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# CD9 correlates with cancer stem cell potentials in human B-acute lymphoblastic leukemia cells

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ABSTRACT

Cancer stem cell (CSC) theory suggests that only a small subpopulation of cells having stem cell-like potentials can initiate tumor development. While recent data on acute lymphoblastic leukemia (ALL) are conflicting, some studies have demonstrated the existence of such cells following CD34-targeted isolation of primary samples. Although CD34 is a useful marker for the isolation of CSCs in leukemias, the identification of other specific markers besides CD34 has been relatively unsuccessful. To identify new markers, we first performed extensive analysis of surface markers on several B-ALL cell lines. Our data demonstrated that every B-ALL cell line tested did not express CD34 but certain lines contained cell populations with marked heterogeneity in marker expression. Moreover, the $CD9^+$ cell population possessed stem cell characteristics within the clone, as demonstrated by *in vitro* and transplantation experiments. These results suggest that CD9 is a useful positive-selection marker for the identification of CSCs in B-ALL.


## Introduction

According to the cancer stem cell (CSC) theory, CSCs are defined as a small subpopulation having self-renewal and differentiation abilities, and are maintained by asymmetric cell division [1]. Engraftment of CSCs can reproduce disease in immunodeficient mice, with important implications for cancer pathogenesis and therapeutic strategies [2,3]. CSCs were first described in acute myeloid leukemia (AML) as a small subpopulation of $CD34^+/CD38^-$ phenotype [4].

CSCs are believed to arise from normal tissue stem cells in many cases, with those of AML thought to derive from a hematopoietic stem cell (HSC) [4,5]. Since tissue stem cells are highly resistant to chemotherapy and since CSCs exhibit common biological properties with normal stem cells, it is believed that CSCs are at least partially responsible for tumor resistance to therapy, leading to recurrence and metastasis.

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of diseases and is considered to represent the clonal proliferation of malignant-transformed lymphoid progenitors. In addition, different subtypes of ALL originate from distinct stages of transformed cells during the hierarchal development of hematopoietic cells [6]. Recent studies using primary samples have shown that ALL cells capable of long-term proliferation *in vivo* were the $CD34^+/CD10^-/CD19^-$ fraction in B-ALL [7] and the $CD34^+/CD4^-/CD7^-$ fraction in T-ALL [8]. Other reports demonstrated that $CD34^+/CD19^-$ cells were candidates for CSCs in t(9;22)-positive ALL and t(4;11)-positive ALL [9]. It is important to note that the markers used to isolate the CSCs in these studies were mostly negative-selection markers except for CD34, a key marker of stemness.

On the other hand, a promising therapeutic strategy for CSCs involves the use of monoclonal antibodies as immunotherapy. If attempts at identifying positive markers expressed only on CSCs but not normal stem cells are successful, antibodies targeting these specific antigens may potentially be future therapeutic options.

While specific positive-selection markers such as CD123 [5] and CD96 [10] have been identified for AML, successful identification of positive-selection markers in lymphoid malignancies has been relatively sparse. We previously used cell lines to perform detailed fluorescence-activated cell sorter (FACS) analysis of CD markers in adult T-cell leukemia/lymphoma (ATLL) [11]. We now employ a similar strategy to search for novel positive-selection markers in B-cell leukemia/lymphoma cell lines.

Among the cell lines examined, we found that three B-lineage precursor leukemia cell lines consisted of distinct cell populations with heterogeneity in cell surface marker expressions, suggesting the existence of a stem cell-like hierarchy system. Moreover, the expression

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of CD9 correlated with stem cell characteristics, with $CD9^+$ cells exhibiting greater tumorigenic potential in immunodeficient mice than $CD9^-$ cells. These results suggest that CD9-expression has significant implications for the biological characteristics of B-ALL and may also serve as a novel therapeutic target in this disease.

## Materials and methods

*Cell lines and culture.* The human pre-B-ALL cell lines, YAMN90 and KOCL45 were established at Yamanashi University. Human pre-B-ALL cell line Reh was obtained from American Type Culture Collection (Rockville, MD, USA). B-lymphoblastoid cell line ARH77, Burkitt's lymphoma cell lines RAJI, RAMOS, and JIYOYE were obtained from European Collection of Cell Culture (Wiltshire, UK). Cells were cultured in RPMI1640 containing 10% fetal bovine serum (FBS) and penicillin/streptomycin.

