression of Tax gene in human $CD34^+/CD38^-$ cells resulted in G_0/G_1 cell-cycle arrest by P21 and P27 up-regulation and the suppression of multilineage hematopoietic differentiation.[44] These results would suggest that the reacquisition of stem cell properties in T cell–committed progenitors might require both *Tax* expression and *CD117* and *Notch1* reexpression.

In the quantitative real-time PCR analysis, contrary to our expectations, we found a strong down-regulation of *Bmi1* in CSCs, which has been shown to be involved in the progression of several malignancies.[27] Recently, Miyazaki et al[45] reported that *Bmi1* is required for the survival, the activation of pre-T cell, and the transition from DN to double-positive cells. Although expression of *Bmi1* was detected in DN-stage, dependent up-regulation of *Bmi1* was observed from DN2 to DN4.[45] These data support our proposal that the CSCs were derived from EPLMs, but the mechanism of *Bmi1* activation in these cells remains to be investigated.

At present, the microenvironment of CSCs has not been clearly identified. Recently, Ishikawa et al reported that acute myeloid leukemia leukemic stem cells engraft within osteoblastic niche of the BM, and it has been suggested that this may afford protection from chemotherapy-induced apoptosis.[46] In the present study, we have identified potential BM niches for our candidate CSCs in the NOD/SCID model. Specifically, we found that CSCs were located both in the osteoblastic and vascular niches, but further studies are required to determine whether the former localization might also contribute to resistance to chemotherapy in our CSCs, and this is currently under investigation.

In conclusion, our transgenic and NOD/SCID animal studies have allowed the identification of candidate CSCs in this model of ATL. We think that our investigations will both inform and provide a basis for similar studies on human disease to determine whether these or equivalent stem cell populations exist and have the same characteristics. If successful, this will potentially allow the development of new anti-CSC therapeutics, which may provide more effective treatment for human disease.

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Authorship

Contribution: J.Y., T.M., and I.H. designed the research; J.Y. performed the majority of FACS and transplantation analysis; T.M. performed the histologic studies, analyzed the data, and wrote the paper; K.T. assisted with cell sorting and transplantation experiments; M.K. performed the quantitative PCR experiments; H.M. and A.M. also performed experiments; Y.A. assisted with the NOD/SCID mouse experiments; H.H. and W.W.H. produced the Tax transgenic mice, developed the NOD/SCID model, and wrote the paper; and H.T. and K.Y. were involved in the design of the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Isao Hamaguchi, Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases 4-7-1, Gakuen, Musashimurayama, Tokyo, 208-0011, Japan; e-mail: 130hama@nih.go.jp.

References

1. Yamaguchi K. Human T-lymphotropic virus type I in Japan. *Lancet.* 1994;343(8891):213-216.
2. Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. *Oncogene.* 2005;24(39):6058-6068.
3. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma: a report from the Lymphoma Study Group (1984-87). *Br J Haematol.* 1991;79(3):428-437.
4. Yamada Y, Kamihira S, Amagasaki T, et al. Adult T cell leukemia with atypical surface phenotypes: clinical correlation. *J Clin Oncol.* 1985;3(6):782-788.
5. Karube K, Ohshima K, Tsuchiya T, et al. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. *Br J Haematol.* 2004;126(1):81-84.
6. Feuer G, Fraser JK, Zack JA, Lee F, Feuer R, Chen IS. Human T-cell leukemia virus infection of human hematopoietic progenitor cells: maintenance of virus infection during differentiation in vitro and in vivo. *J Virol.* 1996;70(6):4038-4044.
7. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer.* 2007;7(4):270-280.
8. Hasegawa H, Sawa H, Lewis MJ, et al. Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. *Nat Med.* 2006;12(4):466-472.
9. Ohsugi T, Kumasaka T, Okada S, Urano T. The Tax protein of HTLV-1 promotes oncogenesis in not only immature T cells but also mature T cells. *Nat Med.* 2007;13(5):527-528.
10. Yamada Y, Tomonaga M, Fukuda H, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol.* 2001;113(2):375-382.
11. Mahieux R, Hermine O. In vivo and in vitro treatment of HTLV-1 and HTLV-2 infected cells with arsenic trioxide and interferon-alpha. *Leuk Lymphoma.* 2005;46(3):347-355.
12. Jordan CT. Unique molecular and cellular features of acute myelogenous leukemia stem cells. *Leukemia.* 2002;16(4):559-562.
13. Holyoake TL, Jiang X, Drummond MW, Eaves AC, Eaves CJ. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia. *Leukemia.* 2002;16(4):549-558.
14. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene.* 2004;23(43):7274-7282.
15. Clarke MF. A self-renewal assay for cancer stem cells. *Cancer Chemother Pharmacol.* 2005; 56[Suppl 1]:64-68.
16. Tang C, Ang BT, Pervaiz S. Cancer stem cell: target for anti-cancer therapy. *FASEB J.* 2007; 21(14):3777-3785.
17. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med.* 1996;183(4):1797-1806.
18. Takemori N, Hirai K, Onodera R, Saito N, Kamiguchi K, Namiki M. Vacuolated glycogen-laden leukemic cells in a case of crisis type chronic adult T-cell leukemia. *Leuk Lymphoma.* 1993;11(3):309-314.
19. Hamaguchi I, Imai J, Momose H, et al. Two vaccine toxicity-related genes Agp and Hpx could prove useful for pertussis vaccine safety control. *Vaccine.* 2007;25(17):3355-3364.

