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LETTER TO THE EDITOR

T-cell immunotherapy with a chimeric receptor against CD38 is effective in eradicating chemotherapy-resistant B-cell lymphoma cells overexpressing survivin induced by BMI-1

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The expression of BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog), a member of the polycomb-group genes (PcG), is well correlated with a poor prognosis and treatment failure among patients with malignancies such as myelodysplastic syndrome, chronic myeloid leukemia, acute myeloid leukemia and lymphoma.[1–3] Recently, we found that BMI-1 renders B-cell lymphoma cells refractory to several anti-cancer drugs by inducing the expression of survivin.[4] There is an urgent clinical need to find therapeutics to treat patients with lymphoma cells overexpressing BMI-1 and survivin. Although in the pre-rituximab era (Rituximab, IDEC Pharmaceuticals, San Diego, CA, USA), the long-term remission rate for patients with diffuse large B-cell lymphoma (DLBCL) was 50–60%, the addition of rituximab has led to an enormous improvement in survival. As rituximab has more significantly improved the overall survival, event-free survival and progression-free survival of patients with non-germinal center B cell-type DLBCL, which have a poor prognosis, than those with germinal center B cell-type DLBCL,[5–9] immunotherapy with an antibody such as rituximab may function by overcoming drug-resistant genes including Bcl-2, Bcl-6 and Bcl-xL.[10] We previously developed T cells with a chimeric antigen receptor (CAR) against CD38 and reported that these CD38-specific T cells effectively eliminated B-cell lymphoma cells *in vitro* and *in vivo*.[11,12] This is because CD38 is widely and highly expressed in B-cell lymphoma cells (40–50% of patients with the B-cell typed),[13] especially in AIDS-associated lymphoma cells[14] and DLBCL cells bearing t(14;18) with aggressiveness (100% of these patients).[15]

We, thus investigated whether T cells bearing an anti-CD38-CAR exerted cytotoxicity against B-cell lymphoma cells expressing BMI-1 and survivin. Here we report that, the CD38-specific T cells efficiently eliminated chemotherapy-resistant B-lymphoma cells overexpressing BMI-1 and survivin, and propose that T-cell immunotherapy with CAR may be useful for treating refractory B-cell lymphoma.

Two lymphoma cell lines (HT and RL) obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C. The cells were transduced with a vesicular stomatitis virus G glycoprotein (VSVG)-pseudotyped retrovirus containing MSCV-*BMI-1-Flag*-IRES-GFP or MSCV-IRES-GFP alone and the GFP-positive cells were sorted by FACS Aria (BD, San Jose, CA, USA). Primary cells were obtained from the lymph node, pleural effusion, and spleen of patients with lymphoma, and peripheral blood cells from healthy donors, and subjected to Ficoll-density centrifugation. Informed consent was obtained from all of the patients and donors. Patients with B-cell lymphoma and donors were examined as approved by the institutional review board at Hiroshima University. The retroviral vector construct consisting of GFP, the transmembrane domain of CD8α, 4-1BB, CD3ζ and anti-CD38 scFv was made previously.[11] Briefly, to generate a RD114-pseudotyped retrovirus, Lipofectamine-Plus reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect 293T cells with the retroviral vector including anti-CD38-CAR, pEQ-PAM3(-E), and pRDF. Conditioned medium containing the retrovirus was harvested after the transfection and stored at −80 °C prior to use. Peripheral blood mononuclear cells were stimulated for 48 h with 7 μg/ml PHA-M (Sigma, St Louis, MO, USA), 200 IU/ml human Interleukin-2 (PeproTech, London, UK), and 10% FCS in RPMI-1640 medium. Cells were transduced in high titer of virus-rich conditioned medium with 4 μg/ml polybrene (Sigma) in a polypropylene tube coated with retroronectin by spinoculation technique. An anti-CD38 antibody (CPK-H; MBL, Nagoya, Japan) was added to protect transduced T cells from auto-lysis through cross-linkage of the anti-CD38-CAR with intrinsic CD38, described previously.[11] For the co-culture experiment, T cells were washed with Phosphate buffered saline several times to eliminate the residual antibodies in the medium. To detect surface expression of the anti-CD38-CAR, cells were stained with a goat anti-mouse $(Fab')_2$ polyclonal antibody conjugated to biotin (Jackson ImmunoResearch, West Grove, PA, USA), followed by streptavidin-PerCP (BD Biosciences, Franklin Lakes, NJ, USA). Antibody staining was detected with a FACS Calibur flow cytometer (BD) as described.[11] The cytotoxicity of the transduced cells was assessed by flow cytometric analysis as described previously.[11] Cells harvested from the cultures were co-incubated in anti-CD38-antibody-PerCP and anti-CD19-antibody-APC for two-color staining. Specific cytotoxicity was evaluated by using the formula (B-A)/B, where A is the number of $CD19^+CD38^+GFP^-$ cells after incubation with the anti-CD38-CAR-expressing T cells, and B is the number of $CD19^+CD38^+GFP^-$ cells after incubation with vector-transduced T cells. Recovery of viable cells (%) was evaluated by using the formula A/B.

We recently demonstrated that expression of survivin, enhanced by BMI-1, is well correlated with drug-resistance against etoposide (Sigma) or platinum-containing drugs such as oxaliplatin (Sigma) and cisplatin (Sigma).[4] We, then, examined whether human T cells with an anti-CD38-CAR effectively kill B-cell lymphoma cells overexpressing BMI-1 as well as survivin. Initially, we confirmed the expression of the anti-CD38-CAR on T cells, freshly isolated from donors and retrovirally transduced (data not shown). We previously reported that survivin expression was enhanced by BMI-1, and confirmed that both BMI-1 and survivin were overexpressed in B-lymphoma cell lines, HT and RL cells, transduced with BMI-1 (designated as HT-BMI-1 and RL-BMI-1, respectively) (Figure 1a). Firstly, we evaluated whether T cells bearing the anti-CD38-CAR eliminate chemotherapy-resistant HT-BMI-1 cells overexpressing survivin and BMI-1. T cells expressing the anti-CD38-CAR were co-cultured with HT-BMI-1 cells at an effector (E):target (T) ratio of 1:2 for 3 days. As shown in Figure 1b, the T cells effectively eliminated the retrovirally transduced B-lymphoma cells in a time-dependent manner. Specific cytotoxicity in the 3-day co-culture was 95.19% ± 0.23% (mean ± s.d.) ($n = 3$) in HT cells transduced with control vector alone (mock) and 95.76% ± 0.05% ($n = 3$) in HT-BMI-1 cells. Next, we tested whether the killing effect was dependent on the dose of the effectors.

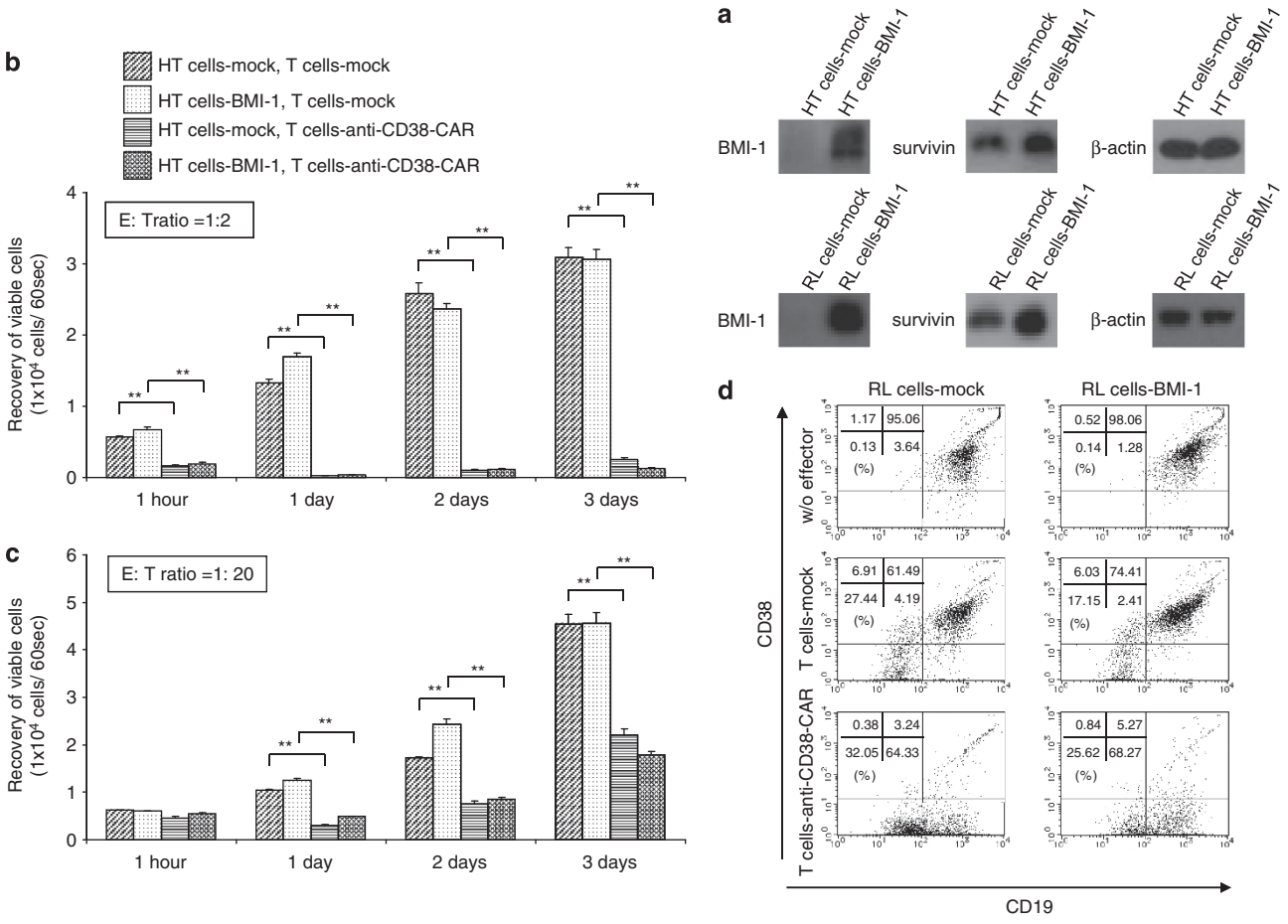

Figure 1. Cytotoxicity of human T cells retrovirally transduced with anti-CD38-CAR against B-lymphoma cells expressing both survivin and BMI-1. (**a**) Expression of BMI-1 and survivin in HT-BMI-1 and RL-BMI-1 cells as well as parental mock-transduced cells respectively as revealed by Western blotting. (**b**) T cells expressing the anti-CD38-CAR were co-cultured with HT-BMI-1 at an effector (E): target (T) ratio of 1 to 2 for 3 days. Cytotoxicity was assessed by flow cytometry with the anti-CD38 antibody-PerCP. T cells bearing the anti-CD38-CAR were highly cytotoxic to HT-BMI-1 cells in a time-dependent manner. (**c**) T cells with the anti-CD38-CAR were co-cultured with HT-BMI-1 at an E: T ratio of 1 to 20 for 3 days. The cytotoxic effect of the transduced T cells on HT-BMI-1 cells was dose-dependent compared with the results in Figure 1b. (**d**) T cells bearing the anti-CD38-CAR were co-cultured with RL cells overexpressing survivin and BMI-1 at an E: T ratio of 1 to 2 for 3 days. Representative results on the cytotoxicity with no effector (the upper panel), mock-transduced T cells (the middle panel) or T cells transduced with the anti-CD38-CAR vector (the lower panel) at an E: T ratio of 1: 2 for 3 days are shown on the left (RL-mock) and right (RL-BMI-1 cells). T cells with the anti-CD38-CAR eliminated RL-BMI-1 cells as effectively as RL-mock cells. The unpaired Students *t*-test was used to evaluate statistical significance. Asterisks indicate statistical significance (**$P < 0.01$).

T cells with the anti-CD38-CAR were co-cultured with HT-BMI-1 cells at an E:T ratio of 1:20 for 3 days. We confirmed that these CD38-specific T cells eliminated HT-BMI-1 cells in a dose-dependent fashion (Figure 1c). Next, we examined whether T cells with the anti-CD38-CAR kill RL-BMI-1 cells in similar experiments. T cells bearing the anti-CD38-CAR were co-cultured with RL cells overexpressing survivin and BMI-1 at an E:T ratio of 1:2 for 3 days. Specific cytotoxicity was 92.26% ± 0.19% ($n = 3$) in RL-mock cells and 93.82% ± 0.11% ($n = 3$) in RL-BMI-1 cells. The results were quite similar to those for HT-BMI cells. Representative flow cytometric data are shown in Figure 1d. These results suggested that T cells with the anti-CD38-CAR functioned successfully in eliminating B-lymphoma cells overexpressing survivin and BMI-1, which are resistant to chemotherapy.

Next, we tested whether T cells with the anti-CD38-CAR exerted cytotoxic activity against B-lymphoma cells harboring both survivin and BMI-1. Six patients with refractory B-cell lymphoma (DLBCL) were studied as shown in Table 1. Western blotting revealed both BMI-1 and survivin to be expressed in all the samples (data not shown). Intriguingly, T cells transduced with the anti-CD38-CAR effectively eliminated B-lymphoma cells from chemotherapy-resistant patients in the co-culturing system *in vitro*. Mean specific cytotoxicity observed at an E:T ratio of 1:2 for 3 days was over 90% ($n = 4$) (Table 1). These

Table 1. Characteristics of patients with DLBCL and cytotoxicity of T cells harboring anti-CD38-CAR against DLBCL cells

Case	*Site*	*Stage*	*IPI*	*Response to chemotherapy*	*Expression of BMI-1/ survivin*	*Specific cytotoxicity (%)*
1	Spleen	IV A	3	CR	−/−	91.21 ± 2.42
2	LN	III A	2	CR	−/−	92.31 ± 0.78
3	LN	III A	3	CR	−/−	94.77 ± 0.78
4	LN	IV B	4	PD	+/+	96.47 ± 2.02
5	LN	IV B	3	PD	+/+	94.30 ± 1.53
6	PE	IV B	5	PD	+/+	98.61 ± 0.26

Abbreviations: DLBCL, diffuse large B-cell lymphoma; LN, lymph node; PE, pleural effusion; IPI, international prognostic index. Results are the mean ± s.d. for four experiments. CR denotes complete response. PD denotes progressive disease.

results showed that CD38-specific T cells efficiently eliminated B-lymphoma cells with BMI-1 and survivin.

Overexpression of anti-apoptotic genes including the *Bcl-2, Bcl-x, XIAP* and survivin genes is one mechanism by which cancer cells become refractory to anti-cancer drugs. We confirmed that HT cells transduced with BMI-1 strongly expressed *Bcl-x, XIAP* and survivin (data not shown). Alternatively, RL cells with BMI-1 expressed Bcl-2, Bcl-x, XIAP and survivin (data not shown). Although we previously showed RL-BMI-1 cells were more resistant to etoposide and platinum-containing anti-cancer drugs like oxaliplatin and cisplatin than HT-BMI-1 cells,[4] CD38-specific T cells exerted a similar cytotoxic effect on HT-BMI-1 and RL-BMI-1, indicating that these autologous T cells bearing the anti-CD38-CAR eliminated B-lymphoma cells even though the cells expressed multiple antiapoptotic genes. Recent publications showed that genetically-engineered T cells reactive to CD19 exerted persistent cytotoxic effects in patients with refractory chronic lymphoid leukemia.[16,17] These results suggest immunotherapy with T cells bearing a CAR to be an important option in treating refractory hematological malignancies.

In this study, we demonstrated that T cells bearing the anti-CD38-CAR were highly cytotoxic to B-cell lymphoma cells expressing BMI-1 and survivin. If autologous T-cell immunotherapy using the anti-CD38-CAR is harnessed more efficiently for the treatment of lymphoma, it might shed new light on a therapeutic strategy for patients with refractory B-lymphoma cells with even multiple anti-apoptotic gene expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

KM designed and performed the experiments, analyzed the data, and wrote the paper; JB performed the experiments; AK and KY in collaboration with YT aided in writing the paper; AK, YT, and TK contributed to the statistical analyses. All authors contributed to the interpretation of the results.

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