*Antibodies.* Monoclonal antibodies that were unlabeled or conjugated with phycoerythrin (PE), fluorescein isothiocyanate (FITC), or allophycocyanin (APC) were obtained as follows. Those recognizing human CD2, CD3, CD5, and CD56: Beckman Coulter (Krefeld, Germany); CD16: Abcam (Cambridge, UK); CD39: Ancell Corp (Bayport, MN, USA); CD48, CD49d, CD49e, and CD126, TCRV beta2: Immunotech (Marseille, Cedex, France); CD52 and CD102: Serotech (Birmingham, AL, USA); CD84: Neo Markers (Fremont, CA, USA); CD133: Miltenyi Biotech (Bergisch Gladbach, Germany); CD155: Lab Vision (Fremont, CA, USA); CD229; Serotec; CD277: eBioscience (San Diego, CA, USA). Anti-CD29, and CD82 were produced in our lab. CD1a, CD1b, CD1d, CD4, CD6, CD7, CD8, CD9, CD10, CD11a, CD14, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD30, CD31, CD32, CD33, CD34, CD35, CD37, CD38, CD40, CD40L, CD43, CD44, CD45, CD45RA, CD45RO, CD47, CD49b, CD49c, CD49f, CD54, CDw60, CD64, CD69, CD70, CD71, CD72, CD74, CD75, CD79a, CD79b, CD80, CD83, CD85, CD86, CD90, CD95, CD98, CD99, CD106, CD110, CD117, CD119, CD121a, CD123, CD124, CD127, CD135, CD138, CD150, CD165, CD166, CD179a, CD180, CD195, CD200, CD210, CD220, CD243, CD318, CD338 and HLA-DR were obtained from BD Pharmingen (San Jose, CA, USA).

*Flow cytometry analysis, cell separation and sorting.* For phenotypic analysis of each cell line, cells were washed and suspended in HBSS medium containing 2% FBS and10 mM Hepes buffer. Cells were stained with the above monoclonal antibodies for 1 h at 4 °C. When unlabeled antibodies were used, FITC-conjugated goat anti-mouse IgG antibody was used as a secondary antibody. After cells were resuspended in 2 μg/ml propidium iodide to identify non-viable cells, cells were analyzed and/or sorted on a FACS Aria fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA, USA). Data analysis was done with Flow Jo software (Tree Star, Ashland, OR, USA). For sorting and culture assays, cells were sorted into $CD9^+$/high or $CD9^-$/low fractions, then cultured for additional weeks.

*Transplantation of leukemia cells into immunodeficient NOG mice.* NOG mice [12] were supplied by the Central Institute for Experimental Animals (Kawasaki, Japan). All *in vivo* studies were approved by the Institute and Animal Care and Use Committee of the University of Tokyo. ARH77, YAMN90, and Reh cells were stained with PE-conjugated CD9 antibodies and separated by FACS. Purity of the sorted cells was confirmed by reanalysis of FACS and all fractions were reproducibly shown to have greater than 99% purity. The sorted $CD9^+$ and $CD9^-$ cells were resuspended in 0.2 ml of PBS and injected into the lateral tail vein of 6 week-old female NOG mice. Mice were routinely evaluated for their general condition, leukemia progression and survival times to determine the leukemic potential of the transplanted cells. When the injected mice showed severe wasting or when the observations were completed, the mice were euthanized and sacrificed. Splenectomy and bone marrow biopsy were performed on the sacrificed mice to collect leukemic cells, with the cells being cultured for subsequent reanalysis, resorting, and serial transplantations. Cumulative proportion survival was assessed by Kaplan–Meier. For serial transplantation assays, leukemic cells were obtained and cultured for three additional days, then sorted by FACS and transplanted into NOG mice as described above.

*Morphological analysis.* The bone marrow, spleen, liver, kidney and lung tissues of recipients were fixed with 4% paraformaldehyde for over night, dehydrated with 70% ethanol, embedded in paraffin and 5 mm sections were prepared. Hematoxylin-eosin staining was performed on each tissue section derived from the recipient mice. For immunostaining, mouse anti-human CD79a primary antibody was used. Positive immunostaining was detected with a streptavidin–biotin immunoperoxidase system (Vector Laboratories) according to manufacture's protocols. Each section was examined using light microscopy to identify infiltrating B-ALL cells. The fresh organs were also minced to obtain floating leukemia cells and evaluated tissue infiltration by FACS.