20. Mizukami T, Kuramitsu M, Takizawa K, et al. Identification of transcripts commonly expressed in both hematopoietic and germ-line stem cells. *Stem Cells Dev.* 2008;17(1):67-80.
21. Kohno T, Yamada Y, Akamatsu N, et al. Possible origin of adult T-cell leukemia/lymphoma cells from human T lymphotropic virus type-1-infected regulatory T cells. *Cancer Sci.* 2005;96(8):527-533.
22. Baba H, Yamada Y, Mori N, et al. Multiple gamma-receptor expression in adult T-cell leukemia. *Eur J Haematol.* 2002;68(6):362-369.
23. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3(7):730-737.
24. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood.* 1992;80(12):3044-3050.
25. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature.* 2004;432(7015):396-401.
26. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature.* 2007; 445(7123):106-110.
27. Lee CJ, Dosch J, Simeone DM. Pancreatic cancer stem cells. *J Clin Oncol.* 2008;26(17):2806-2812.
28. Lam WK, Watkins DN. Lung cancer: future directions. *Respirology.* 2007;12(4):471-477.
29. Hadnagy A, Gaboury L, Beaulieu R, Balicki D. SP analysis may be used to identify cancer stem cell populations. *Exp Cell Res.* 2006;312(19):3701-3710.
30. Gibbs CP, Kukekov VG, Reith JD, et al. Stem-like cells in bone sarcomas: implications for tumorigenesis. *Neoplasia.* 2005;7(11):967-976.
31. Atlasi Y, Mowla SJ, Ziaee SA, Bahrami AR. OCT-4, an embryonic stem cell marker, is highly expressed in bladder cancer. *Int J Cancer.* 2007; 120(7):1598-1602.
32. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. *Nat Rev Cancer.* 2008;8(5):341-350.
33. Cox CV, Evely RS, Oakhill A, Pamphilon DH, Goulden NJ, Blair A. Characterization of acute lymphoblastic leukemia progenitor cells. *Blood.* 2004;104(9):2919-2925.
34. Cox CV, Martin HM, Kearns PR, Virgo P, Evely RS, Blair A. Characterization of a progenitor cell population in childhood T-cell acute lymphoblastic leukemia. *Blood.* 2007;109(2):674-682.
35. Plasschaert SL, Kamps WA, Vellenga E, de Vries EG, de Bont ES. Prognosis in childhood and adult acute lymphoblastic leukaemia: a question of maturation? *Cancer Treat Rev.* 2004;30(1):37-51.
36. Kayo H, Yamazaki H, Nishida H, Dang NH, Morimoto C. Stem cell properties and the side population cells as a target for interferon-alpha in adult T-cell leukemia/lymphoma. *Biochem Biophys Res Commun.* 2007;364(4):808-814.
37. Massa S, Balciunaite G, Ceredig R, Rolink AG. Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. *Eur J Immunol.* 2006;36(3):508-511.
38. Agosti V, Corbacioglu S, Ehlers I, et al. Critical role for Kit-mediated Src kinase but not PI 3-kinase signaling in pro T and pro B cell development. *J Exp Med.* 2004;199(6):867-878.
39. Parrott JA, Kim G, Skinner MK. Expression and action of kit ligand/stem cell factor in normal human and bovine ovarian surface epithelium and ovarian cancer. *Biol Reprod.* 2000;62(6):1600-1609.
40. Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood.* 2002;99(7):2369-2378.
41. Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. *Nat Immunol.* 2004;5(3):247-253.
42. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science.* 2004;306(5694): 269-271.
43. Masuda K, Kakugawa K, Nakayama T, Minato N, Katsura Y, Kawamoto H. T cell lineage determination precedes the initiation of TCR beta gene rearrangement. *J Immunol.* 2007;179(6):3699-3706.
44. Tripp A, Liu Y, Sieburg M, Montalbano J, Wrzesinski S, Feuer G. Human T-cell leukemia virus type 1 tax oncoprotein suppression of multilineage hematopoiesis of CD34+ cells in vitro. *J Virol.* 2003;77(22):12152-12164.
45. Miyazaki M, Miyazaki K, Itoi M, et al. Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. *Immunity.* 2008;28(2):231-245.
46. Ishikawa F, Yoshida S, Saito Y, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol.* 2007;25(11): 1315-1321.

