shown in Figure 5C, CD109 mRNA was weakly expressed in fetal skin, spleen, liver, lung, kidney, heart, pancreas and colon tissues. In adult tissues, CD109 mRNA was also weakly expressed in the lung, heart, small intestine, and testis. However, CD109 protein was expressed in alveoli of the lung, but not in heart and colon tissues (Figure 5D). These results suggested that CD109 could be an antigen highly expressed in various human sarcomas.

### CD109 is associated with cancer-initiating ability and TGFβ/Smad signaling

The proportion of CD109-positive cells was 0.2% in ESX (Figure 6A), which was lower than that of $ALDH^{high}$ cells (Figure 1A). However, higher expression of ALDH1 mRNA was detected in CD109-positive cells than in CD109-negative cells (Figure 6B). Moreover, the number of spherical colonies derived from CD109-positive cells was higher than that from CD109-negative cells (Figure 6, C and D). In addition, the expression of stemness-related genes was lower in CD109-positive cells than in CD109-negative cells (Figure 6E). These features of CD109-positive cells were similar to those of $ALDH^{high}$ cells and suggested that CD109 might regulate cancer-initiating ability in ESX.

Next, we examined the effect of CD109 knockdown on cell proliferation. ESX cells were treated with siCD109, trypsinized and counted after 48 hr, 72 hr and 120 hr. Expression of both mRNA and protein of CD109 was downregulated (Figure S4, A, B and C). As shown in Figure 7A, siCD109 significantly inhibited cell proliferation ($p<0.05$). These results also supported the idea that CD109 plays an important role in cancer-initiating ability.

CD109 is a component of the TGF-β1 receptor 1 complex and negatively regulates TGF-β/Smad signaling [22]. Therefore, we hypothesized that CD109 could positively regulate ALDH1 activity and negatively regulate TGFβ1R1 expression in the TGF-β/Smad signaling pathway. We examined whether downregulation of ALDH1A1 and upregulation of TGFβ1R1 were induced by silencing of CD109. As shown in Figure 7B, ALDH1A1 mRNA was downregulated in siCD109-transfected ESX cells. On the other hand, TGFβ1R1 mRNA was upregulated in the siCD109-transfected cells (Figure 7C). Moreover, silencing of CD109 reduced the $ALDH^{high}$ population in ESX cells (Figure 7, D and E). These findings suggested that CD109 positively regulated ALDH1A1 activity and negatively regulated the TGF-β/Smad signaling pathway.

### CD109 protein is associated with poor prognosis in soft tissue sarcoma patients

To determine the clinical relevance of CD109 expression in soft tissue sarcomas (STS), we evaluated CD109 expression by immunohistochemistry in the primary extremity lesions of 80 STSs. The clinical characteristics of these patients are summarized in Table S3. Representative staining patterns with the anti-CD109 antibody are shown in Figure 8A. Positive expression of CD109 protein was identified in 18% (15/80) of the STSs. Higher expression of CD109 was significantly associated with histologic grade, tumor stage, and distant metastases (p=0.021, 0.0012, and 0.0003 respectively). However, no other significant correlation was found between CD109 expression and other clinical parameters of the STSs. As shown in Figure 8B and 8C, positive CD109 expression, including in well-differentiated liposarcomas, was significantly associated with decreased probabilities of overall survival (OS) and disease-free survival (DFS) ($P=8.3\times10^{-5}$ and $4.5\times10^{-4}$, respectively). Moreover, excluding well-differentiated liposarcomas, positive CD109 expression was also significantly associated with decreased probabilities of OS and DFS (P=0.006 and 0.049, respectively). The OS rates at 5 years were 46.7% for CD109-positive patients and 85.3% in those who were CD109 negative. Several variables were tested to assess whether they had an impact on survival. Univariate and multivariate analyses revealed that the histologic grade and expression of CD109 were independent risk factors for poor outcome (Table 1, Table 2). The hazard ratio (HR) of OS for the CD109-positive group was 3.85 (95% confidence interval CI, 1.40-10.55). Thus, CD109 might be a predictive biomarker for both distant metastasis and the prognosis of STSs in the clinical setting.

## Discussion

In the present study, we (i) established and characterized the new ES cell line ESX; (ii) demonstrated that the $ALDH^{high}$ population of ESX contained CSCs/CICs showing *in vitro and in vivo* tumorigenesis; (iii) found that high expression of CD109 in $ALDH^{high}$ cells was important to maintain tumorigenesis as the important feature of CSCs/CICs; and (iv) showed the prognostic impact of CD109 expression on patients with STSs. ES is a rare, slow-growing malignant tumor. With an infiltrative growth pattern and a propensity for extension along fascial planes, and nerves, it is characteristically associated with multiple local recurrences and late metastasis [23]. The effects of multiagent chemotherapy and radiotherapy remain unclear; therefore, novel therapeutic options need to be developed. The establishment of an experimental model is imperative to investigate the biological characteristics of ES and develop novel therapeutic options. To the best of our knowledge, only 11 human ES cell lines have been reported to date [10,24-33]. ESX might therefore facilitate further studies on the biological characteristics of this rare tumor entity.

Using ESX, we tried to identify CSCs/CICs of ES. Previously, we performed side population (SP) analysis to identify CSCs/CICs of sarcomas [16]. However, SP cells were hardly detected in ESX (data not shown). Therefore, we used the ALDEFLUOR assay. In this study, we demonstrated that an $ALDH^{high}$ population existed in ESX, and that the $ALDH^{high}$ cells possessed repopulating capacity and high tumor-forming ability *in vitro and in vivo*. ALDH is a cytosolic isoenzyme involved in the detoxification of intracellular aldehydes by oxidation and conversion of retinol to retinoic acid, and it confers resistance to chemotherapeutic agents such as cyclophosphamide [34]. Therefore, it makes sense that $ALDH^{high}$ cells contain CSCs/CICs. Visus et al. were the first to describe the use of ALDH1A1 as a putative CSC/CIC marker in head and neck squamous cell carcinomas [35]. In the field of sarcoma, Awad

Figure 6

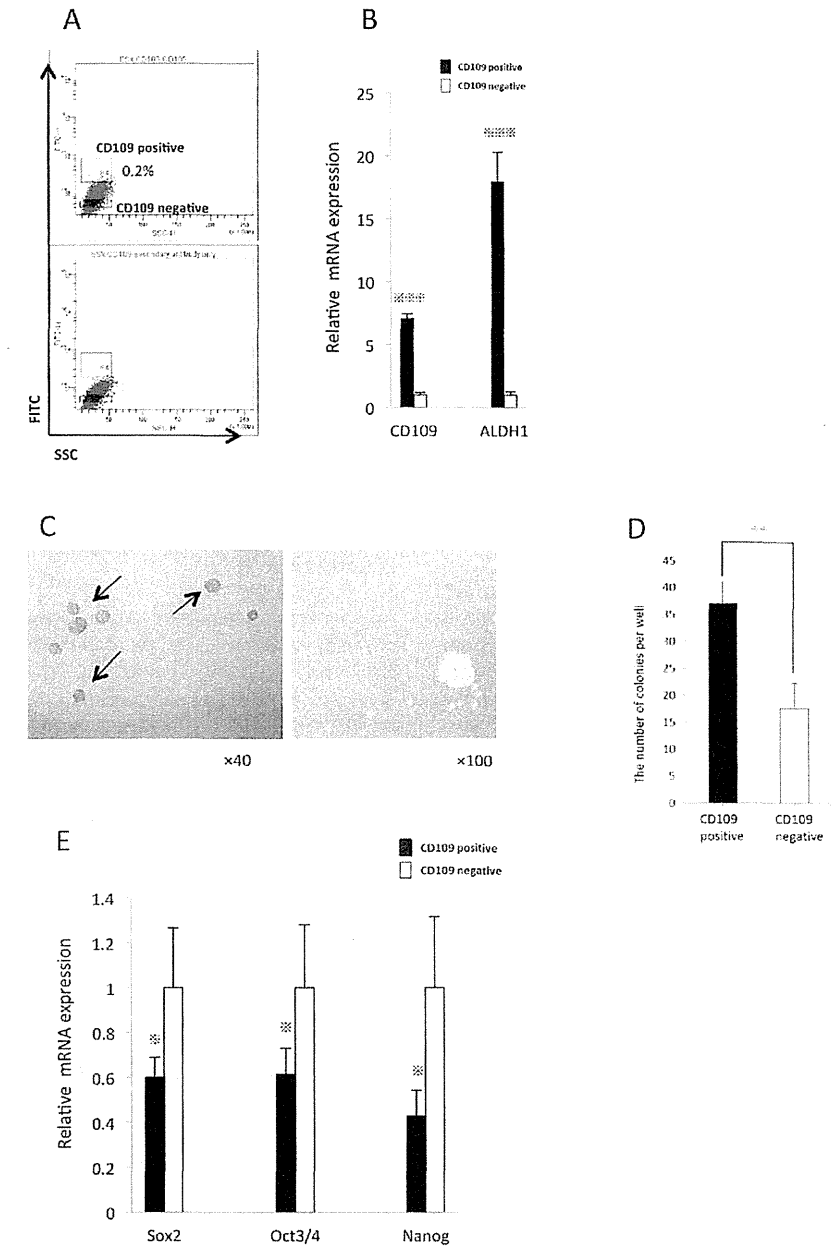



**Figure 6. CD109 is a representative marker for the features of cancer-initiating ability.** A. FACS analysis of CD109 positive cells in ESX was shown.

B. The mRNA expression of CD109 and ALDH1A1. Bars represent mean±SEM. ***$p$<0.001, determined by the Mann-Whitney test.

C. The features of spherical colonies (indicated by arrows) derived from resultant CD109-positive cells and CD109-negative cells of ESX (original magnification ×40).

D. The number of spherical colonies from CD109-positive cells and CD109-negative cells of ESX. Bars represent mean±SEM (n=3). **$p$<0.01, determined by the Mann-Whitney test.

E. The mRNA expression of stem/progenitor cell-related genes. Bars represent mean±SEM. *$p$<0.05, determined by the Mann-Whitney test.

doi: 10.1371/journal.pone.0084187.g006

# Figure 7

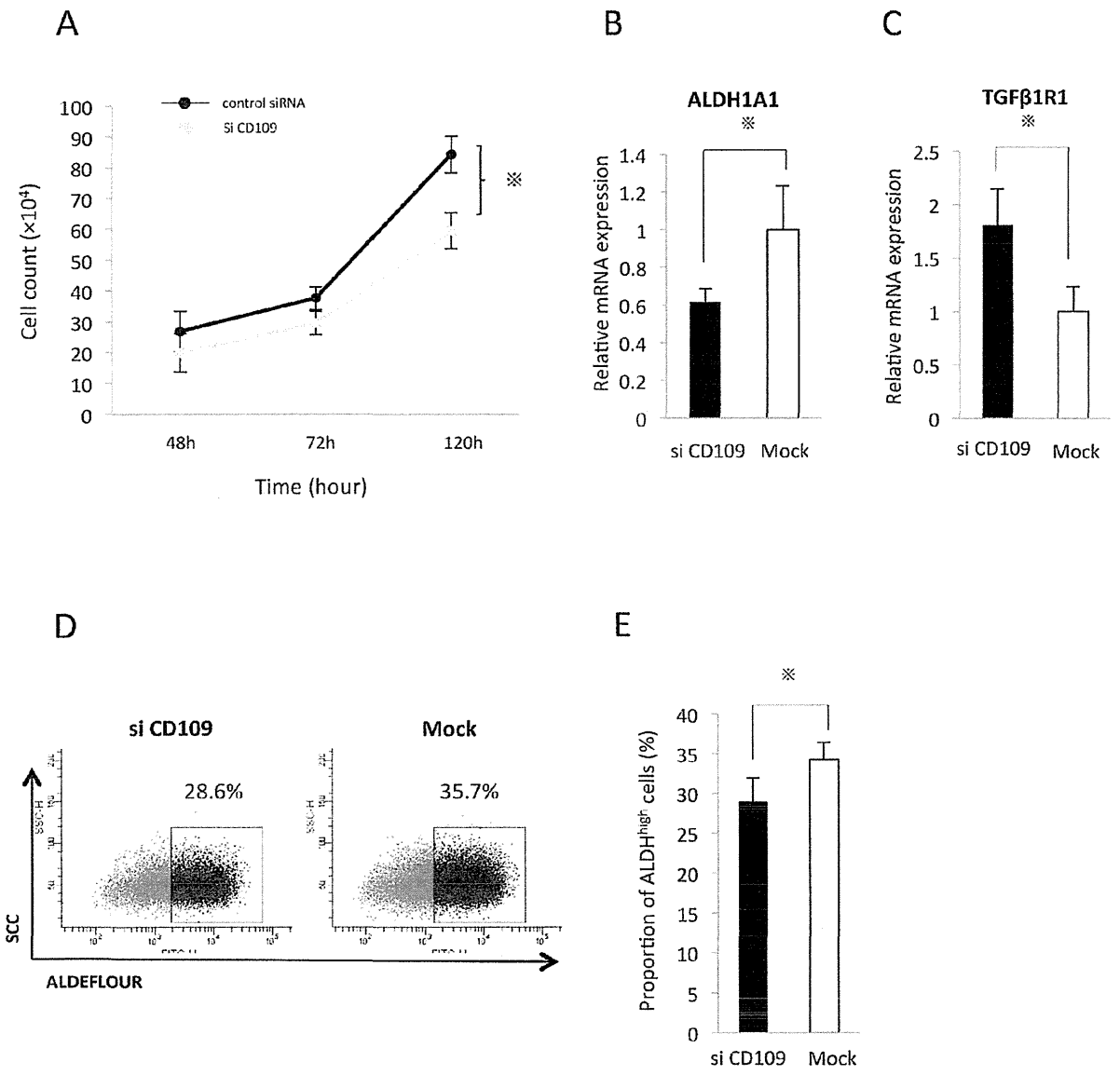



**Figure 7. CD109 positively regulates ALDH activity and negatively regulates the TGFβ/Smad signaling pathway.** A. The cell proliferation curve of ESX treated with SiCD109 or control siRNA. Bars represent mean±SEM (n=4). *$p$<0.05, determined by the Mann-Whitney test.

B, C. mRNA expression of ALDH1A1 (D) and TGFβ1R1 (E). The expression of ALDH1A1 and TGFβ1R1 was evaluated by real-time PCR two days after transfection.

D. FACS analysis of ALDH activity.

E. The proportion of ALDH$^{high}$ cells. Bars represent mean±SEM (n=4). *$p$<0.05, determined by the Mann-Whitney test.

doi: 10.1371/journal.pone.0084187.g007

Figure 8

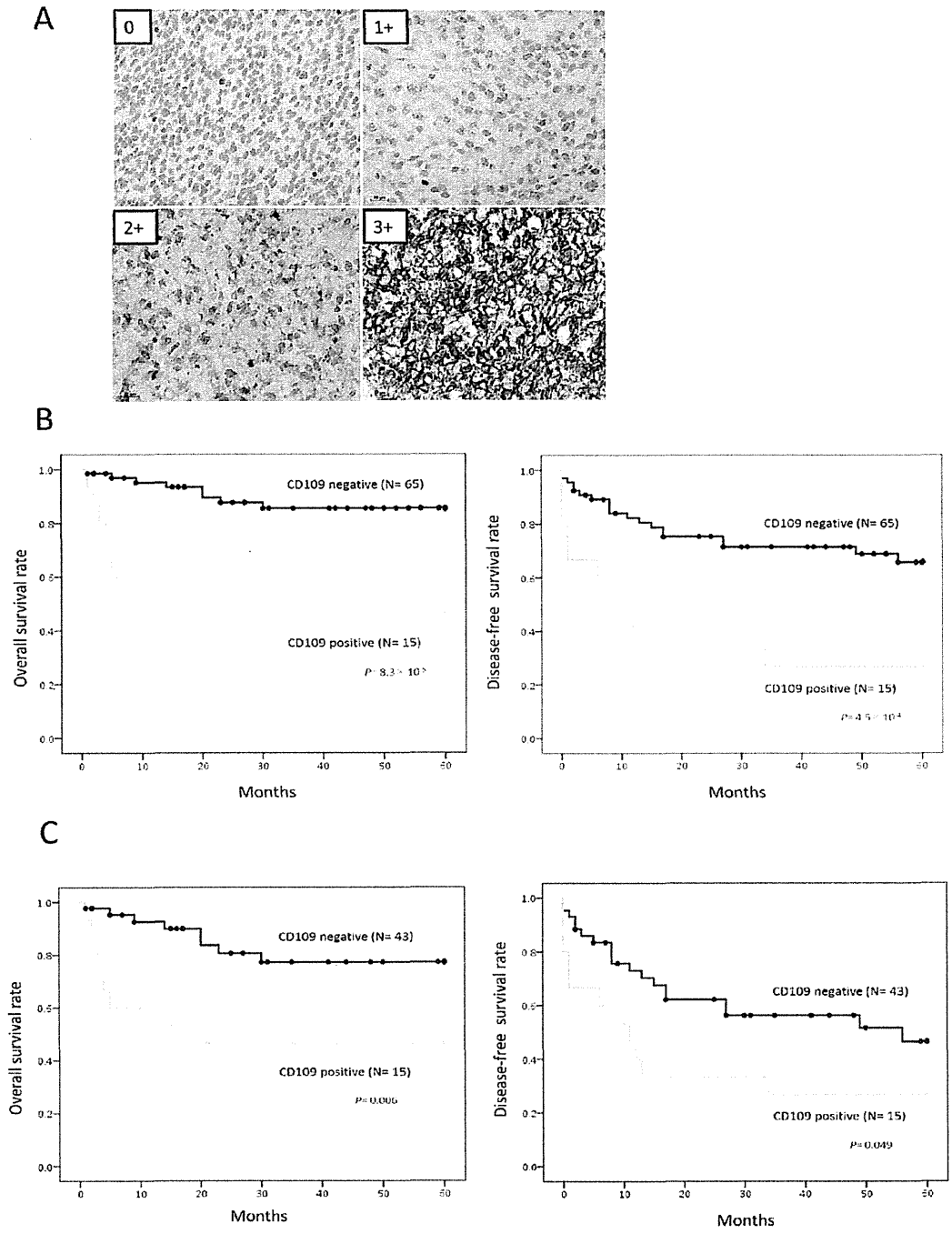



**Figure 8. Expression of CD109 protein is associated with poor prognosis in soft tissue sarcoma patients.** A. Representative immunostaining of CD109 (original magnification x400).
B, C. Prognosis estimated by Kaplan-Meier plots for patients with soft tissue sarcoma including (B) and excluding (C) the patients with well-differentiated liposarcoma.
doi: 10.1371/journal.pone.0084187.g008

**Table 1.** Univariate analysis of the variables in overall survival.

| Variable | Hazard ratio | 95% CI | *P* value |
|---|---|---|---|
| Tumor size | | | |
| <5cm | 1 | | |
| 5-10cm | 3.38 | 0.70-16.2 | 0.129 |
| >10cm | 2.21 | 0.46-10.6 | 0.321 |
| Tumor depth | | | |
| Superficial | 1 | | |
| Deep | 24.0 | 0.024-24154 | 0.367 |
| Histologic grade | | | |
| I | 1 | | |
| II | 3.18 | 0.28-35.1 | 0.344 |
| III | 20.4 | 2.66-157 | 0.004 |
| CD109 expression | | | |
| Negative | 1 | | |
| Positive | 5.72 | 2.13-15.3 | 0.001 |

CI: confidence interval

doi: 10.1371/journal.pone.0084187.t001

**Table 2.** Multivariate analysis of the variables in overall survival.

| Variable | Hazard ratio | 95% CI | *P* value |
|---|---|---|---|
| Histologic grade | | | |
| I | 1 | | |
| II | 2.03 | 0.177-23.39 | 0.570 |
| III | 13.9 | 1.75-111.7 | 0.013 |
| CD109 expression | | | |
| Negative | 1 | | |
| Positive | 3.85 | 1.40-10.55 | 0.009 |

CI: confidence interval

doi: 10.1371/journal.pone.0084187.t002

et al. tested ALDH activity in Ewing sarcoma, defining CSCs/CICs as those cells that showed the highest ALDH activity [36]. However, there are no other reports regarding CSCs/CICs of sarcoma. This is the first report identifying and characterizing of CSCs/CICs of ES. The proportion of $ALDH^{high}$ cells in ES cell lines was clearly higher than in the other sarcoma cell lines (Figure S2). These results were compatible with the chemotherapy-resistant characteristics of ES.

Expression of the stem cell-related genes *Sox2*, *Oct3/4*, and *Nanog* is also used to characterize CSCs/CICs [37]. These are essential for the maintenance of pluripotent embryonic stem cells and germ cells, as well as CSCs/CICs [38]. Twist and Snail, which could promote EMT, are also considered to be key factors in the maintenance of CSCs/CICs. Twist and Snail induce not only increased potential for invasiveness and metastases, but also increased ability to form spheres and generate tumors in xenografts [39]. In this study, the $ALDH^{high}$ population expressed *Twist 1 and Snail 1* at higher levels than $ALDH^{low}$ cells. $ALDH^{high}$ cells of ESX had higher invasive ability, which was considered to be compatible with previous reports [7,8]. On the other hand, expression of *Sox2, Oct3/4* and *Nanog* was lower in $ALDH^{high}$ cells. The reason for this discrepancy in the expression status between $ALDH^{high}$ and $ALDH^{low}$ cells in the case of ESX remains unknown. Velcheti et al. reported that high expression of Sox2 was correlated with good prognosis in patients with non-small cell lung carcinomas [40]. These results suggested that Sox2 was not necessarily related to tumorigenicity and malignant potential. Alternatively, we speculate that CSCs/CICs of ES might have more differentiated characteristics with downregulation of Sox2 than those of carcinomas.

Using a cDNA microarray, we found that CD109 was upregulated in $ALDH^{high}$ cells. CD109, a GPI-anchored glycoprotein, was originally identified as a leukemia antigen. It has been reported that CD109 is expressed on activated T lymphocytes and platelets, endothelial cells and a subpopulation of CD34+ hematopoietic stem and progenitor cells [20,21,41]. CD109 is also expressed in keratinocytes and contributes to the inhibition of extracellular matrix production in scleroderma [42]. In addition, it is also expressed in malignancies of the lung, esophagus, cervix, urinary tract and breast and plays a role in the tumor growth of oral cancer [43]. The present study also demonstrated that CD109 was highly expressed in sarcoma but not in normal tissues. The expression status suggested that CD109 might be a candidate therapeutic target not only for sarcoma but also for epithelial cancer.

CD109 is a TGF-β co-receptor, a component of the TGF-β1 receptor 1 (TGFβ1R1) complex. It accelerates TGF-β receptor degradation and negatively regulates TGF-β/Smad signaling [44,45]. In some human cancers, CD109 actually impairs TGF-β/Smad signaling [46]. TGF-β can play both tumor-suppressive and tumor-promoting roles. Especially in the early phase of cancer initiation, TGF-β acts as an anti-oncogenic factor [47]. In this study, $ALDH^{high}$ cells of ESX showed higher tumorigenicity and higher expression of CD109. In addition, silencing of CD109 upregulated TGFβ1R1 mRNA in ESX cells. These findings suggested that CD109 expressed in $ALDH^{high}$ cells of ESX promoted cancer-initiating ability as the result of the CD109-mediated inhibition of TGF-β.

CD109-positive cells of ESX highly expressed ALDH1 and showed higher tumorigenicity than CD109-negative cells. Furthermore, the expression status of stemness-related genes of CD109-positive cells was similar to that of $ALDH^{high}$ cells of ESX. Therefore, we considered that CD109 could be a representative molecule of $ALDH^{high}$ cells. In addition, knockdown of CD109 decreased ALDH1 activity in ESX cells. These results suggested that CD109 might regulate ALDH1 activity and confer the characteristics of CSCs/CICs in ESX cells. However, the proportion of CD109-positive cells was lower than that of $ALDH^{high}$ cells of ESX. Therefore we hypothesized that other factors might also regulate ALDH1 activity.

The evaluation of the clinical specimens of STSs revealed a strong correlation between CD109 expression and DFS and OS, suggesting that CD109 could be a promising prognostic biomarker in STSs. Although it is reported that CD109 is preferentially expressed in the early stage of tumorigenesis in

oral tumor and urothelial carcinomas [43,46], we demonstrated that high expression of CD109 was significantly associated with advanced stage in STSs. These results suggest that the pathophysiological function of CD109 protein in sarcomas is different from that in carcinomas. Therefore, we speculate that CD109 expression in STSs is deeply involved in invasion and metastasis.

In conclusion, we established the novel ES cell line ESX. Next, we investigated CICs/CSCs in ES cell lines and isolated CSCs/CICs based on ALDH activity. Finally, we demonstrated that CD109 is a potential CSC/CIC marker, prognostic factor and molecular target for STSs, including ES.

## Supporting Information

**Table S1. List of commercial sources of the antibodies used in the study.**
(DOC)

**Table S2. List of the 37 membrane protein-related related upregulated (rate ≥2.0) genes in ALDH$^{high}$ cells of ESX.**
(DOC)

**Table S3. Association between CD109 expression and clinical variables.**
(DOC)

**Figure S1. Clinical characteristics of the origin of the new epithelioid sarcoma cell line.** A. Magnetic resonance imaging reveals a subcutaneous tumor (3×3 cm) located in the left thigh (left panel, arrow) and lymph node metastases in the inguinal region (right panel, arrow).
B. H&E staining of the primary tumor from a resected specimen reveals typical features of epithelioid sarcoma, showing central necrosis with peripheral palisading of epithelioid cells around necrotic areas (scale bars, left 500μm, right 50μm).
C. Immunohistochemical analysis of the primary tumor for AE1/AE3, vimentin, CD34, CA125, S-100 and INI1 expression (scale bar, 50μm).
D. Fluorescence in situ hybridization (FISH) analysis using the INI1/CEP22 deletion probe performed according the protocol we previously described [48]. Heterozygous deletion demonstrated by the lack of one red signal (indicating the INI1 region) was detected (indicated by red arrow). Green signals indicate the centromeric region of chromosome 22.
(TIF)

**Figure S2. The proportions of ALDH$^{high}$ cells in the sarcoma cell lines.** FACS analysis of ALDH1 activities of the cell lines of osteosarcoma (U2OS and OS2000), synovial sarcoma (Fuji and HS-SYII), Ewing sarcoma (WES and RD-ES) and malignant fibrous histiocytoma (MFH2003 and MFH2004) with and without DEAB control.
(TIF)

**Figure S3. The mRNA expression of stem/progenitor cell-related genes in epithelioid sarcoma cell lines, VA-ES-BNJ and FU-EPS-1.** RNA was isolated from freshly sorted spheroid cells ($1x10^5$) on day 7. Bars represent mean±SEM. $^*p<0.05$, determined by the Mann-Whitney test.
(TIF)

**Figure S4. CD109 knockdown using siRNA.** A. Real-time PCR analysis of CD109 mRNA expression of ESX after transfection of siCD109 or mock siRNA on day 2. Bars represent mean±SEM. $^{***}p<0.001$ was determined by Student's t-test.
B. Western blot analysis of siCD109 cells. Cell lysate with Nonident P-40 detergent solution was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred onto polyvinylidene fluoride membranes and probed with a mouse anti-CD109 antibody (H-7; Santa Cruz Biotechnology, USA). β-Actin was used as a loading control. The anti-CD109 antibody was used at 100-fold dilution. The membrane was visualized with Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) according to the manufacturer's protocol and analyzed using Odyssey Fc Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Anti-β-actin was used as an internal control.
C. Real-time PCR analysis of CD109 mRNA expression of ESX after transfection of siCD109 on days 3, 5, 7 and 10.
(TIF)

## Author Contributions

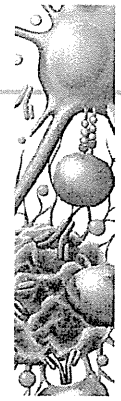
Conceived and designed the experiments: ME T. Tsukahara VK T. Torigoe TW TY NS. Performed the experiments: ME T. Tsukahara MM M. Kano KM AT TK HA KY. Analyzed the data: ME T. Tsukahara TS TH NS. Contributed reagents/materials/analysis tools: M. Kaya SN JN HI. Wrote the manuscript: ME T. Tsukahara NS.

## References


1. Weiss SW, Goldblum JR (2008) Enzinger and Weiss's soft tissue tumors. Mosby, Inc.
2. Jordan CT, Guzman ML, Noble M (2006) Cancer stem cells. N Engl J Med 355: 1253-1261. doi:10.1056/NEJMra061808. PubMed: 16990388.
3. Sládek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol 17: 7-23. doi:10.1002/jbt.10057. PubMed: 12616643.
4. Vasiliou V, Pappa A, Petersen DR (2000) Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. Chem Biol Interact 129: 1-19. doi:10.1016/S0009-2797(00)00211-8. PubMed: 11154732.
5. van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Lippitt JM et al. (2010) High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. Cancer Res 70: 5163-5173. doi:10.1158/0008-5472.CAN-09-3806. PubMed: 20516116.
6. Cheung AM, Wan TS, Leung JC, Chan LY, Huang H et al. (2007) Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. Leukemia 21: 1423-1430. doi:10.1038/sj.leu.2404721. PubMed: 17476279.
7. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J et al. (2009) Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung

cancer. Mol Cancer Res 7: 330-338. doi: 10.1158/1541-7786.MCR-08-0393. PubMed: 19276181.
8. Li T, Su Y, Mei Y, Leng Q, Leng B et al. (2010) ALDH1A1 is a marker for malignant prostate stem cells and predictor of prostate cancer patients' outcome. Lab Invest 90: 234-244. doi:10.1038/labinvest.2009.127. PubMed: 20010854.
9. Ishibe T, Nakayama T, Okamoto T, Aoyama T, Nishijo K et al. (2005) Disruption of fibroblast growth factor signal pathway inhibits the growth of synovial sarcomas: potential application of signal inhibitors to molecular target therapy. Clin Cancer Res 11: 2702-2712. doi: 10.1158/1078-0432.CCR-04-2057. PubMed: 15814652.
10. Nishio J, Iwasaki H, Nabeshima K, Ishiguro M, Naumann S et al. (2005) Establishment of a new human epithelioid sarcoma cell line, FU-EPS-1: molecular cytogenetic characterization by use of spectral karyotyping and comparative genomic hybridization. Int J Oncol 27: 361-369. PubMed: 16010416.
11. Wada T, Uede T, Ishii S, Matsuyama K, Yamawaki S et al. (1988) Monoclonal antibodies that detect different antigenic determinants of the same human osteosarcoma-associated antigen. Cancer Res 48: 2273-2279. PubMed: 2450650.
12. Nabeta Y, Kawaguchi S, Sahara H, Ikeda H, Hirohashi Y et al. (2003) Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. J Orthop Sci 8: 554-559. doi:10.1007/s00776-003-0663-5. PubMed: 12898310.
13. Tsukahara T, Kawaguchi S, Ida K, Kimura S, Tamura Y et al. (2006) HLA-restricted specific tumor cytolysis by autologous T-lymphocytes infiltrating metastatic bone malignant fibrous histiocytoma of lymph node. J Orthop Res 24: 94-101. doi:10.1002/jor.20019. PubMed: 16419974.
14. Kimura S, Kozakai Y, Kawaguchi S, Tsukahara T, Ida K et al. (2008) Clonal T-cell response against autologous pleomorphic malignant fibrous histiocytoma antigen presented by retrieved HLA-A*0206. J Orthop Res 26: 271-278. doi:10.1002/jor.20497. PubMed: 17853492.
15. Tsukahara T, Kawaguchi S, Torigoe T, Kimura S, Murase M et al. (2008) Prognostic impact and immunogenicity of a novel osteosarcoma antigen, papillomavirus binding factor, in patients with osteosarcoma. Cancer Sci 99: 368-375. doi:10.1111/j.1349-7006.2008.00695.x. PubMed: 18271936.
16. Murase M, Kano M, Tsukahara T, Takahashi A, Torigoe T et al. (2009) Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. Br J Cancer 101: 1425-1432. doi:10.1038/sj.bjc.6605330. PubMed: 19826427.
17. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D et al. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95: 379-391. doi: 10.1016/S0092-8674(00)81769-9. PubMed: 9814708.
18. Chambers I, Colby D, Robertson M, Nichols J, Lee S et al. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643-655. doi:10.1016/S0092-8674(03)00392-1. PubMed: 12787505.
19. Yasuda K, Torigoe T, Morita R, Kuroda T, Takahashi A et al. (2013) Ovarian cancer stem cells are enriched in side population and aldehyde dehydrogenase bright overlapping population. PLOS ONE (in press).
20. Sutherland DR, Yeo E, Ryan A, Mills GB, Bailey D et al. (1991) Identification of a cell-surface antigen associated with activated T lymphoblasts and activated platelets. Blood 77: 84-93. PubMed: 1984805.
21. Murray LJ, Bruno E, Uchida N, Hoffman R, Nayar R et al. (1999) CD109 is expressed on a subpopulation of CD34+ cells enriched in hematopoietic stem and progenitor cells. Exp Hematol 27: 1282-1294. doi:10.1016/S0301-472X(99)00071-5. PubMed: 10428505.
22. Finnson KW, Tam BY, Liu K, Marcoux A, Lepage P et al. (2006) Identification of CD109 as part of the TGF-beta receptor system in human keratinocytes. FASEB J 20: 1525-1527. doi:10.1096/fj.05-5229fje. PubMed: 16754747.
23. Hollmann TJ, Hornick JL (2011) INI1-deficient tumors: diagnostic features and molecular genetics. Am J Surg Pathol 35: e47-e63. doi: 10.1097/PAS.0b013e31822b325b. PubMed: 21934399.
24. Reeves BR, Fisher C, Smith S, Courtenay VD, Robertson D (1987) Ultrastructural, immunocytochemical, and cytogenetic characterization of a human epithelioid sarcoma cell line (RM-HS1). J Natl Cancer Inst 78: 7-18. PubMed: 2432306.
25. Kelland LR, Bingle L (1988) Radiosensitivity and characterisation of a newly established cell line from an epithelioid sarcoma. Br J Cancer 58: 322-325. doi:10.1038/bjc.1988.211. PubMed: 3179184.
26. Stenman G, Kindblom LG, Willems J, Angervall L (1990) A cell culture, chromosomal and quantitative DNA analysis of a metastatic epithelioid sarcoma. Deletion 1p, a possible primary chromosomal abnormality in epithelioid sarcoma. Cancer 65: 2006-2013. doi: 10.1002/1097-0142(19900501)65:9. PubMed: 2196989.
27. Gerharz CD, Moll R, Ramp U, Mellin W, Gabbert HE (1990) Multidirectional differentiation in a newly established human epithelioid sarcoma cell line (GRU-1) with co-expression of vimentin, cytokeratins and neurofilament proteins. Int J Cancer 45: 143-152. doi:10.1002/ijc.2910450126. PubMed: 1688830.
28. Roché H, Julia AM, Jozan S, Muller C, Dastugue N et al. (1993) Characterization and chemosensitivity of a human epithelioid sarcoma cell line (SARCCR 2). Int J Cancer 54: 663-668. doi:10.1002/ijc.2910540423. PubMed: 8099901.
29. Sonobe H, Furihata M, Iwata J, Oka T, Ohtsuki Y et al. (1993) Morphological characterization of a new human epithelioid sarcoma cell line, ES020488, in vitro and in vivo. Virchows Arch B Cell Pathol Incl Mol Pathol 63: 219-225. doi:10.1007/BF02899265. PubMed: 7685133.
30. Cordoba JC, Parham DM, Meyer WH, Douglass EC (1994) A new cytogenetic finding in an epithelioid sarcoma, t(8;22)(q22;q11). Cancer Genet Cytogenet 72: 151-154.
31. Helson C, Melamed M, Braverman S, Traganos F, Preti R et al. (1995) Va-es-bj - an epithelioid sarcoma cell-line. Int J Oncol 7: 51-56. PubMed: 21552805.
32. Iwasaki H, Ohjimi Y, Ishiguro M, Isayama T, Kaneko Y et al. (1996) Epithelioid sarcoma with an 18q aberration. Cancer Genet Cytogenet 91: 46-52. doi:10.1016/S0165-4608(95)00315-0. PubMed: 8908166.
33. Kusakabe H, Sakatani S, Yonebayashi K, Kiyokane K (1997) Establishment and characterization of an epithelioid sarcoma cell line with an autocrine response to interleukin-6. Arch Dermatol Res 289: 224-233. doi:10.1007/s004030050184. PubMed: 9143739.
34. Russo JE, Hilton J (1988) Characterization of cytosolic aldehyde dehydrogenase from cyclophosphamide resistant L1210 cells. Cancer Res 48: 2963-2968. PubMed: 3365687.
35. Visus C, Ito D, Amoscato A, Maciejewska-Franczak M, Abdelsalem A et al. (2007) Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. Cancer Res 67: 10538-10545. doi:10.1158/0008-5472.CAN-07-1346. PubMed: 17974998.
36. Awad O, Yustein JT, Shah P, Gul N, Katuri V et al. (2010) High ALDH activity identifies chemotherapy-resistant Ewing's sarcoma stem cells that retain sensitivity to EWS-FLI1 inhibition. PLOS ONE 5: e13943. doi:10.1371/journal.pone.0013943. PubMed: 21085683.
37. Nakatsugawa M, Takahashi A, Hirohashi Y, Torigoe T, Inoda S et al. (2011) SOX2 is overexpressed in stem-like cells of human lung adenocarcinoma and augments the tumorigenicity. Lab Invest 91: 1796-1804. doi:10.1038/labinvest.2011.140. PubMed: 21931300.
38. Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132: 567-582. doi: 10.1016/j.cell.2008.01.015. PubMed: 18295576.
39. Schatton T, Frank MH (2008) Cancer stem cells and human malignant melanoma. Pigment. Cell - Melanoma Res 21: 39-55.
40. Velcheti V, Schalper K, Yao X, Cheng H, Kocoglu M et al. (2013) High SOX2 levels predict better outcome in non-small cell lung carcinomas. PLOS ONE 8: e61427. doi:10.1371/journal.pone.0061427. PubMed: 23620753.
41. Hwang SM, Kim MJ, Chang HE, Hong YJ, Kim TS, et al. (2013) Human platelet antigen genotyping and expression of CD109 (human platelet antigen 15) mRNA in various human cell types. Biomed Res Int 2013: 946403
42. Man XY, Finnson KW, Baron M, Philip A (2012) CD109, a TGF-beta co-receptor, attenuates extracellular matrix production in scleroderma skin fibroblasts. Arthritis Res Ther 14: R144. doi:10.1186/ar3877. PubMed: 22694813.
43. Hagiwara S, Murakumo Y, Sato T, Shigetomi T, Mitsudo K et al. (2008) Up-regulation of CD109 expression is associated with carcinogenesis of the squamous epithelium of the oral cavity. Cancer Sci 99: 1916-1923. PubMed: 19016750.
44. Hagiwara S, Murakumo Y, Mii S, Shigetomi T, Yamamoto N et al. (2010) Processing of CD109 by furin and its role in the regulation of TGF-beta signaling. Oncogene 29: 2181-2191. doi:10.1038/onc.2009.506. PubMed: 20101215.
45. Bizet AA, Liu K, Tran-Khanh N, Saksena A, Vorstenbosch J et al. (2011) The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. Biochim Biophys Acta 1813: 742-753. doi:10.1016/j.bbamcr.2011.01.028. PubMed: 21295082.
46. Hagikura M, Murakumo Y, Hasegawa M, Jijiwa M, Hagiwara S et al. (2010) Correlation of pathological grade and tumor stage of urothelial carcinomas with CD109 expression. Pathol Int 60: 735-743. doi: 10.1111/j.1440-1827.2010.02592.x. PubMed: 20946523.
47. Ikushima H, Miyazono K (2012) TGF-beta signal transduction spreading to a wider field: a broad variety of mechanisms for context-

dependent effects of TGF-beta. Cell Tissue Res 347: 37-49. doi: 10.1007/s00441-011-1179-5. PubMed: 21618142.
48. Miura Y, Keira Y, Ogino J, Nakanishi K, Noguchi H et al. (2012) Detection of specific genetic abnormalities by fluorescence in situ hybridization in soft tissue tumors. Pathol Int 62: 16-27. doi:10.1111/j.1440-1827.2011.02739.x. PubMed: 22192800.





# Cytotoxic T lymphocytes: the future of cancer stem cell eradication?

**Hiroshi Saijo**
*Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan and Third Department of Internal Medicine, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan*

**Yoshihiko Hirohashi**
*Author for correspondence: Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan hirohashi@sapmed.ac.jp*

**Toshihiko Torigoe**
*Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan*

**Vitaly Kochin**
*Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan*

**Hiroki Takahashi**
*Third Department of Internal Medicine, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan*

**Noriyuki Sato**
*Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan*

*"Cancer stem-like cells/cancer-initiating cells are immunogenic to cytotoxic T lymphocytes and express several tumor-associated antigens that can be recognized by cytotoxic T lymphocytes."*

**KEYWORDS: cancer stem cell ▪ cytotoxic T lymphocyte ▪ immunotherapy ▪ tumor antigen**

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are a hot topic in cancer research since they are highly tumorigenic and are resistant to standard cancer therapies. Recent studies have revealed that cancer immunotherapy is a possible and promising candidate to target CSCs/CICs. Among the various immunological effector cells, cytotoxic T lymphocytes (CTLs) are a good candidate for CSC/CIC-targeted immunotherapy as CTLs are antigen-specific effector cells. In this article, we summarize advances in studies on CTLs and discuss the future of CSC/CIC-targeted therapy.

Since the first identification of leukemia stem cells from an acute myeloid leukemia sample, studies of CSCs/CICs have made great advances [1]. CSCs/CICs are a small population of cancer cells that have tumor-initiating, self-renewal and differentiation abilities. Recent studies have revealed that CSCs/CICs are resistant to cancer therapies because they are in a quiescent cell-cycle state, they express high levels of transporters and apoptosis inhibitors, and they express low levels of reactive oxygen species. CSCs are therefore regarded as major causes of cancer recurrence, distant metastasis and treatment resistance; studies of CSCs/CICs have been focusing on how CSCs/CICs can be targeted efficiently.

## Cancer immunotherapy: a possible option for CSC/CIC-targeted therapy

Cancer immunotherapy is expected to become the fourth main cancer therapy following surgery, chemotherapy and radiotherapy. Several cancer immunotherapy protocols have been tested in clinical trials, and the first cancer immunotherapy drug Provenge® (Dendreon, WA, USA) was approved by the US FDA in 2010 for treatment of advanced prostate cancer. It had not been clear whether the immune system can recognize therapy-resistant CSCs/CICs, but recent studies have revealed that both the innate and acquired immune system can recognize CSCs/CICs [2]. CTLs, NK cells, γδT cells and antibodies have been shown to be able to target CSCs/CICs. CTLs, NK cells and γδT cells kill target cells by cytotoxic granules including perforin and granzymes, therefore treatment-resistant CSCs/CICs would be susceptible to death. CTLs are a key player in the aquired immune system and recognize target cells in an antigen-specific manner. On the other hand, NK cells and γδT cells are players in the innate immune system and are not specific for antigens. Therefore, CTLs might be useful for CSC/CIC-targeted immune therapy.

## CTLs: key player for eradicating CSCs/CICs

Since CTLs recognize antigenic peptides presented by MHCs, it is essential that CSCs/CICs express both MHCs and tumor-associated antigens (TAAs). A previous study revealed that colon cancer stem cells isolated as side population cells express MHC molecules at levels similar to those in non-CSCs/CICs [3]. In a recent study, Carbone's group found that colon CSCs/CICs derived as spheroid cultures express lower levels of MHC molecules than parental colon tumor cells, which enables NK cells to recognize CSCs/CICs [4]. They reported that NK cells could recognize colon CSCs/CICs because they express higher levels of ligands of NK cell-activating receptors, including NKp30 and NKp44. However, colon CSCs/CICs used in the study expressed MHC molecules to some extent, which may be sufficient to be recognized by CTLs. In fact, we and other groups have reported that CSCs/CICs can be recognized by CTLs [3,5,6] and CSCs/CICs might

Future Medicine part of fsg

therefore express sufficient levels of MHCs to be recognized by CTLs.

CSCs/CICs have been shown to express several TAAs. We previously classified TAAs into three groups according to expression profiles in CSCs/CICs and non-CSCs/CICs [7]. These three groups are: CSC/CIC antigens, which are expressed preferentially in CSCs/CICs; shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs; and non-CSC/CIC antigens, which are expressed preferentially in non-CSCs/CICs. Thus, CSC/CIC antigens and shared antigens are expressed in CSCs/CICs. The CSC/CIC antigens so far reported include MAGEA3, MAGEA4, DNAJB8, SOX2, OCT3/4, BMI1 and ALDH1A1 [6,8–11]. SOX2, OCT3/4 and ALDH1A1 are also expressed in the normal stem cell fraction, whereas MAGEA3, MAGEA4 and DNAJB8 are cancer testis (CT) antigens that are expressed in CSCs/CICs and normal testis cells, but not in normal stem cells. SOX2, OCT3/4 and ALDH1A1 can be candidates for CSC/CIC-targeted immunotherapy; however, there is a risk of also targeting normal stem cells, which may make the patient's condition severe. Therefore, CT antigens may be good candidates for CSC/CIC-targeted immunotherapy. Interestingly, large numbers of CT antigens are preferentially expressed in CSCs/CICs [12]. The testis is an 'immunologically privileged organ', and CT antigens are regarded as immunogenic TAAs [13]. The biological significance of testis gene products in CSCs/CICs is still elusive; however, they should be better candidates for CSC/CIC-targeted immunotherapy as they are not expressed in normal stem cells.

> *"...both the innate and acquired immune system can recognize cancer stem-like cells/cancer-initiating cells."*

Since both CSC/CIC antigens and shared antigens are expressed in CSCs/CICs, it raises the question, which one is better for targeting CSCs/CICs? We have identified a novel CSC/CIC antigen, DNAJB8, which is expressed in kidney CSCs/CICs [11]. To answer the above question, we compared the potency of DNAJB8 with that of survivin, which is a well-established TAA and is a shared antigen [14]. Interestingly, DNAJB8 was more potent than survivin in a tumor prophylactic DNA vaccination model. However, it may be too early to reach a conclusion; these results suggest that CSC/CIC antigens are better at targeting cancers than shared antigens. Several other studies have demonstrated the potency of CSC/CIC-targeted immunotherapy by *in vivo* animal models [5,6,15]. Therefore, CSC/CIC-targeted immunotherapy using CSC/CIC antigens is a feasible and promising approach.

> *"...cancer stem-like cell/cancer-initiating cell antigens are better at targeting cancers than shared antigens."*

There are several molecular mechanisms by which CSCs/CICs may escape from CTLs. Heinberger's group reported that glioma stem cells express high levels of immunosuppressive molecules, including B7-H1 and soluble galectin-3 [16]. Kim's group reported that CSCs/CICs express high levels of NANOG, which induces CTL resistance by activation of Nanog/Tcl1a/Akt signaling [17]. The results of these two studies demonstrate the molecular mechanisms of suppression of CTL induction phase and suppression of CTL effector phase, respectively. Since CSCs/CICs express high levels of CT antigens, which are regarded as highly immunogenic TAAs, CSCs/CICs must be a relatively immunogenic cancer cell population. However, CSCs/CICs in clinical cancer specimens survive immune pressure, indicating that there are mechanisms for CSCs/CICs to escape the immune system. These two mechanisms may be aspects of CSC/CIC immune escape. Therefore, to overcome the problem of immune escape of CSCs/CICs and target CSCs/CICs in cancer immunotherapy, an immune potentiator (adjuvant) may be necessary to achieve significant antitumor effects.

## Conclusion

CSCs/CICs are immunogenic to CTLs and express several TAAs that can be recognized by CTLs. CSC/CIC-targeted immunotherapy using these TAAs might be feasible. Antigenic peptide vaccination and CTL adoptive transfer are possible approaches to target CSCs/CICs. In the near future, chemotherapy- and radiotherapy-resistant CSCs/CICs may be able to be targeted by CTLs in clinical settings.

**Financial & competing interests disclosure**

*This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant numbers 16209013, 17016061 and 15659097), for Practical Application Research from the Japan Science and Technology Agency, and for Cancer Research (15-17 and 19-14) from the Ministry of Health, Labor and Welfare of Japan, Ono*

*Cancer Research Fund (to N Sato) and Takeda Science Foundation (to Y Hirohashi). This work was supported in part by the National Cancer Center Research and Development Fund (23-A-44). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

*No writing assistance was utilized in the production of this manuscript.*

## References

1 Clevers H. The cancer stem cell: premises, promises and challenges. *Nat. Med.* 17, 313–319 (2011).

2 Hirohashi Y, Torigoe T, Inoda S *et al.* Immune response against tumor antigens expressed on human cancer stem-like cells/tumor-initiating cells. *Immunotherapy* 2, 201–211 (2010).

3 Inoda S, Hirohashi Y, Torigoe T *et al.* Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. *Am. J. Pathol.* 178, 1805–1813 (2011).

4 Tallerico R, Todaro M, Di Franco S *et al.* Human NK cells selective targeting of colon cancer-initiating cells: a role for natural cytotoxicity receptors and MHC class I molecules. *J. Immunol.* 190, 2381–2390 (2013).

5 Xu Q, Liu G, Yuan X *et al.* Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. *Stem Cells* 27, 1734–1740 (2009).

6 Visus C, Wang Y, Lozano-Leon A *et al.* Targeting ALDH (bright) human carcinoma-initiating cells with ALDH1A1-specific CD8+ T cells. *Clin. Cancer Res.* 17, 6174–6184 (2011).

7 Hirohashi Y, Torigoe T, Inoda S, Morita R, Kochin V, Sato N. Cytotoxic T lymphocytes: sniping cancer stem cells. *Oncoimmunology* 1, 123–125 (2012).

8 Schmitz M, Temme A, Senner V *et al.* Identification of SOX2 as a novel glioma-associated antigen and potential target for T cell-based immunotherapy. *Br. J. Cancer* 96, 1293–1301 (2007).

9 Steele JC, Torr EE, Noakes KL *et al.* The polycomb group proteins, BMI-1 and EZH2, are tumour-associated antigens. *Br. J. Cancer* 95, 1202–1211 (2006).

10 Dhodapkar KM, Feldman D, Matthews P *et al.* Natural immunity to pluripotency antigen OCT4 in humans. *Proc. Natl Acad. Sci. USA* 107, 8718–8723 (2010).

11 Nishizawa S, Hirohashi Y, Torigoe T *et al.* HSP DNAJB8 controls tumor-initiating ability in renal cancer stem-like cells. *Cancer Res.* 72, 2844–2854 (2012).

12 Yamada R, Takahashi A, Torigoe T *et al.* Preferential expression of cancer/testis (CT) genes in cancer stem-like cells: proposal of a novel sub-category, cancer/testis/stem (CTS) gene. *Tissue Antigens* doi:10.1111/tan.12113. (2013) (Epub ahead of print).

13 Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun.* 4, 1 (2004).

14 Andersen MH, Svane IM, Becker JC, Straten PT. The universal character of the tumor-associated antigen survivin. *Clin. Cancer Res.* 13, 5991–5994 (2007).

15 Ning N, Pan Q, Zheng F *et al.* Cancer stem cell vaccination confers significant antitumor immunity. *Cancer Res.* 72, 1853–1864 (2012).

16 Wei J, Barr J, Kong LY *et al.* Glioma-associated cancer-initiating cells induce immunosuppression. *Clin. Cancer Res.* 16, 461–473 (2010).

17 Noh KH, Kim BW, Song KH *et al.* Nanog signaling in cancer promotes stem-like phenotype and immune evasion. *J. Clin. Invest.* 22(11), 4077–4093 (2012).

# Chapter 28

# Production of Multiple CTL Epitopes from Multiple Tumor-Associated Antigens

**Rena Morita, Yoshihiko Hirohashi, Munehide Nakatsugawa, Takayuki Kanaseki, Toshihiko Torigoe, and Noriyuki Sato**

## Abstract

Identification of antigenic peptides derived from tumor-associated antigens (TAA) enables cancer vaccine therapy using antigenic peptides. Here, we summarize the design of antigenic peptides and induction of cytotoxic T lymphocytes (CTL) using antigenic peptides and validation of CTL.

**Key words** Tumor-associated antigen, Cytotoxic T lymphocyte, Antigenic peptide, HLA

## 1 Introduction

In recent years, immunotherapies for malignant diseases have been regarded as the fourth strategy following surgery, chemotherapy, and radiotherapy. The molecular biological characteristics of immunotherapies have been analyzed and have been partially applied in clinical settings. Previous studies showed that antigen-specific immunotherapies such as peptide vaccine therapy were less effective and successful in vivo than in vitro [1, 2]. These results might be due to various escape mechanisms from the immune system, including antigen molecules targeted by immune cells, actions of immune suppression, e.g., regulatory T lymphocytes, or inhibiting cytokines and loss of human leukocyte-associated antigen (HLA) and β2-microglobulin. It is essential to design antigenic peptides to prevent escape from the immune system [3]. Loss of antigens is thought to be one of the main causes of escape from the immune system, therefore, functional antigens are thought to be suitable targets.

An antigen derived from the melanoma-associated antigen (MAGE) family that was recognized by cytotoxic T lymphocytes from a human melanoma patient was discovered in 1991 [4]. Since then, many tumor-associated antigens (TAA) have been identified and analyzed. Various methods have been used for identifying

Michael J.P. Lawman and Patricia D. Lawman (eds.), *Cancer Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1139, DOI 10.1007/978-1-4939-0345-0_28, 

candidate TAA, including cDNA expression cloning, cDNA microarray, DNA subtraction methods, serological identification of antigens by recombinant expression cloning (SEREX methods), and a reverse-immunogenetical approach [4–7]. Although cancer cell-specific proteins are potential immunological targets, it is necessary to determine whether a peptide from a candidate protein can induce a CTL response. In this chapter, we summarize (1) prediction of antigenic peptides, (2) generation of CTL, and (3) validation of CTL and establishment of CTL clones.

## 2 Materials

### 2.1 Selection of HLA-Restricted Peptides Derived from Candidate Antigens

1. Putative antigenic peptides can be designed by several website programs (e.g., BIMAS, SYFPEITHI, CTLPred, ProPred1, MAPPP, nHLAPred, LPPEP, SVMHC, NetMHC, MHCPred, Epitope binding, MMPRED, and PREDEP) (Fig. 1) (*see* **Note 1**) [8, 9].
2. Synthetic peptides.
3. Dimethyl sulfoxide (DMSO).
4. T2 cells cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10 % FBS (*see* **Note 2**).
5. Phosphate-buffered saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$ on ice.
6. Opti-MEM® (Life Technologies, Inc., Carlsbad, CA, USA).
7. Anti-HLA-class I monoclonal antibody (mAb) (*see* **Notes 3** and **4**).
8. ITC-conjugated rabbit antimouse IgG+IgM (KPL, Gaithersburg, MD, USA).

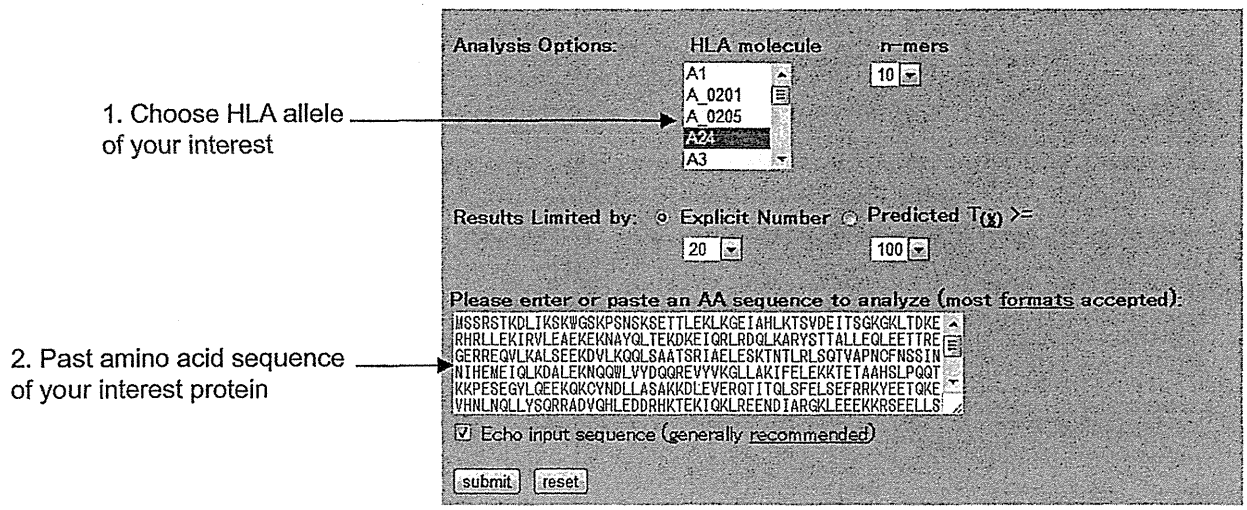



**Fig. 1** Representative prediction of antigenic peptides by BIMAS website. BIMAS website: http://www-bimas.cit.nih.gov/molbio/hla_bind/

9. PBS containing 1 % formaldehyde.
10. Disposable pipettes and Pasteur pipettes (sterile).
11. Sterile tubes for flow cytometry.
12. Sterile micropipettors and tips.
13. Centrifuge (refrigerated) with swing-out rotor and appropriate carriers.
14. Hemocytometer and microscope for cell counting.
15. 5 % $CO_2$ incubator at 26 and 37 °C.
16. Flow cytometer.

### 2.2 Preparation of APC and CD8+ T Cells Isolated from Peripheral Blood Mononuclear Cells (PBMC)

1. Blood sample (*see* **Note 4**).
2. Lymphoprep (Nycomed, Oslo, Norway).
3. Anticoagulant agent, e.g., heparin sodium, EDTA, and sodium citrate.
4. PBS with 2 mM EDTA, at room temperature.
5. AIM-V medium.
6. 2-Mercaptoethanol.
7. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES).
8. Human recombinant interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN, USA).
9. Human recombinant interleukin-4 (IL-4) (R&D Systems).
10. Human granulocyte/macrophage-colony stimulating factor (GM-CSF) (R&D Systems).
11. Tumor necrosis factor-α (TNFα) (R&D Systems).
12. Phytohaemagglutinin (PHA-P).
13. MACS separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD8 mAb coupled with magnetic microbeads.
14. Sterile disposable pipettes and Pasteur pipettes.
15. Sterile 50-mL high-clarity polypropylene conical centrifuge tube.
16. Sterile micropipettors and tips.
17. Centrifuge (not refrigerated) with swing-out rotor and appropriate carriers.
18. Sterile 10-cm culture flasks (dish) and 24-well plates.

### 2.3 Induction of CTL

1. Synthesized peptides dissolved in 20 mg/mL of DMSO.
2. β2-Microglobulin.
3. AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, and HEPES buffer.

4. Human recombinant IL-2 (R&D Systems).
5. Human recombinant interleukin-7 (IL-7) (R&D Systems).
6. Human AB serum.
7. Complete RPMI-1640 medium, i.e., RPMI-1640 supplemented with 10 % fetal bovine serum (FBS).
8. Sterile disposable pipettes and Pasteur pipettes.
9. Sterile 24-well plates.
10. Enzyme-linked immunospot (ELISPOT) Human interferon-γ (IFN-γ) ELISPOT set (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).
11. Sterile micropipettors and tips.
12. KS ELISPOT assay system (Carl Zeiss, Oberkochen, Germany).
13. Gamma counter (PerkinElmer, Waltham, MA, USA).
14. X-ray irradiation device for cells (SOFTEX, Tokyo, Japan).

### 2.4 Establishment of CTL Clone

1. $CD8^+$ T cells (CTL).
2. PBMC from donors.
3. AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, and 10 mM HEPES.
4. Human recombinant IL-2 (R&D Systems).
5. PHA-P.
6. Human AB serum.
7. Sterile disposable pipettes and Pasteur pipettes.
8. Sterile 96-, 48-, and 24-well plates.
9. Sterile micropipettors and tips.
10. X-ray irradiation device for cells (SOFTEX, Tokyo, Japan).

## 3 Methods

### 3.1 Selection of HLA-Restricted Peptides Derived from Candidate Antigens

1. Predict putative antigenic peptides with protein sequence (*see* **Note 1**). The synthesized peptides should be dissolved in DMSO and stored at −80 °C before use (*see* **Note 2**).
2. After incubation of T2 cells in RPMI-1640 culture medium supplemented with 10 % FBS at 26 °C for 18 h, wash the cells with ice-cold PBS (*see* **Note 5**).
3. For flow cytometric analysis, divide the cells equally into two sterile tubes and suspend T2 cells with 1 mL of Opti-MEM® with or without 100 μg of peptide, followed by incubation at 26 °C for 3 h and then at 37 °C for 3 h.

4. After washing 1× with ice-cold PBS, incubate the cells with anti-HLA-class I mAb at 4 °C for 30 min (*see* **Notes 3** and **4**).
5. After washing with ice-cold PBS 1×, incubate the cells with FITC-conjugated rabbit antimouse IgG+IgM at 4 °C for 30 min.
6. Then suspend the cells with 1 mL of PBS containing 1 % formaldehyde and analyze the cells by flow cytometry.
7. Binding affinity is evaluated by comparing mean fluorescence intensity of HLA-class I expression in the presence of peptide with mean fluorescence intensity in the absence of the peptide (*see* **Note 4**).

### *3.2 Preparation of APC and CD8⁺ T Cells from PBMC (Summarized in Fig. 3) [10–12]*

1. Obtain 50 mL of a blood sample with an anticoagulant agent, e.g., heparin sodium, EDTA, and sodium citrate (*see* **Note 6**).
2. Add 15 mL of Lymphoprep to each of two 50-mL high-clarity polypropylene conical centrifuge tubes. Carefully add 25 mL of a blood sample onto the top of Lymphoprep and centrifuge at 780×*g* for 20 min at room temperature using a swing-out rotor (*see* **Note 7**).
3. After centrifugation, the PBMC form a white–light yellow band at the plasma/Lymphoprep interface as shown in Fig. 2. Obtain the PBMC layer from the interface using micropipettors without removing the upper plasma layer and transfer the PBMC to a fresh 50-mL conical centrifuge tube (*see* **Note 8**).
4. Add 1× or more volume of PBS with EDTA to the tube with the isolated PBMC cells. Pellet PBMC by centrifugation at 630×*g* for 7–10 min at room temperature using a swing-out rotor.
5. Following centrifugation, wash the pellet 2× with 10 mL of PBS with EDTA (centrifugation at 440×*g* for 5 min at room temperature).
6. Incubate PBMC in AIM-V medium supplemented with 50 μM 2-mercaptoethanol and 10 mM HEPES for 2–24 h at 37 °C in a culture flask to separate adherent cells and nonadherent cells (*see* **Note 9**).
7. To generate dendritic cells (DC) from adherent PBMC, incubate adherent cells in AIM-V medium supplemented with 1,000 U/mL of IL-4 and 1,000 U/mL of GM-CSF for 5 days and then add 10 ng/mL TNFα to facilitate maturation of monocyte-derived DC (*see* **Note 10**).
8. Isolation of $CD8^+$ T lymphocytes and $CD8^-$ lymphocytes from nonadherent cells: $CD8^+$ T lymphocytes are isolated from nonadherent cells utilizing the MACS separation system with anti-CD8 mAb coupled with magnetic microbeads according to the manufacturer's instructions.

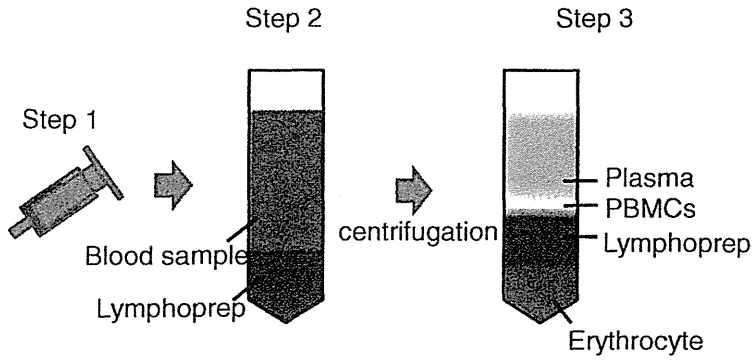



**Fig. 2** Isolation of peripheral blood mononuclear cells (PBMC) from peripheral blood

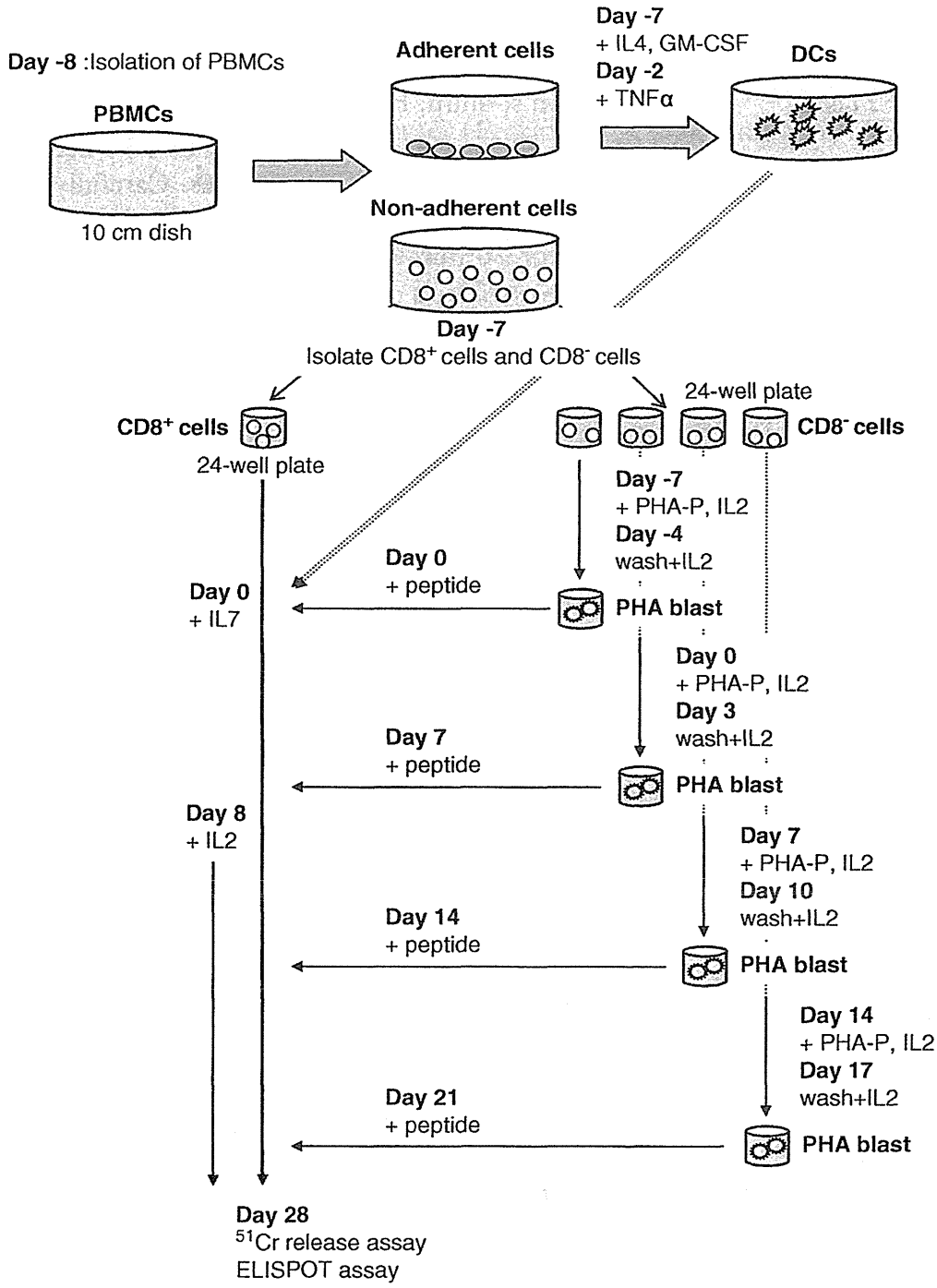



**Fig. 3** Summary of CTL induction from PBMC

9. Generation of PHA-blasts from nonadherent PBMC: $CD8^-$ cells derived from nonadherent PBMC are seeded into four wells of a 24-well plate and cultured in AIM-V medium containing 1 μg/mL of PHA-P and 100 U/mL of IL-2 for 3 days, followed by washing with AIM-V medium and culture in AIM-V medium supplemented with 100 U/mL of IL-2 for 4 days (*see* **Note 11**).

### 3.3 Induction of CTL (Summarized in Fig. 3)

1. On day 0, autologous APC (DC or PHA-blasts) are incubated at room temperature for 2 h in AIM-V medium with 2.5 μg/mL of β2-microglobulin and 50 μg/mL of synthetic peptide. APC are then irradiated (100 Gy) using an X-ray irradiation device and washed with AIM-V medium. One hundred thousand peptide-pulsed-irradiated APC are incubated with $1 \times 10^6$ $CD8^+$ cells in 2 mL of AIM-V medium supplemented with 10 % human AB serum, recombinant 10 ng/mL of IL-7, 50 μM 2-mercaptoethanol, and 10 mM HEPES in 1 well of a 24-well plate (*see* **Note 11**).
2. On day 7, autologous PHA-blasts are incubated at room temperature for 2 h in AIM-V medium with 50 μg/mL peptide. PHA-blasts are then irradiated (100 Gy) and washed with AIM-V medium. One million $CD8^+$ T cells are stimulated with $2 \times 10^5$ peptide-pulsed PHA-blasts in 2 mL of AIM-V medium.
3. On day 8, add IL-2 to each well at a concentration of 50 U/mL.
4. The peptide stimulation procedure using PHA-blasts is repeated every 7 days. During CTL induction, cells are fed with fresh AIM-V medium supplemented with 10 % human serum, 50 U/mL of IL-2, 50 μM 2-mercaptoethanol, and 10 mM HEPES every 2–5 days (*see* **Note 12**).
5. On day 28, $CD8^+$ T cell reactivity is assessed by IFN-γ ELISPOT assay or conventional 6-h $^{51}Cr$ release assay (see below).

### 3.4 IFN-γ ELISPOT Assay

Multiscreen 96-well plates are coated with 100 μL/well of 5 μg/mL of anti-IFN-γ capture antibody in PBS at 4 °C overnight. Plates are washed once with 200 μL/well of complete RPMI-1640 and blocked with 200 μL/well of complete RPMI-1640 at room temperature for 2 h. Then, $2 \times 10^5$ $CD8^+$ T cells are incubated with $5 \times 10^4$/well T2 cells pulsed with each peptide at 50 μg/mL. After 40 h of incubation at 37 °C, IFN-γ spots are developed and counted as per the manufacturer's instructions (*see* **Note 13**).

### 3.5 $^{51}Cr$ Release Assay

Target cells are labeled with 100 μCi of $^{51}Cr$ for 1 h at 37 °C, washed 3×, and resuspended in AIM-V medium. $^{51}Cr$-labeled target cells at $2 \times 10^3$ cells/well are incubated with various numbers of effector cells for 6 h at 37 °C in 96-well microtiter plates. Radioactivity of the culture supernatant is measured using a gamma counter. The percentage of cytotoxicity is calculated as follows:

% cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100 (*see* **Note 14**).

### 3.6 Establishment of CTL Clone [13]

1. Seed CTL at 0.3–30/well by limiting dilution in a 96-well round-bottom plate. Fifty thousand irradiated PBMC derived from three healthy donors are placed in each well in 200 μL of AIM-V medium with 10 % normal human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, 10 mM HEPES, and 5 μg/mL of PHA-P (*see* **Note 15**).
2. The cells are fed with fresh AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, and 10 mM HEPES every 7 days.
3. Growing wells can be observed on days 14–20. Transfer the cells to a 48- or 24-well plate. $CD8^+$ cell reactivity is assessed by a cytotoxicity assay such as $^{51}Cr$ release or ELISPOT assays.

## 4 Notes

1. We usually use the BIMAS website: (http://www-bimas.cit.nih.gov/molbio/hla_bind/) (Fig. 1). A peptide with a score of more than 100 binds strongly to HLA molecules in the described HLA-binding assay.
2. Predicted peptides are often hydrophobic and are very difficult to dissolve in PBS or water. Therefore, DMSO is recommended to dissolve synthetic peptides.
3. Use an appropriate anti-HLA mAb, e.g., anti-HLA-A2 mAb clone BB7.2 is available for detection of HLA-A2.
4. The use of an appropriate positive control peptide (HLA-binding) and negative control peptide (HLA-nonbinding) is necessary. We use HLA-A2-restricted influenza peptide (GILGFVFTL), HLA-A24-restricted HIV peptide (RYLRDQQLLGI), and EBV peptide (TYGPVFMSL) as positive controls for HLA-A2 and -A24, respectively [14–16].
5. T2 cells lack the transporter associated with antigen transport, and thus endogenous peptide loading onto HLA molecules is extensively impeded and the expression level of HLA molecules on the cell surface is very low. Cell culture at a lower temperature (26 °C) facilitates the expression of antigenic peptide unbound to HLA molecules. After incubation with exogenous synthetic peptides, peptide-HLA complex is stabilized

even under a normal temperature condition (37 °C). The genotype of T2 cells is HLA-A*0201/ B*5101/ Cw*0102, and T2 cells can therefore be used for HLA-A*0201/ B*5101/ Cw*0102-binding peptides. Furthermore, since other HLA molecules are expressed on T2 cells, they are also available for detection of those HLA types, e.g., T2-A24 cells for detection of HLA-A24-binding peptides [12, 13, 17].

6. Before acquiring blood samples, it is necessary to obtain informed consent from all patients and volunteer donors according to the guidelines of the Declaration of Helsinki.
7. This is a very delicate procedure and care should be taken not to disturb the surface of separation between the blood sample and Lymphoprep. For centrifugation, minimum acceleration and deceleration are highly recommended to avoid disturbing the surface of separation.
8. Obtain monocyte layer carefully not to disturb the layer. Eliminate contamination of Plasma layer and Lymphoprep layer as possible.
9. Adherent cells can be washed gently 2× or 3× in PBS to eliminate contamination of nonadherent cells.
10. Dendritic cells are floating cells that have many dendrites. Some adherent cells can be observed after 7 days of culture. These adherent cells are macrophages. DO NOT use these adherent cells as APC. Macrophages inhibit CTL induction in the described CTL induction procedure.
11. DC are commonly used to induce CTL; however, we could induce CTL more efficiently by PHA-blasts as APC than by DC in our experiments. Thus, we strongly recommend the use of PHA-blasts rather than DC as APC.
12. Viability of $CD8^+$ cells is critical for generation of CTL. Highly viable $CD8^+$ are small with a round shape (Fig. 4). Activated $CD8^+$ cells form clusters (Fig. 4).
13. We usually stimulate $CD8^+$ cells with multiple peptides-pulsed PHA-blasts. Following stimulation, the reactivities to peptides are screened using the ELISPOT assay.
14. Both peptide-pulsed T2 cells and cancer cells are available as target cells. For peptide-pulsed T2 cells, add 10 μg/mL of peptide onto T2 cells for 1 h and wash with PBS before the assay. For cancer cells, it is recommended to add 100 units/mL of IFN-γ in the culture for 48–72 h to increase the expression of HLA.
15. For feeder cells, we use a mixture of PBMC from three different donors. A mixture yields higher CTL clone establishment efficiency than that of feeder cells from a single donor. The reason for this is unknown.