## Results

### *Extensive cell surface antigen marker analysis of B-hematologic malignancy cell lines*

To efficiently isolate CSCs in B-hematologic malignancies, identification of novel specific positive-selection markers other than CD34 is essential. We first analyzed a total of seven B-hematologic malignancy cell lines (YAMN90, ARH77, Reh, KOCL45, RAJI, RAMOS, and JIYOYE) using 101 antibodies against hematopoietic stem cell (HSC) and lymphocyte-lineage differentiation cell surface antigen markers by FACS (see Materials and methods). Overall, most of the cell lines express cell surface antigen markers in patterns similar to those seen in immature B-lineage cells, with their expression profiles being quite homogeneous (data not shown). However, heterogeneous expressions of several markers were observed within the clone in three B-ALL cell lines; ARH77 (normal karyotype), YAMN90 (t(1;19) positive), and Reh (TEL/AML1, (t(12;21)) positive). The observed markers were CD9/38/90/123 in ARH77, CD9/CD52 in YAMN90, and CD9 in Reh (Fig. 1A). Summary of hematopoietic stem cell/lymphoid stem cell (common lymphoid progenitor cell) and B-precursor cell markers are listed in Fig. 1B. Intriguingly, while all the cell lines were positive for CD19 but negative for CD34, CD9 was commonly expressed at high level of heterogeneity in these three cell lines. These results suggest that these B-ALL cell lines are composed of heterogeneous cell populations, and a hierarchy-like system similar to HSCs exists even in the established cell lines. We thus performed additional analysis characterizing stem cell properties of these three cell lines.

### *In vitro assays of stem cell properties of B-ALL cell lines*

Stem cell potentials are defined by the self-renewal capacity and differentiation ability which are maintained by asymmetric cell division. Cells from the B-ALL cell lines ARH77, YAMN90, and Reh were stained for the heterogeneously expressed markers described above, and each population was separated according to the intensity of expression (~10% of high/+ and low/− populations) by FACS. Each cell population was then cultured for several additional weeks, and the expression patterns of the cell surface markers were subsequently reanalyzed. Among the markers examined, we found that asymmetric cell division-like proliferation in each cell line was observed only in the populations with high expressions of CD9 (Fig. 1C). The populations with high expression of

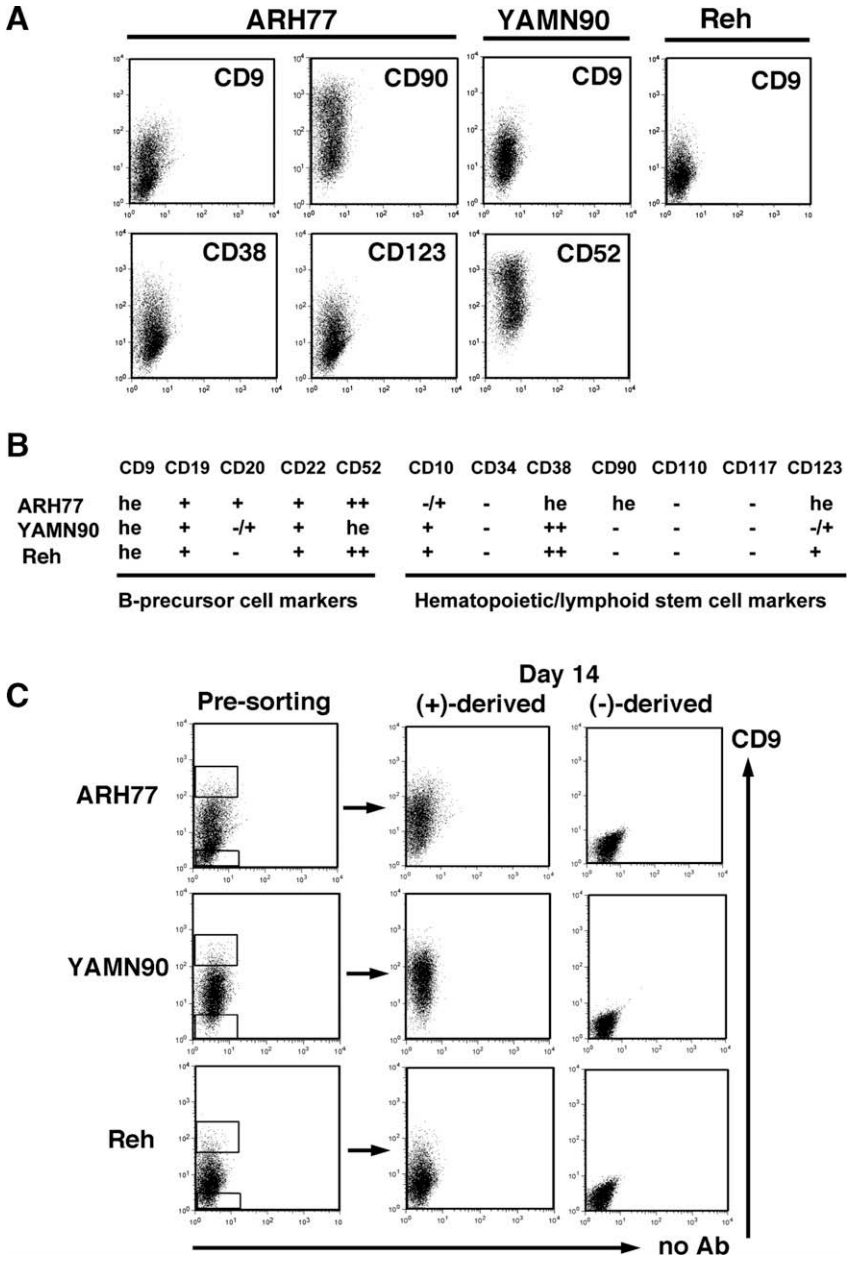


**Fig. 1.** Cell surface antigen marker analysis in three B-ALL cell lines. (A) CD9/38/90/123 in ARH77, CD9/CD52 in YAMN90, and CD9 in Reh are heterogeneously expressed. (B) Summary of the expression of hematopoietic/lymphoid stem cell and B-precursor cell markers in the B-ALL cell lines. Note that CD9 is commonly expressed in a heterogeneous pattern. he, heterogeneous expression; −, negative expression; −/+, partial positive expression; +, medium expression; ++, high expression. (C) CD9 based-isolation and culture assay of B-ALL cell lines. Cells were sorted according to the marker expression (upper squares, $CD9^+$/high population; lower squares, $CD9^-$/low population) by FACS and cultured for several additional weeks. The expression patterns of CD9 were reanalyzed again at day 14. $CD9^-$ cells generated only $CD9^-$ cells, whereas long-term culture of $CD9^+$ cells led to the repopulation of $CD9^+$ and $CD9^-$ cells.

CD9 ($CD9^+$) generated both $CD9^+$ and $CD9^-$ low expression ($CD9^-$) cells to repopulate the original pattern, whereas $CD9^-$ cells repopulated mostly only $CD9^-$ cells. These findings suggest that these B-ALL cell lines consist of heterogeneous populations, and only the $CD9^+$ population has the ability to generate heterogeneous cells by asymmetric cell division-like proliferation. It is therefore our conclusion that the stem cell-like populations are significantly enriched in the $CD9^+$ fraction.

*In vivo assays for stem cell properties in B-ALL cell lines*

Our *in vitro* data suggested that CD9 may serve as a candidate to be a novel positive-selection marker for CSCs of B-ALL. To confirm this possibility, we examined the *in vivo* tumorigenic potential of $CD9^+$ cells by transplantation assays into immunodeficient NOG mice. To evaluate the development of leukemia and its effect on survival, we have performed preliminary experiments and shown that transplantation of unsorted cells from YAMN90 ($1 \times 10^5$ cells), ARH77 ($1 \times 10^6$ cells), and Reh ($1 \times 10^5$ cells) into NOG mice caused leukemic death within 30–50 days. $CD9^+$ and $CD9^-$ cells ($1 \times 10^6$ cells; ARH77, $1 \times 10^5$ cells; YAMN90 and Reh) were then injected intravenously into the NOG mice, and the animals were then observed until either leukemic death or 100 days after transplantation.

The Kaplan–Meier survival curves demonstrated a clear difference between the two groups (Fig. 2A). Mice injected with $CD9^+$ cells

died from leukemia within 30–45 days of cell-injections, while most of the mice injected with CD9$^-$ cells survived more than 3 months. Pathological analysis revealed that the mice with CD9$^+$ cells had marked splenomegaly (YAMN90 and Reh) and bone marrow involvement (all lines), but the mice with CD9$^-$ cells generally had no splenomegaly or bone marrow involvement, except for two cases of ARH77. Even a reduced dose of $2 \times 10^4$ YAMN90-derived CD9$^+$ cells exhibited greater tumorigenic potential than CD9$^-$ cells, leading eventually to death of all mice (Table 1).

Next, serial transplantation experiments of CD9$^+$ cells were performed for further confirmation of their *in vivo* stem cell properties (Fig. 2B). Following the first transplantation, enlarged spleens (YAMN90 and Reh) or bone marrows (ARH77) were removed from the euthanized mice and minced to obtain tumor cells, which were then cultured for three additional days to reduce the contamination of the recipient mouse cells and sorted again for a second transplantation. FACS analysis of the leukemic cells revealed that CD9$^+$ cells generated both CD9$^+$ and CD9$^-$ cells in each cell line (Fig. 2B, left panels). Following the same procedure, the leukemic cells from the second recipient mice were cultured and analyzed (middle panels). Then the CD9$^+$ cells were subsequently transplanted into the third recipient mice, resulting in the reconstitution of the same expression pattern of CD9 in each cell line (right panels). The results of our serial transplantation experiments are summarized in Table 1. Thus, the serial transplantation capacity and higher tumorigenic potential, essential characteristics for CSCs, were demonstrated in the CD9$^+$ population of each cell line.

*Analysis of tissue infiltration by CD9$^-$ sorted cells*

During the transplantation assay, we evaluated peripheral blood, bone marrow, and spleen engraftment kinetics in each recipient until the recipients were sacrificed (0, 1, 2, 3 and 4th weeks) (Table 2). CD9$^+$ or CD9$^-$ cells of Reh and YAMN90 were injected into the NOG mice and human cells were detected with anti-human CD19 and CD45 antibodies. In the experiment of Reh, no or only trace number of human cells was detected in the bone marrow and spleen within the 1st week of CD9$^+$ cell transplantation. Human cell presence in the bone marrow and spleen were then detected from the 2nd week onward, with the relative proportion increasing toward the later time points. In YAMN90 experiment, significant number of human cells appeared suddenly from the 4th week. However, only trace human cells were detected in the peripheral blood of both Reh and YAMN90 experiments. In the case of CD9$^-$, no clear involvement of human cells was detected in each experiment.

Proportions of infiltrated cells were also examined in the liver, kidney and lung. These tissues were dissected by scissors and filtrated, then floating cells were analyzed by FACS in a similar manner as previously described. As summarized in Table 2, infiltration of leukemia cells in solid organs and hematopoietic organs was clearly correlated with each other.

Simultaneously, we also performed histological analysis of the recipient organs to confirm the infiltration of human leukemia cells. In the bone marrow, spleen, liver, kidney and lung, infiltra-

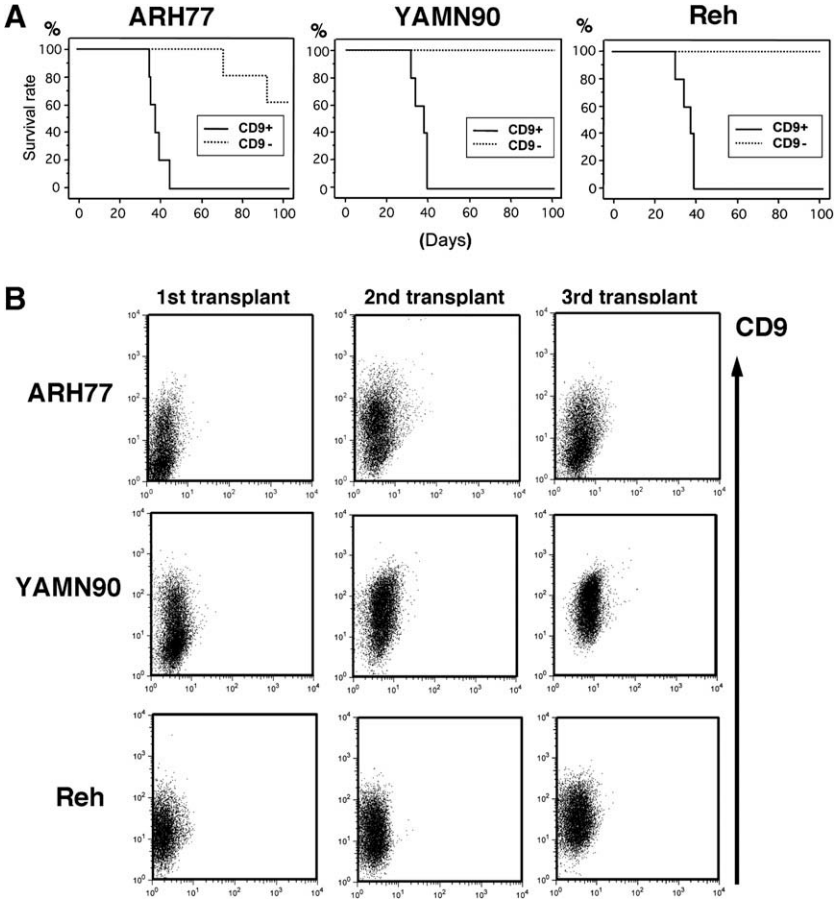


**Fig. 2.** Transplantation of CD9$^+$ and CD9$^-$ cells of the cell lines. (A) Survival curves relating to transplantation of CD9-isolated cells. The cells (CD9$^+$ and CD9$^-$ cells) were transplanted intravenously into five mice in each experiment. All of the mice injected with CD9$^+$ cells died within 45 days but most the mice with CD9$^-$ cells survived for more than 3 months in each experiment. (B) Serial transplantation experiments of CD9$^+$ cells. After the first transplantation, tumor cells were obtained from the leukemic mice injected with CD9$^+$ cells. The cells were then analyzed by FACS (first transplant, left panels). CD9$^+$ cells were sorted again for a second transplantation (middle panels) and a third transplantation (right panels).

**Table 1**
Summary of the serial transplantation experiments.

| Cell line | Cell type | | Cell number (cells/mouse) | Number of injection | Survival (days) | |
|---|---|---|---|---|---|---|
| | | | | | Mean | Range |
| ARH77 | $CD9^+$ | 1st transplant | $1 \times 10^6$ | 5 | 38 | 35–45 |
| | | 2nd transplant | $1 \times 10^6$ | 5 | 34 | 29–41 |
| | | 3rd transplant | $1 \times 10^6$ | 5 | 35 | 31–40 |
| YAMN90 | $CD9^+$ | 1st transplant | $1 \times 10^5$ | 5 | 35 | 31–37 |
| | | 2nd transplant | $1 \times 10^5$ | 5 | 31 | 26–33 |
| | | 3rd transplant | $1 \times 10^5$ | 5 | 31 | 26–33 |
| | | 1st transplant | $2 \times 10^4$ | 5 | 54 | 44–64 |
| Reh | $CD9^+$ | 1st transplant | $1 \times 10^5$ | 5 | 37 | 30–39 |
| | | 2nd transplant | $1 \times 10^5$ | 5 | 30 | 26–35 |
| | | 3rd transplant | $1 \times 10^5$ | 5 | 32 | 25–34 |

tion of monomorphic mononuclear cells was detected with hematoxylin-eosin staining in the recipients transplanted with $CD9^+$ cells (4th week samples) of Reh (Fig. 3) and YAMN90 (data not shown), whereas no infiltration was observed in the recipient mice with $CD9^-$ cells of both cell lines at the same time point. These cells also stained with anti-human CD79a (anti-pan B), suggesting that they are human leukemia cells. No infiltrating $CD79a^+$ cells were detected in the mice transplanted with $CD9^-$ cells. These results suggest that systemic infiltration of leukemia cells in not only hematopoietic organs but also other tissues caused leukemic death in the recipient mice.

## Discussion

CD9 is a 24–27 kDa cell surface glycoprotein which belongs to a tetraspanin superfamily, characterized as having four transmembrane domains, and is expressed in a variety of blood cells including pre-B lymphocytes but not in HSCs. It is also expressed in many types of solid tumors, and is involved in a various kinds of cell processes, such as cell adhesion, motility, and signaling events through an association with integrin family proteins [13].

For example, it has been reported that down-regulation of CD9 was associated with cancer progression, metastasis, and clinical outcome [14,15]. Others have demonstrated that up-regulation of CD9 and its interaction with endothelial cells were involved in trans-endothelial invasion and spreading of cancer cells [16].

On the other hand, recent trends in leukemia therapy rely on defining the molecular pathways underlying the pathogenesis of specific diseases. Several monoclonal antibodies have recently been incorporated into various anti-leukemic regimens due to their activities against certain leukemia-associated antigens. Especially, the addition of rituximab (anti-CD20) [17] to chemotherapy regimens significantly improves the clinical outcome of patients with B-cell hematologic malignancies. Therefore, therapeutic strategies involving the development of novel antibodies against CD9-expressing cells may lead to improved clinical outcome, since the search for novel antigens selectively expressed on CSCs can lead to the future development of therapeutic antibodies.

The correlation between CD9-expression and clinical prognosis in B-ALL should also be evaluated in the future. Several studies demonstrated that minimal residual disease detected by flow cytometry is an independent adverse prognostic factor in pediatric ALL [18]. Therefore, flow cytometric analysis of CD9-expression may be helpful in monitoring disease progression. The presence of minimal residual disease is associated with a high risk of relapse and may be an independent risk factor for disease relapse. The future use of antibodies against CD9 may maximize the ability to detect microscopic disease and help in the clinical management of leukemias.

In our study, expression of CD9 is well-correlated with CSC potentials in B-ALL cell lines and can serve as a useful positive-selection marker. Therefore, future combination of CD9 with other novel markers should make it possible to identify CSCs in B-ALL

**Table 2**
Infiltration of human-derived cells in the organs of NOG mice.

| | % Of human $CD19^+/CD45^+$ cells | | | | | |
|---|---|---|---|---|---|---|
| | PB | BM | Spleen | Liver | Kidney | Lung |
| *Reh* | | | | | | |
| $CD9^+$ | | | | | | |
| 1 week | 0 | 0 | 0 | – | – | – |
| 2 week | 0 | 26 | 3.9 | 0.5 | 0.1 | 0.1 |
| 3 week | 0 | 99 | 96 | 84 | 62 | 26 |
| 4 week | 0.1 | 98 | 76 | 31 | 8 | 17 |
| $CD9^-$ | | | | | | |
| 3 week | 0 | 0.2 | 0.1 | 0 | 0 | 0 |
| 4 week | 0 | 0 | 0 | 0 | 0 | 0 |
| *YAMN90* | | | | | | |
| $CD9^+$ | | | | | | |
| 1 week | 0 | 0 | 0 | – | – | – |
| 2 week | 0 | 0.3 | 0.7 | 0.1 | 0.3 | 0.2 |
| 3 week | 0.1 | 0.3 | 0.1 | 0 | 0 | 0.1 |
| 4 week | 0.1 | 35 | 39 | 81 | 54 | 11 |
| $CD9^-$ | | | | | | |
| 3 week | 0 | 0.2 | 0.1 | 0 | 0 | 0.2 |
| 4 week | 0 | 0 | 0 | 0 | 0 | 0 |

*Note:* B, peripheral blood; BM, bone marrow.

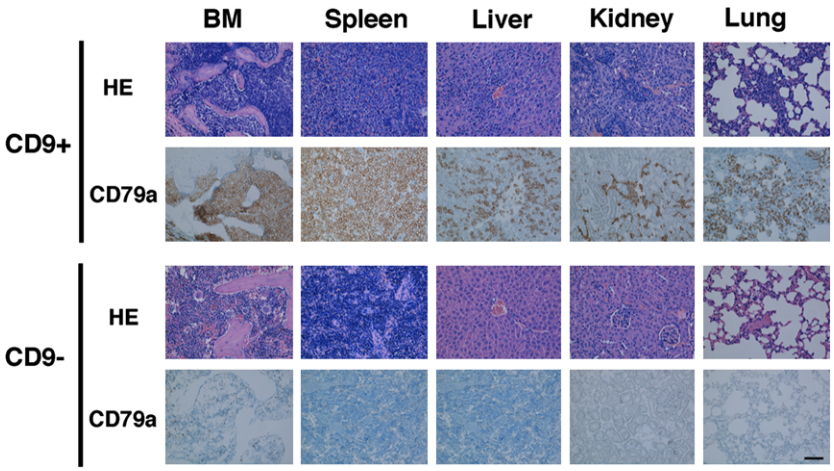


**Fig. 3.** Histological analysis of the mice injected with Reh cells. $CD9^+$ or $CD9^-$ cells were injected into the NOG mice and human cells were stained with Hematoxylin-eosin (HE) and anti-human CD79a antibody. In the 4th week samples of $CD9^+$ cell-injection, severe infiltration of leukemic cells was observed in each organ, but no significant change was observed in the $CD9^-$ samples similar to the pre-injection samples. BM, bone marrow. Scale bar, 50 μm.

more efficiently, while targeted therapy against CD9-expressing cells can be a novel therapeutic strategy to eradicate CSCs.

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