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ORIGINAL ARTICLE

Adhesion-dependent growth of primary adult T cell leukemia cells with down-regulation of HTLV-I p40Tax protein: a novel in vitro model of the growth of acute ATL cells

Kazuhiro Nagai · Itsuro Jinnai · Tomoko Hata · Tetsuya Usui · Daisuke Sasaki · Kunihiro Tsukasaki · Kazuyuki Sugahara · Yoshitaka Hishikawa · Yasuaki Yamada · Yuetsu Tanaka · Takehiko Koji · Hiroyuki Mano · Shimeru Kamihira · Masao Tomonaga

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Abstract In order to better understand the biology of adult T cell leukemia (ATL), we aimed to establish a novel method, which allows the primary growth of ATL cells using a co-culture system with murine bone marrow-derived stromal cells, MS-5. ATL cells grew in close contact with MS-5 layers and formed so-called "cobblestone areas" (CAs) without the addition of IL-2. In clinical samples, eight of ten (80.0%) cases of acute or lymphoma type ATL cells formed CAs. The frequency of CA forming cells in ATL cells ranged from 0.03 to 1.04%. The morphology, immunophenotyping, and DNA analysis indicated that cells composing CA were compatible with ATL cells, and clonally identical to primary CD4-positive ATL cells. Furthermore, in ATL cells composing CA, the expression of p40Tax was down-regulated in transcriptional and translational level, while that of HTLV-I basic leucine zipper factor (HBZ) gene was comparable to the level of primary ATL cells, resembling expression pattern of proviral genes in in vivo ATL cells. By microarray analysis, several genes which coded products involved in cell–cell interaction, and cellular survival and proliferation, were differentially expressed in ATL cells composing CA compared with primary samples. In conclusion, our co-culture system allows for the first time the growth of primary ATL cells in vitro, and might be useful as an in vitro assay for biological and clinical studies to develop molecular targeting drugs against ATL.

Keywords Adult T cell leukemia · Microenvironment · In vitro co-culture system · Cell adhesion

K. Nagai (✉) · S. Kamihira
Transfusion Service, Nagasaki University Hospital of Medicine and Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: agwkn@nagasaki-u.ac.jp

I. Jinnai
Division of Hematology, Department of Internal Medicine, Saitama Medical School, Saitama, Japan

T. Hata · K. Tsukasaki · M. Tomonaga
Department of Hematology, Molecular Medicine Unit, Atomic Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

T. Usui · D. Sasaki · K. Sugahara · Y. Yamada · S. Kamihira
Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Y. Hishikawa · T. Koji
Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Y. Tanaka
Department of Immunology, Graduate School and Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

H. Mano
Division of Functional Genomics, Jichi Medical University, CREST, Japan Science and Technology Agency, Tochigi, Japan

1 Introduction

Adult T cell leukemia (ATL) is a lymphoma of mature CD4-positive T cells with frequent leukemic manifestation. Patients with ATL show variable clinical manifestations and a diverse prognosis, being classified into four subtypes; smoldering, chronic, lymphoma, and acute [1, 2]. The disease is also molecularly characterized by monoclonal integration of the provirus of human T-lymphotropic virus type 1 (HTLV-1), which has been

accepted as an etiologic agent of ATL [3, 4]. However, since only a small proportion of HTLV-I carriers develop ATL [5], and the latent period from the initial infection of HTLV-1 until the onset of this disease is over 40–60 years [2], it is believed that, in addition to HTLV-1 viral proteins, as yet undetermined multi-step accumulations of leukemogenic changes in cellular genes and dysregulation of transcription by epigenetic changes may have great roles in providing a growth advantage to the malignant clone and in the evolution to the fully aggressive phase of this neoplastic disease [6].

A few culture systems have allowed primary ATL cells to grow in vitro [7–9], although they showed only transient and cytokine-dependent proliferation. Furthermore, in these reports, it was demonstrated that once placed under in vitro culture conditions, especially when stimulated with interleukin-2 (IL-2), ATL cells began to abundantly express HTLV-1 viral protein p40Tax, a protein encoded by the pX regulatory region of the HTLV-1 genome [10]. The interactions of Tax with a number of cellular molecules, transcriptional factors or modulators of cellular functions, have been reported, resulting in the trans-activation or -repression of many specific cellular genes involved in the control of cell growth or apoptosis [11–13], and it is considered to contribute to the initial immortalization and transformation of HTLV-1-infected T lymphocytes. However, ATL cells do not produce any distinct amount of Tax proteins in vivo [3], although the expression of the *tax* gene has been detected using highly sensitive RT-PCR or RT-PCR in situ hybridization in several previous reports [14, 15]. These findings suggest that in vitro growth in cultures supported with IL-2 does not necessarily reflect the growth mechanism of ATL cells in vivo.

To address this issue, we aimed at establishing a novel method to allow the primary growth of ATL cells using a co-culture system with a stromal cell layer to provide various factors to support proliferation of target cells. Previously, using long-term cultures of stromal cells obtained from lymph nodes (LNs) of ATL patients, we observed that a number of cytokines were produced which might contribute to several clinical manifestations [16]. Thus, we have speculated that there might be some important interactions between the lymphohematopoietic microenvironment of LN or bone marrow (BM) and ATL cells. It has been demonstrated that by co-culturing with the murine BM-derived stromal cell line MS-5, it is possible to support the long-term proliferation of primitive hematopoietic progenitors, B-cell progenitor cells, and acute lymphoblastic leukemia (ALL) blasts [17, 18]. Thus, the system may be assumed to have properties by which human neoplastic lymphoid cells are able to grow without any exogenous cytokine.

Fig. 1 Characterization of cobblestone areas (CAs) formed by primary ATL cells using a co-culture system of ATL cells with a murine stroma cell line, MS-5. **a** Scheme of co-culture experiments: ATL cells from patients were cultured and observed as described in "Sect. 2". **b** Phase-contrast micrographs (×200) indicate time-course changes in CAs of primary ATL cells. ATL cells from a patient (UPN007) were cultured and observed on days 3, 7, 14, and 28, as indicated, respectively. **c** Photographs indicate representative results obtained from UPN 1; May-Giemsa staining (×600), immunostaining for CD4, and PCNA (×600), respectively, left to right. **d** Integration of HTLV-1 proviral DNA; Clonality of primary ATL cells and ATL cells composing CA of UPN001 and UPN005 was examined by SBH using whole HTLV-I probes[26]. Restriction enzyme (*EcoR*I or *Pst*I) was indicated at the bottom of the figure. *Lanes* 1 and 15: marker, 2: ST-1, 3: MS-5 co-cultured with MT-2, 4: MS-5 co-cultured with HUT-102, 5 and 11: primary ATL cells of UPN001, 6 and 12: ATL cells in CA of UPN001, 7 and 13: primary ATL cells of UPN005, 8 and 14: ATL cells in CA of UPN005, 9: HUT-102, 10: MT-2. **e** HTLV-1 proviral load was quantified using real-time PCR method to test involvement of HTLV-1 genome to MS-5 stromal layer[27]. Light and dark gray bars indicate the results of the proviral load in co-cultured MS-5 and original HTLV-1 related cell lines (MT-2 and HUT-102), respectively. **f** Comparison of capacity of CA formation in co-culture experiments with different types of stromal layer. 1×10^5 ATL cells from UPN001, 003, 005, and 010 were co-cultured with stromal layer of MS-5 (*filled diamond*), HESS-5 (*filled square*), and HUVEC (*filled triangle*), in triplicate. Culture dishes were observed daily, and the numbers of CAs were counted at days 3, 5, 7, 10, 14, and 21. Values represent the means plus or minus standard error of the mean

In the present study, primary ATL cells were co-cultured with MS-5 cells and the usefulness of this co-culture system was demonstrated also for ATL cells. The significance of this novel stroma cell-dependent in vitro clonal culture system as a new model of primary ATL cell growth will be discussed.

2 Materials and methods

2.1 Cell lines and primary ATL cells

Four human HTLV-1-infected T cell lines established from normal lymphocytes (MT-2 and HUT-102) and one IL-2-independent (OMT) and three IL-2-dependent (ST-1, KOB, and KK-1) ATL cell lines established from clinical cases were used [19]. With informed consent according to the Helsinki Declaration, clinical samples of ATL cells were obtained from 20 patients, and normal $CD4^+$ T lymphocytes were obtained from peripheral blood of two healthy volunteers. The diagnosis and subtypes of ATL were defined as described by Shimoyama et al. [20]. Mononuclear cells (MNCs) were isolated from samples by Ficoll Paque gradient centrifugation (Amersham Pharmacia Biotech. NJ). ATL cells or normal $CD4^+$ T lymphocytes were enriched by labeling with magnetic bead-conjugated anti-CD4 monoclonal antibody (CD4 MicroBead; Miltenyi Biotec, Auburn, CA, USA), and then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec).