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Long-term eradication of extranodal natural killer/T-cell lymphoma, nasal type, by induced pluripotent stem cell-derived Epstein-Barr virus-specific rejuvenated T cells *in vivo*

Miki Ando,[1,2] Jun Ando,[1] Satoshi Yamazaki,[2] Midori Ishii,[1] Yumi Sakiyama,[2] Sakiko Harada,[1] Tadahiro Honda,[1] Tomoyuki Yamaguchi,[2] Masanori Nojima,[3] Koichi Ohshima,[4] Hiromitsu Nakauchi,[2,5*] and Norio Komatsu[1*]

[1]Department of Hematology, Juntendo University School of Medicine, Tokyo, Japan; [2]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [3]Center for Translational Research, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [4]Department of Pathology, School of Medicine, Kurume University, Fukuoka, Japan and [5]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA

**HN and NK are co-last authors*

ABSTRACT

Functionally rejuvenated induced pluripotent stem cell (iPSC)-derived antigen-specific cytotoxic T lymphocytes (CTL) are expected to be a potent immunotherapy for tumors. When L-asparaginase-containing standard chemotherapy fails in extranodal natural killer/T-cell lymphoma, nasal type (ENKL), no effective salvage therapy exists. The clinical course then is miserable. We demonstrate prolonged and robust eradication of ENKL *in vivo* by Epstein-Barr virus-specific iPSC-derived antigen-specific CTL, with iPSC-derived antigen-specific CTL persisting as central memory T cells in the mouse spleen for at least six months. The anti-tumor response is so strong that any concomitant effect of the programmed cell death 1 (PD-1) blockade is unclear. These results suggest that long-term persistent Epstein-Barr virus-specific iPSC-derived antigen-specific CTL contribute to a continuous anti-tumor effect and offer an effective salvage therapy for relapsed and refractory ENKL.

Correspondence:

MIKI ANDO
m-ando@juntendo.ac.jp

HIROMITSU NAKAUCHI
nakauchi@stanford.edu

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Introduction

ENKL, a highly aggressive disease, is relatively common in Asia and South America. Necrosis is extensive and dissemination to various sites is rapid. The outcome is miserable.[1,2] Expressing high concentrations of multidrug-resistance P-glycoprotein, ENKL cells resist anthracycline-based standard chemotherapy.[3] L-asparaginase selectively induces apoptosis in ENKL,[4] and the L-asparaginase-containing regimen SMILE (dexamethasone ["steroids"], methotrexate, ifosfamide, L-asparaginase, etoposide) prolongs survival in advanced ENKL.[5,6] However, even with SMILE, 5-year overall survival is 47%.[5] No effective salvage regimen exists. Development of such a regimen is thus an urgent issue.

Antigen-specific CTL therapy can induce durable remission in selected tumors such as melanomas.[7-10] ENKL cells are invariably infected by Epstein-Barr virus (EBV) with type II latency; they express the EBV antigens latent membrane protein (LMP) 1 and LMP2 (LMP1/2). As T cells specific for these antigens are infrequent and often are anergic in the tumor microenvironment, ENKL should be a good target for CTL therapy directed against LMP1 and LMP2.[11-16] However, CTL continuously exposed to viral or tumor antigens become exhausted.[17]

Exploiting fully rejuvenated CTL innovatively overcomes CTL exhaustion. We generated antigen-specific CTL directed against LMP1 and LMP2 from iPSC established from peripheral blood-derived antigen-specific CTL.[18-20] The iPSC-derived CTL have the same antigen specificity as the original CTL. As these redifferentiated CTL have a higher proliferative capacity, younger memory phenotype, and

longer telomeres than the original CTL, iPSC-derived CTL are functionally rejuvenated T cells (rejT).[17] The rejT that we generated have strong anti-tumor effects against EBV-infected lymphoblastoid cells (LCL) *in vivo* and in mice they confer a survival advantage compared to mice treated using original CTL.[19] Hence, rejT therapy directed against LMP1 and LMP2 is expected to be useful as a salvage therapy for ENKL in which SMILE has failed.

Another factor in ENKL prognosis is the PD-1 pathway for immunoevasion.[21-23] EBV-associated lymphoma cells often express the PD-1 ligand (PD-L1).[24-26] The complete remission (CR) rate is high after PD-1 blockade with pembrolizumab in ENKL in which L-asparaginase therapy has failed.[27]

We sought to demonstrate the effectiveness of rejT therapy targeting ENKL. We also investigated additive anti-tumor effects of PD-1 blockade in conjunction with rejT therapy. Both rejT and original CTL showed robust tumor-suppressive effects against ENKL *in vitro* and *in vivo*. However, only LMP2-specific rejT significantly prolonged long-term survival in ENKL-bearing mice. This effect was so strong that any additive anti-tumor effect of PD-1 blockade was overwhelmed. LMP1- and LMP2-specific rejT therapy seems a promising salvage therapy for ENKL refractory to SMILE.

Methods

More detailed information can be found in the *Online Supplementary Data.*

Patients and samples

We reviewed 28 biopsy samples from 24 patients diagnosed with ENKL at the Juntendo University School of Medicine, Department of Hematology, between 2006 and 2017. The use of the material and clinical information was approved by the Research Ethics Committee for the Faculty of Medicine, Juntendo University, and was in accordance with the Declaration of Helsinki.

Immunohistochemical staining

Tissue samples were fixed in formalin and embedded in paraffin. Anti-PD-L1 rabbit monoclonal antibodies (EPR1161[2]; 1:200 dilution; ab174838, Abcam, Cambridge, MA), anti-PD-1 mouse monoclonal antibodies (NAT105; 1:100 dilution; ab52587, Abcam), and anti-CD3 rabbit monoclonal antibodies (SP7; 1:50 dilution; ab16669, Abcam) were used for immunostaining.

Generation of LMP1/2-specific CTL and establishment of T-iPSC

LMP1/2-specific CTL were generated using peripheral blood mononuclear cells (PBMC) obtained from two human leukocyte antigen (HLA)-A*2402-expressing healthy donors and one HLA-A*0201-expressing ENKL patient. Selected clones were transduced with Sendai virus vectors to establish T-iPSC.

T-cell differentiation from T-iPSC

To differentiate human iPSC into hematopoietic cells, small clumps of iPSC were transferred onto C3H10T1/2 cells. Hematopoietic cells collected from iPSC sac contents were transferred onto DL1/4-expressing C3H10T1/2 feeder cells. T-lineage cells were then harvested and stimulated. The antigen specificity of LMP1/2-specific rejT was determined by staining with LMP1/2 tetramer.

Cell lines

ENKL cells (NK-YS and SNK-6 lines) were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of interleukin-2 (IL-2) and in NS-A2 (GreenDay) supplemented with 100 U/mL of IL2, respectively.[4,28]

^{51}Cr release assays

Cytotoxic specificity of EBV-CTL and EBV-rejT directed at LMP1/2 antigen was analyzed in a standard 4-hour ^{51}Cr- release assay at different effector : target ratios (E:T; 40:1, 20:1, 10:1 and 5:1) and using a γ counter (PerkinElmer, Waltham, MA).[29] To elucidate whether PD-L1 blockade can enhance the killing potential of EBV-CTL and EBV-rejT against ENKL, NK-YS cells were cultured with 10 μg/mL of anti-PD-L1 antibody (Ultra-LEAF™ purified anti-human CD274, BioLegend) for three days preceding the assay.

Antitumor activity *in vivo* model

To evaluate the antitumor effects of LMP2-CTL and LMP2-rejT against ENKL, cells from an HLA class I-matched ENKL line, NK-YS, that had been transduced with a γ-retroviral vector encoding the fusion protein GFP/FFluc were sorted for GFP expression by flow cytometry. Six-week-old female NOD/Shi-scid, IL-2RγKO Jic (NOG) mice (In-Vivo Science, Tokyo, Japan) were engrafted intraperitoneally with NK-YS (1×10^5 cells/mouse) and tumor growth was monitored using the Xenogen-IVIS Imaging System (Xenogen, Alameda, CA, USA). Once a progressive increase of bioluminescence occurred, usually four days after tumor inoculation, mice were treated intraperitoneally with three once-weekly doses of 5×10^6 LMP2-rejT ± 50 μg of anti-PD-1 Ab or with 5×10^6 original LMP2-CTL ± 50 μg of anti-PD-1 Ab (In VivoMAb anti-h PD-1, BioXCell, West Lebanon, NH, USA).

PCR and sequencing

EBV strain typing of the NK-YS cells in ascites was performed by PCR using LMP2-specific primers 5'-TATGAATCCAGTATGCCTGC-3' and 5'-CGCAGTAAGCACTGTCACCG-3' as described[29] to detect LMP2 epitopes that are associated with HLA-A*2402.

Results

Distinct PD-L1 expression was significantly associated with poor prognosis in ENKL

To understand the microenvironment of ENKL cells, ENKL cells and tumor-infiltrating lymphocytes (TIL) were analyzed by immunohistochemical staining for PD-L1 and PD-1 receptor expression respectively. As EBV *in situ* hybridization demonstrated infection in all 28 cases, we initially expected high PD-L1 expression. However, the proportion of ENKL cells (PD-L1 expression ratio, lymphoma cell : macrophage) was 5-10% (+, positive) in only three samples, 2-3% (+/-, weakly positive) in seven, 1% (-/+, slightly positive) in one, and negative in 17 (Figure 1 A-B).

The presence of many PD-1-expressing TIL is associated with favorable overall survival (OS) in patients with diffuse large B-cell lymphoma.[26] It is noteworthy that PD-1$^+$ TIL were rarely observed in ENKL, with only 1% of PD-1$^+$ TIL seen in only two of 28 samples (Figure 1 C-D). Table 1 summarizes the characteristics of patients diagnosed with ENKL in our institute. Three patients whose lymphoma cells expressed PD-L1 (5-10%) were in stage IV

(3 of 3). Of these, two were refractory to initial therapy. They survived for only one month (2 of 3) and 4 months (1 of 3). The lymphoma cells of these three patients did not express LMP1. Statistical analysis showed that distinct PD-L1 expression of ENKL was significantly correlated with poor prognosis (P=0.001). Even in advanced-stage disease, PD-L1 expression was significantly associated with poor OS (P=0.031) (Figure 1E). Another factor conferring poor OS in ENKL was the lack of LMP1 expression (P=0.009) (Figure 1F). Treatment with SMILE was associated with favorable OS in advanced-stage disease (P=0.008) (Figure 1G), whereas plasma EBV DNA positivity did not significantly affect OS in these ENKL patients (P=0.181) possibly due to the small number of EBV-DNA negative group (Figure 1H). Table 2 shows the results of cross-tabulation analysis of the correlations between PD-L1 expression and variables including age, sex, clinical stage, EBV DNA titre, SMILE history, CR, and LMP1

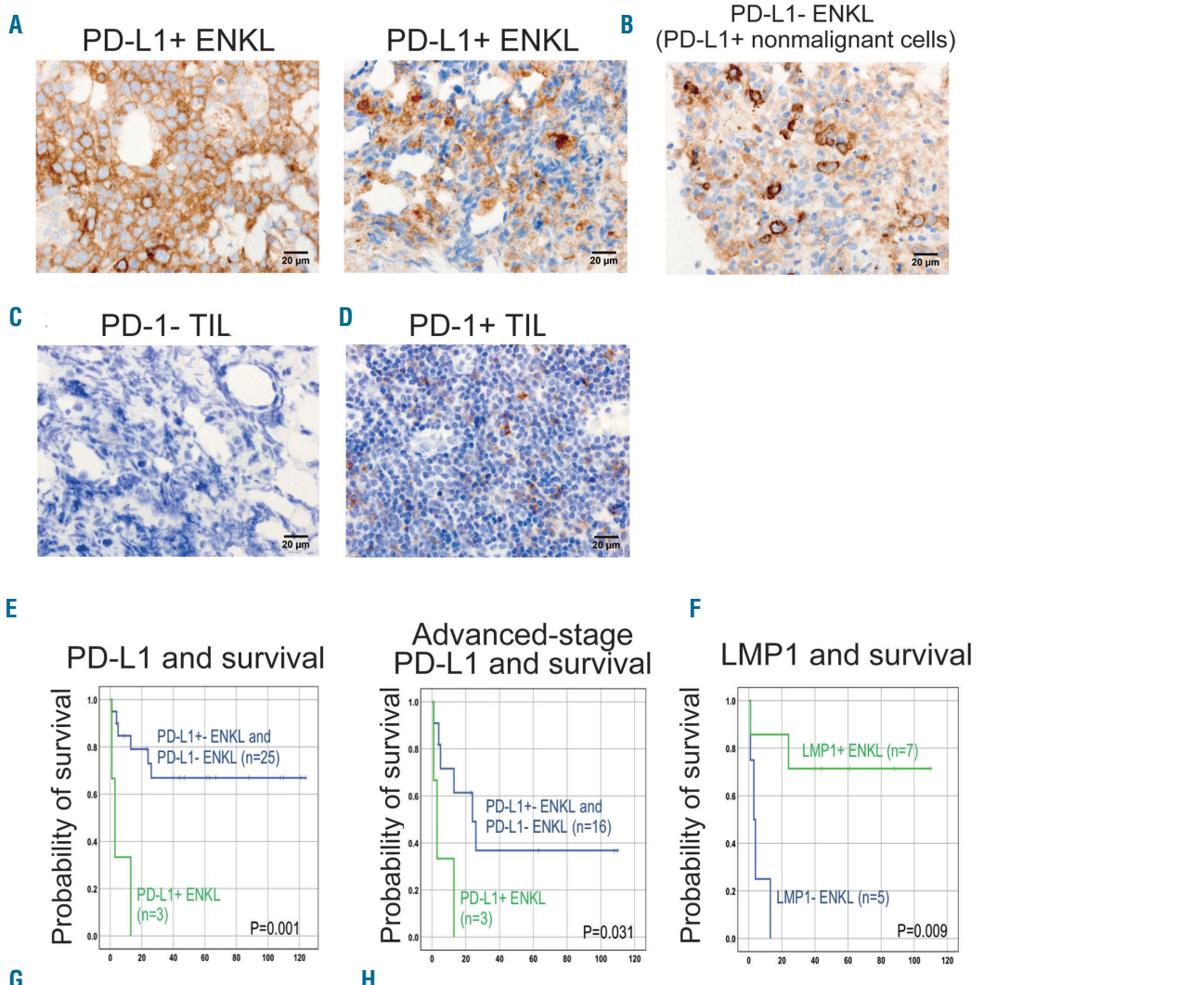

Figure 1. Representative immunohistochemical features, analysis of programmed death-ligand 1 expression in extranodal NK/T-cell lymphoma, nasal type (ENLK), and overall survival in patients with ENKL according to various factors. (A) PD-L1$^+$ ENKL cells expressed PD-L1 (brown). (B) PD-L1$^+$ nonmalignant cells were identified. ENKL cells did not express PD-L1. (C) PD-1+ TILs were not observed in most ENKL cases. (D) PD-1+ TILs (1%) were observed only in two ENKL cases. (E) Overall survival (OS) of patients among the two PD-L1 expressing groups (positive group vs weakly positive and negative groups) (the entire study cohort: left; advanced stage cohort: right). (F) OS of patients according to LMP1 positivity. (G) OS of patients in advanced stage who received dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE) therapy. (H) OS of patients according to EBV DNA positivity. PD-L1: programmed death-ligand 1; TIL: tumor-infiltrating lymphocytes; PD-1: programmed cell death 1; LMP: latent membrane protein; EBV: Epstein-Barr virus.

expression. In ENKL cells, lack of LMP1 expression was significantly correlated with PD-L1 expression.

These analyses revealed that distinct PD-L1 expression by ENKL was strongly associated with poor prognosis. CTL therapy directed at LMP1/2 and combined with PD-1/PD-L1 axis blockade is therefore suggested as useful for the treatment of refractory ENKL.

Both LMP1-specific and LMP2-specific rejT showed strong cytotoxicity against ENKL *in vitro*

To examine the cytotoxicity of EBV-specific rejT against ENKL, we first determined the HLA type of the ENKL cell line NK-YS (HLA-A*2402) and SNK6 (HLA-A*02). A*2402- and A*0201-restricted LMP1/2-specific CTL clones were generated from healthy donors expressing A*2402 and an ENKL patient (Pt) (Pt 6) who expresses HLA-A*0201. T-iPSC were established from these clones and were redifferentiated into rejT with antigen specificities that matched those of the respective clones (Figure 2 A-B). We assayed the cytotoxicity of A*2402-restricted LMP2-rejT (TYGPVFMSL), A*0201-restricted LMP1-rejT (YLQQNWWTL), and A*0201-restricted LMP2-rejT (FLYALAL) against HLA-matched ENKL by ^{51}Cr release. Strong killing by A*2402-restricted LMP2-rejT was shown against HLA-A*2402^{+} NK-YS cells (85.3%, 84.7%, 85.2%, and 83.7%; effector : target [E:T] ratios of 40:1, 20:1, 10:1 and 5:1), but not against HLA-A*2402^{-} tumors (18.4%, 4.8%, 1.9%, and 2.4%; E:T ratios of 40:1, 20:1, 10:1 and 5:1). A*0201-restricted LMP1-rejTs also demonstrated strong cytotoxic activity against HLA-A*02^{+} SNK6 cells (72.0%, 69.9%, 70.4%, and 65.1%; E:T ratios of 40:1, 20:1, 10:1 and 5:1), but not against HLA-A*02^{-} tumors (-2.3%, -1.7%, 1.3%, and 4.8%; E:T ratios of 40:1, 20:1, 10:1 and 5:1). HLA-A*0201-restricted LMP2-rejT exhibited 76.6% and 24.7% killing at E:T ratios of 40:1 and 20:1 against HLA-A*02^{+} SNK6 cells, with 14.9% and 14.6% killing for

Table 1. Extranodal NK/T-cell lymphoma, nasal type: patient characteristics.

	Age (Years)	Sex	Stage	EBV-DNA (copies/mL)	Treatment	Response to treatment	Survival duration (months)	PD-L1 lymphoma cells	PD-L1 macrophages	PD-1 TILs	LMP1	EBNA2
1	41	F	IVB	1.9x10^{3}	No treatment	No treatment	1	+	+ +	−	−	−
2	29	M	IVB	2.0x10^{6}	SMILE	refractory	4	+	+	−	−	−
3	74	M	IVA	2.3x10^{2}	DEX, VP-16	refractory	1	+	+ −	−	−	−
4	73	F	IVA	2.4x10^{1}	RT-DeVIC, SMILE	CR	>63	+ −	+ −	−	N.D.	−
5	32	M	IVB	not detected	SMILE, Allo BMT	CR	>24	+ −	+	−	N.D.	N.D.
6	74	M	II A	not detected	RT-DeVIC	CR	>10	+ −	+ +	1 %	N.D.	N.D.
7	53	M	IVA	N.D.	RT-DeVIC, ICE	refractory	24	+ −	+ −	−	+	−
8		M	IVA	8.1x10^{2}	MILD, Allo BMT, DLI	refractory		+ −	+ −	−	−	−
9	32	M	II A	N.D.	RT-DeVIC	CR	>60	+ −	+	−	N.D.	N.D.
10	32	M	IVB	not detected	SMILE, Auto PBSCT	CR	>110	+ −	+	−	+	N.D.
11	71	F	I B	N.D.	RT-DeVIC	CR	>124	− +	+ −	−	N.D.	N.D.
12	31	M	II A	2.5x10^{1}	SMILE, RT	CR	>44	−	+ −	−	+	+
13	57	F	II A	1.7x10^{2}	RT-DeVIC	CR	>61	−	+ −	−	+	−
14	29	M	IVA	N.D.	SMILE, Auto PBSCT	CR	4	−	+ −	−	N.D.	N.D.
15	41	M	IVA	5.3x10^{5}	RT-DeVIC, MILD	refractory	6	−	+ −	−	N.D.	N.D.
16		M						−	+ −	−	N.D.	N.D.
17		M		9.9x10^{2}				−	+ −	−	N.D.	N.D.
18	78	M	IVA	1.1x10^{4}	MILD	unknown	unknown	−	−	−	N.D.	N.D.
19		M	IVA					−	+	−	N.D.	N.D.
20	84	F	IVB	4.5x10^{3}	RT-DeVIC	unknown	1	−	+	−	+	N.D.
21	46	M	IVB	1.3x10^{4}	SMILE, Allo BMT	CR	>108	−	+ −	−	N.D.	N.D.
22	32	F	I A	2.3x10^{1}	RT-DeVIC	CR	>66	−	+ −	−	N.D.	N.D.
23	33	M	IVA	N.D.	SMILE, Auto PBSCT	CR	24	−	+	−	N.D.	−
24	28	M	I A	N.D.	RT-DeVIC	CR	>40	−	+	−	+	−
25	73	M	I A	N.D.	RT-DeVIC	CR	>88	−	+	−	+	−
26	62	F	IVA	N.D.	RT-DeVIC, HD-MTX	refractory	12	−	− +	1 %	N.D.	N.D.
27	71	F	IVB	N.D.	ESHAP, CHOP	refractory	4	−	+ −	−	−	−
28	65	M	I A	N.D.	RT-DeVIC	CR	>120	−	+ +	−	N.D.	N.D.

ENKL: extranodal NK/T cell lymphoma, nasal type; EBV: Epstein-Barr virus; PD-1: programmed cell death 1; PD-L1: programmed death-ligand 1; LMP: latent membrane protein; EBNA: Epstein-Barr nuclear antigen; SMILE: dexamethasone, methotrexate, ifosfamide, L-asparaginase and etoposide; DEX: dexamethasone; VP-16, etoposide; RT: radiation therapy; DeVIC: dexamethasone, etoposide, ifosfamide and carboplatin; Allo BMT: allogenetic bone marrow transplantation; ICE: ifosfamide, carboplatin and etoposide; Auto PBSCT: autologous peripheral blood stem cell transplantation; MILD: methotrexate, ifosfamide, L-asparaginase and dexamethasone; HD-MTX: high dose methotrexate; ESHAP: etoposide, methylprednisolone, high dose cytarabine and cisplatin; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone; CR: complete remission; N.D., not done.

HLA-A*02$^-$ tumors. These results indicated that LMP1-specific and LMP2-specific rejT generated from multiple donors, including an ENKL patient, had strong antigen-specific cytotoxicity against ENKL.

LMP1-specific and LMP2-specific rejT tended to show stronger cytotoxicity against EBV$^+$ tumor cells than the original CTL clone *in vitro*

We next compared rejT cytotoxicity against EBV$^+$ tumor cells with that of original CTL by ^{51}Cr release assay. A*2402-restricted LMP2-rejT generated from a healthy donor more efficiently killed autologous EBV-infected LCL (70.4% and 65.4%; E:T ratios of 20:1 and 10:1) compared to the original CTL clone (51.7% and 49.4%; E:T ratios of 20:1 and 10:1). A*0201-restricted LMP1-rejT generated from an ENKL patient showed somewhat stronger cytotoxicity against autologous EBV-infected LCL (90.3%, 90.0%, 77.8%, and 58.8%; E:T ratios of 40:1, 20:1, 10:1 and 5:1) than the original CTL clone (77.8%, 61.9%, and 43.8%; E:T ratios of 20:1, 10:1 and 5:1). The cytotoxicity of A*0201-restricted LMP2-rejT against autologous EBV-infected LCL (44.0% and 34.5%; E:T ratios of 40:1 and 20:1) was almost the same as that of the original CTL clone (40.4% and 33.7%; E:T ratios of 40:1 and 20:1). We further compared PD-1 expression of peripheral blood-derived original EBV-CTL and of rejT from the same LMP2-CTL clone, measured by flow cytometry: values were 15.3% for EBV-CTL and undetectable for rejT (Figure 3B). PD-L1 expression of ENKL cells, measured by flow cytometry, was 57.4% (Figure 3C). Although both LMP2-specific rejT and original LMP2-CTL (PYLFWLAAI) powerfully killed HLA class I-matched NK-YS cells, rejT cytotoxicity tended to be stronger (60.8%, 52.6%, 52.6%, and 40.1%; E:T ratios of 40:1, 20:1, 10:1 and 5:1 specific ^{51}Cr release, respectively) than that of the original CTL (59.7%, 50%, 40.9%, and 39.8%; E:T ratios of 40:1, 20:1, 10:1 and 5:1 specific ^{51}Cr release, respectively) (Figure 3D). To elucidate whether PD-L1 blockade can enhance the potential to kill ENKL of the original CTL that express PD-1$^+$ and of rejT that do not express PD-1$^-$, ENKL cells were cultured with 10 μg/mL of anti-PD-L1 antibody for three days immediately preceding the assay. Anti-PD-L1 antibody did not clearly enhance killing by either PD-1$^+$ original CTL or PD-1$^-$ rejT (Figure 3D). Our results demonstrated definite cytotoxic activity against EBV-infected tumor cells *in vitro* for both EBV-specific original CTL and rejT, with cytotoxicity of rejT against ENKL cells stronger than that of original CTL and without killing enhancement in original CTL or rejT cultured with anti-PD-L1 antibody.

Robust anti-ENKL effect and marked survival improvement of LMP2-rejT *in vivo*

ENKL cells express not only PD-L1 but also PD-L2 (Figure 3C). Anti-PD-1 antibody that can block both PD-L1 and PD-L2 was thus potentially more effective than anti-PD-L1 antibody as therapy for ENKL. Using PD-1 blockade to observe whether EBV-specific rejT exert ENKL-suppressive effects *in vivo* and anti-PD-1 antibody has an additive antitumor effect, ENKL cells labeled with retrovirus-derived firefly luciferase were intraperitoneally engrafted into NOG mice ($1x10^5$ cells/mouse). Light emission was monitored as an indicator of tumor growth. Four days after tumor inoculation, these mice were divided into two control groups and four treatment groups. No treatment was given in one control group (n=6) and only anti-PD-1 antibody was given in the other (200 μg per dose, three doses) (n=6). The treatment groups consisted of mice treated with original LMP2-CTL ($5x10^6$ per dose, three doses) (n=6); mice treated with original LMP2-CTL and anti-PD-1 antibody ($5x10^6$ of original CTL and 200 μg of anti-PD-1 antibody per dose, three doses) (n=6); mice treated with LMP2-specific rejT ($5x10^6$ per dose, three doses) (n=6); and mice treated with LMP2-specific rejT and anti-PD-1 antibody ($5x10^6$ of rejT and 50 μg of anti-PD-1 antibody per dose, three doses) (n=5).

By day 21, bioluminescence had progressively increased in the control-group mice (no treatment, 176.8-fold, range 7.33-326.0; anti-PD-1 Ab, 53.3-fold, range 15.5-101.0) (Figure 4A-B). In contrast, tumor suppressive effects were observed in the original CTL group (6.025-fold, range 1.27-10.4) and in the original CTL with anti-PD-1 Ab group (17.5-fold, range 0.71-51.1), with and even stronger suppressive effects in the rejT group (0.505-fold, range 0.31-0.816) as well as in the rejT with anti-PD-1 Ab group (0.71-fold, range 0.50-1.10). Tumor signals regressed further in the four groups treated with original CTL, original CTL with anti-PD-1 antibody, rejT, or rejT with anti-PD-1 antibody than in the untreated group (original CTL, P=0.0001; original CTL with anti-PD-1 antibody, P=0.0003; rejT, P<0.0001, and rejT with anti-PD-1 anti-

Table 2. PD-L1$^+$, PD-L1$^{+/-}$, and PD-L1$^-$ extranodal NK/T-cell lymphoma nasal type: patient characteristics.

	PD-L1 + N=3		PD-L1 +/- N=8		PD-L1 - N=17		*P* value
	mean/N	SD/%	mean/N	SD/%	mean/N	SD/%	
Age	48.00	23.30	52.43	20.35	52.14	20.12	0.945
Sex	1/3	33.3%	2/8	25.0%	5/17	29.4%	1.000
Advanced	3/3	100.0%	5/8	62.5%	11/17	64.7%	0.566
EBV DNA-positive	3/3	100.0%	2/5	40.0%	8/8	100.0%	0.707
SMILE	1/3	33.3%	5/8	62.5%	9/17	52.9%	1.000
CR	0/3	0.0%	6/8	75.0%	9/17	52.9%	0.590
LMP1	0/3	0.0%	2/3	66.7%	5/6	83.3%	0.038
Observation period (month)	5.67	6.43	57.14	44.69	44.77	41.48	

Programmed death ligand-1 (PD-L1); ENKL: extranodal NK/T-cell lymphoma, nasal type; EBV: Epstein-Barr virus; SMILE: dexamethasone, methotrexate, ifosfamide, L-asparaginase and etoposide; CR: complete remission; PD-L1: programmed death-ligand 1; LMP: latent membrane protein; SD: standard deviation.

body, *P*=0.0002; one-way ANOVA). Treatment enhancement was not clearly observed with concomitant anti-PD1 antibody therapy either in the original CTL group (*P*>0.99, one-way ANOVA) or in the rejT group (*P*>0.99, one-way ANOVA). These three-week observations *in vivo* demonstrated the tumor suppressive effects against ENKL cells of the original CTL treatment, with more pronounced anti-tumor effects of the rejT treatment.

On long-term observation, original CTL did not prolong survival *versus* no-treatment controls (*P*=0.09). Treatment with rejT markedly prolonged survival (mean 239 days, 58-296 days) compared to treatment with original CTL (*P*=0.03, mean 74.5 days, 58-140 days), with original CTL with anti-PD-1Ab (*P*=0.01, mean 45.5 days, 34-98 days), and with no treatment (*P*=0.01) (Figure 4C). RejT with anti-PD-1 Ab also significantly improved survival (mean 134 days, 72-204 days) compared to treatment with original CTL with anti-PD-1 Ab (*P*=0.008) and with no treatment (*P*=0.004, mean 58 days, 42-84 days).

No significant survival advantage was observed for the

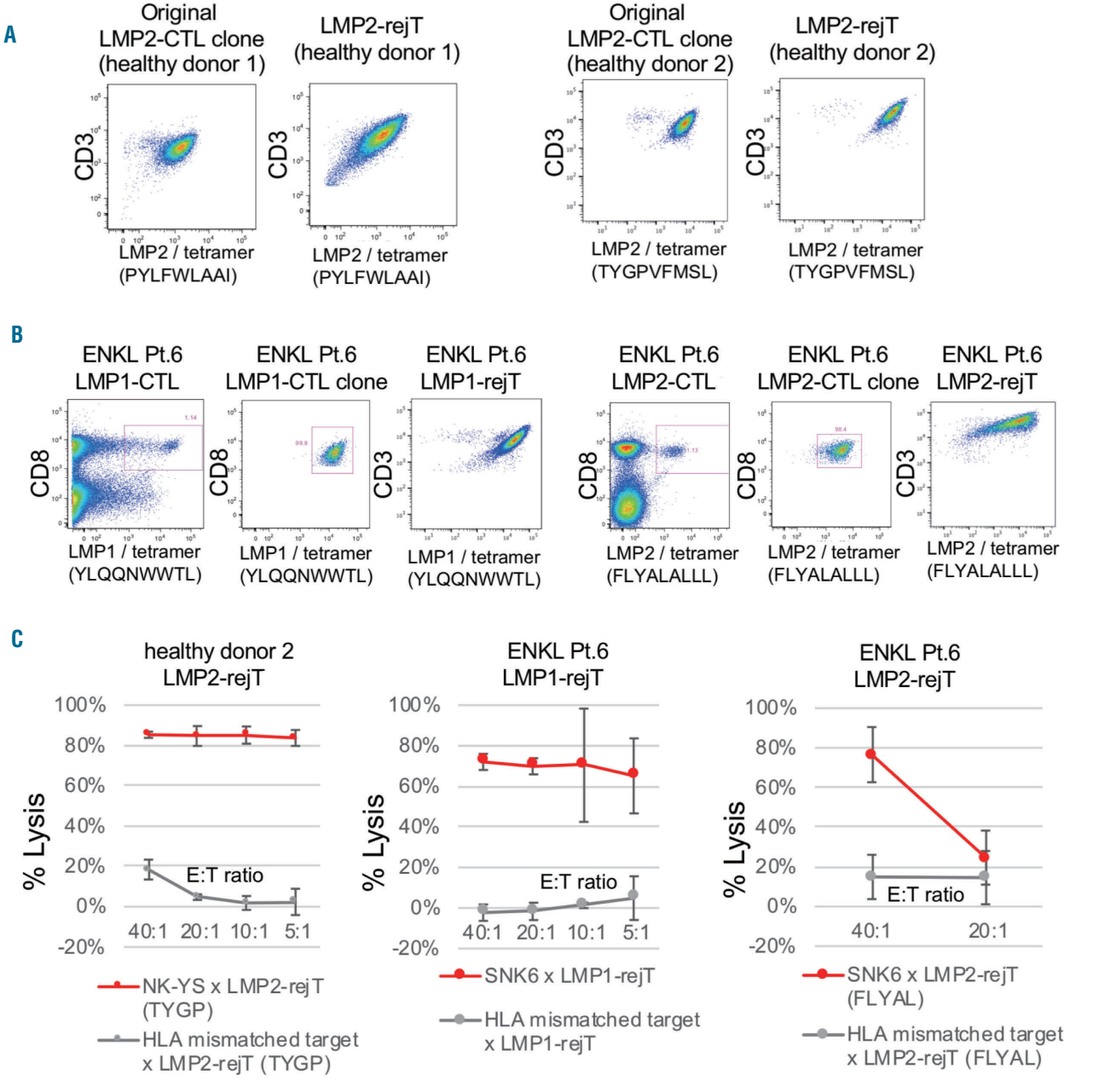

Figure 2. Extranodal NK/T-cell lymphoma, nasal type cell lines are sensitive to killing by induced pluripotent stem cell-derived LMP1- and LMP2-rejT *in vitro*. (A) Flow cytometric EBV LMP2 tetramer analysis of original peripheral blood-derived EBV-CTL and iPSC-derived-EBV-rejT generated from healthy donors. (B) Flow cytometric EBV LMP1 and LMP2 tetramer analysis of original peripheral blood-derived EBV-CTL and iPSC-derived-EBV-rejT generated from an ENKL patient (Pt 6). (C) *In vitro* ^{51}Cr release assay of LMP1- and LMP2-rejT (effectors) against ENKL (targets) and HLA mismatched LCL (control targets). ENKL: extranodal NK/T-cell lymphoma, nasal type; iPSC: induced pluripotent stem cell; LMP: latent membrane protein; rejT: rejuvenated cytotoxic T lymphocytes; EBV: Epstein-Barr virus; Pt: patient; CTL: cytotoxic T lymphocytes; HLA: human leukocyte antigen; LCL: EBV-infected lymphoblastoid cells.

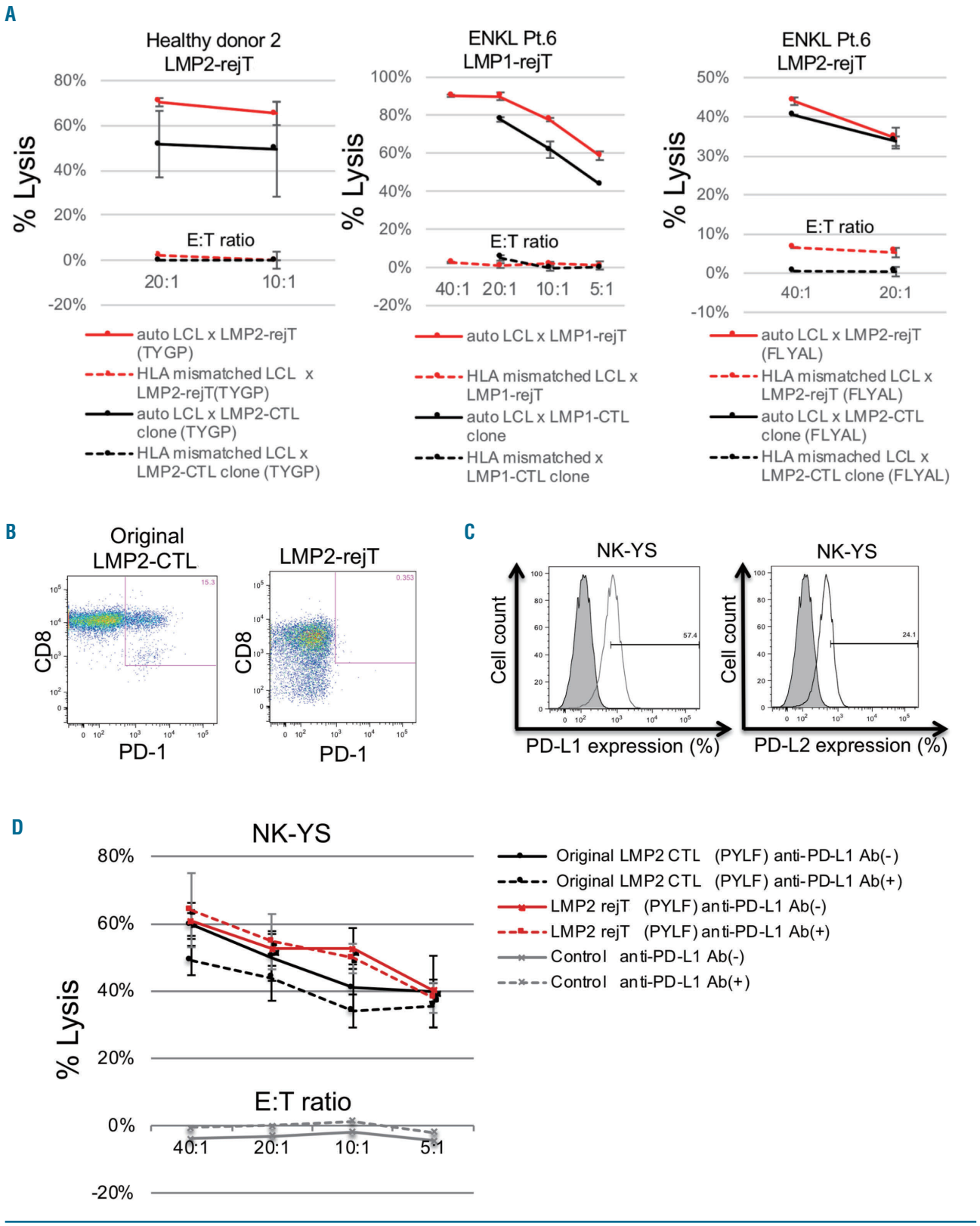

Figure 3. Induced pluripotent stem cell-derived LMP1$^-$ and LMP2-rejT show cytotoxicity against Epstein-Barr virus-infected-infected tumors equal to or greater than original cytotixic T lymphocytes *in vitro*. (A) *In vitro* ^{51}Cr release assay of LMP1- and LMP2-rejT (effectors) and original CTL clones (effectors) against auto LCL (targets) and HLA-mismatched LCL (control targets). (B) Flow cytometric analysis of PD-1 expression of original LMP2-CTLs and iPSC-derived LMP2-rejT. (C) Flow cytometric analysis of PD-L1 and PD-L2 expression on the ENKL cell line NK-YS. (D) *In vitro* ^{51}Cr release assay of original LMP2-CTL, LMP2-rejT (effectors), and HLA-mismatched T cells (control effector) against ENKL (targets). Anti-PD-L1 Ab(+), ENKL cells were cultured with 10 μg/mL of anti-PD-L1 antibody for three days until the assay was conducted. Anti-PD-L1 Ab(-), ENKL cells were cultured without anti-PD-L1 antibody. Data are presented as mean ± SD and represent at least three independent experiments. E:T ratio: effector : target ratio; iPSC: induced pluripotent stem cell; LMP: latent membrane protein; rejT: rejuvenated cytotoxic T lymphocytes; ENKL: extranodal NK/T-cell lymphoma nasal type; CTL: cytotoxic T lymphocytes; Pt: patient; HLA: human leukocyte antigen; LCL: EBV-infected lymphoblastoid cells; PD-1: programmed cell death 1; PD-L1: programmed death-ligand 1; PD-L2: programmed death-ligand 2; Ab: antibody; SD: standard deviation.

combined use of anti-PD-1 Ab with either original CTL (P=0.13) or rejT (P=0.37). Collectively, although both original CTL and rejT exhibited strong anti-tumor effects against ENKL during the three-week observation period, only LMP2-rejT, whether or not anti-PD-1 Ab was given, distinctly improved long-term survival in ENKL-bearing mice.

LMP2-rejT treatment completely eliminated ENKL and rejT persisted in long-term survivor mice

We euthanized mice engrafted with ENKL tumors and that had survived 211 days after the fisrt injection of LMP2-rejT ± anti-PD-1 Ab. The heart, lungs, esophagus, stomach, pancreas, liver, colon, kidneys, spleen, and bone

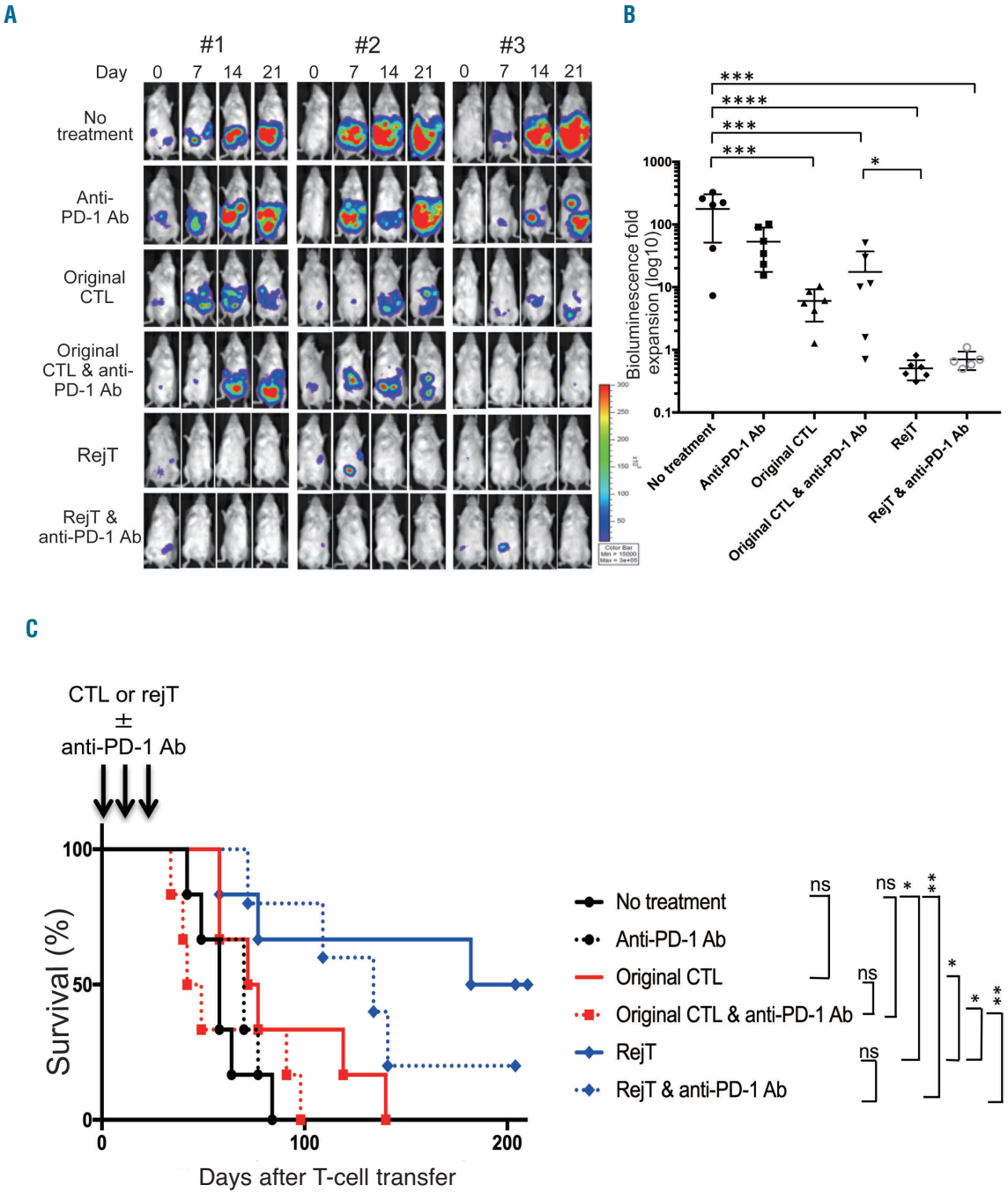

Figure 4. Induced pluripotent stem cell-derived-derived LMP2-rejT display superior anti-extranodal NK/T-cell lymphoma, nasal type activity *in vivo*. (A) Bioluminescence imaging of mice treated either with original LMP2-CTL or LMP2-rejT. FFluc+ENKL-bearing mice were divided into six groups that received no treatment (n=6), anti-PD-1 Ab (n=6), original CTL (n=6), original CTL + anti-PD-1 Ab (n=6), RejT (n=6), or RejT + anti-PD-1 Ab (n=5). Images of three representative mice from each group are shown. (B) Quantification of total tumor growth on day 21 after treatment is represented as log10 signal change. Error bars represent ± SD. ****P<0.0001, ***P<0.001 and *P<0.05 by one-way ANOVA. (C) Kaplan-Meier survival curves representing percentage survival for treated and control mice: tumor only or treated with anti-PD-1 Ab, original CTL, original CTL + anti-PD-1 Ab, RejT, or RejT + anti-PD-1 Ab. **P<0.01 and *P<0.05 by the log-rank test. iPSC: induced pluripotent stem cell; LMP: latent membrane protein; rejT: rejuvenated cytotoxic T lymphocytes; ENKL: extranodal NK/T-cell lymphoma, nasal type; FFluc: firefly luciferase; CTL: cytotoxic T lymphocytes; PD-1: programmed cell death 1; Ab: antibody; SD: standard deviation.

marrow from each mouse were histopathologically examined. All were tumor free in gross and microscopic analysis (Figure 5A, upper row of panels).

After only three rejT injections ENKL tumors were entirely extirpated. By contrast, tissues from a mouse injected with original CTL that died 58 days after the first injection were densely infiltrated by ENKL cells: original LMP2-CTL could not eradicate ENKL cells *in vivo* long-term. To determine whether injected rejT or original CTL persisted long-term, spleen sections were evaluated immunohistochemically. Spleens of euthanized mice that had been treated with rejT, with or without anti-PD-1 Ab, were well-populated by human CD3$^+$ T cells. The spleen of the mouse treated with original CTL and dead at 58

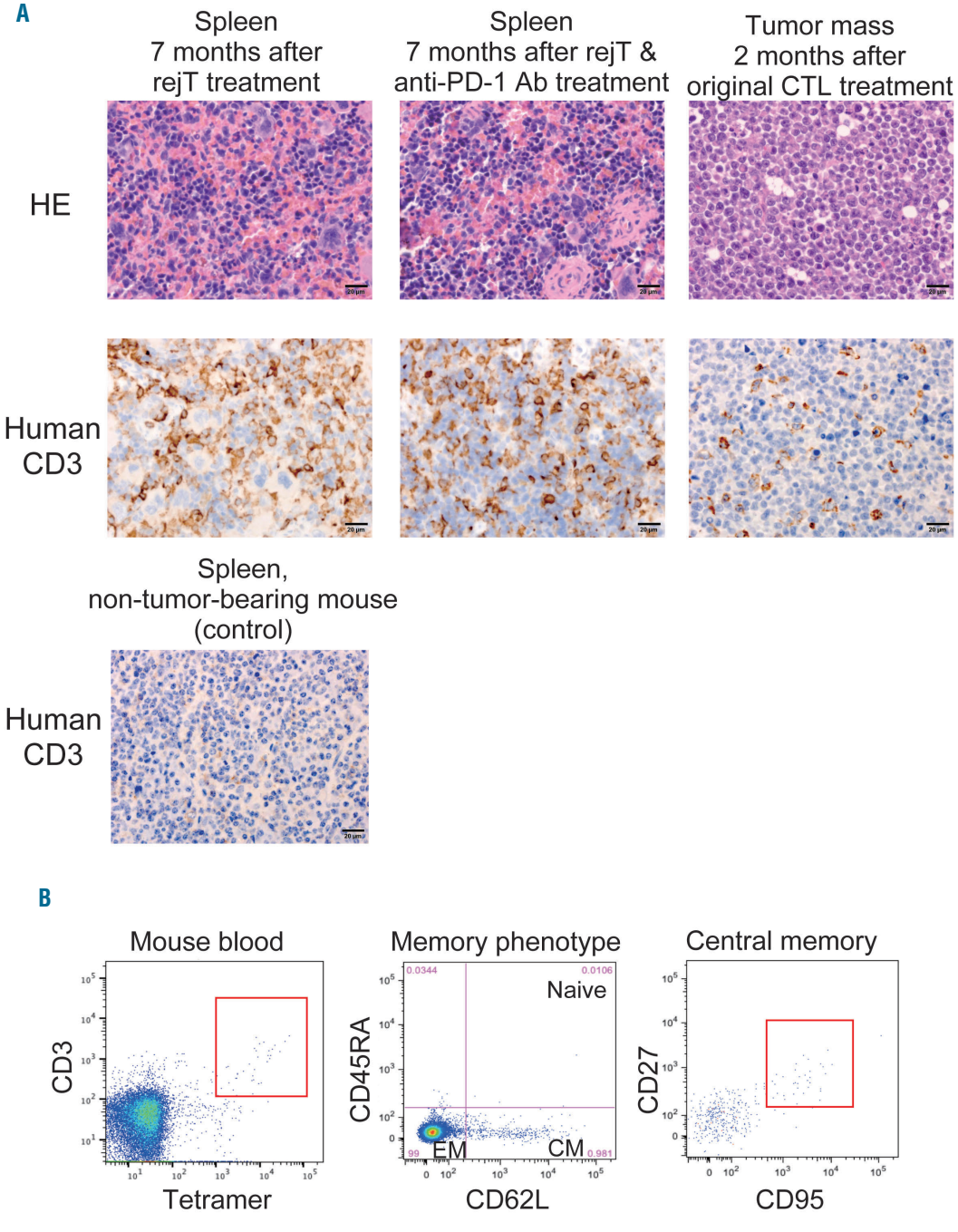

Figure 5. LMP2-rejT persisted in the spleen of long-surviving extranodal NK/T- cell lymphoma, nasal type-bearing mice. (A) Representative HE-stained sections of the spleens of ENKL-bearing mice treated with LMP2-rejT (upper left, center) and tumor mass of ENKL-bearing mice treated with original LMP2-CTL (upper right). Immunohistochemical study shows human CD3$^+$ T-cell infiltration of the spleen in ENKL-bearing mice treated with LMP2-rejT (lower left, center) and of tumor mass in ENKL-bearing mice treated with original CTL (lower right). Spleen sections from untreated mice without ENKL were used as negative control. The scale bar represents 20 µm. (B) Flow cytometric analysis of tetramer+CD3$^+$ LMP2-rejT population from peripheral blood. Central memory phenotype (CD45RA$^-$, CD62L$^+$, CD27$^+$, CD95$^+$) EBV-rejT are maintained in peripheral blood. LMP: latent membrane protein; rejT: rejuvenated cytotoxic T lymphocyte; ENKL: extranodal NK/T-cell lymphoma, nasal type; HE: hematoxylin and eosin; CTL: cytotoxic T lymphocyte; PD-1: programmed cell death 1; Ab: antibody; EBV: Epstein-Barr virus.

days, by contrast, contained many fewer human CD3+ T cells (Figure 5A, lower row of panels). Flow cytometry also was used to detect LMP2-rejT in the peripheral blood of ENKL-bearing mice treated using LMP2-rejT and surviving long-term. LMP2-tetramer – expressing CD3+ human T cells were present. Among them were effector memory (CD45RA-, CD62L-) and central memory phenotype T cells (CD45RA-, CD62L+, CD27+, CD95+) (Figure 5B).

Four of six mice that received rejT injections survived 182 to 296 days after treatment. Two relapsed, developing ascites before day 100. Tumor cells in ascitic fluid retained HLA class I expression (Figure 6A) without mutation in LMP2 (Figure 6B). Resistance to LMP2-rejT therapy in ascitic-fluid tumor cells was evaluated by ^{51}Cr release assay. EBV-rejT specific for LMP2 showed robust cytotoxicity (77%, 71.6%, 67.1%, and 51.2%; E:T ratios of 40:1, 20:1, 10:1 and 5:1 specific ^{51}Cr release, respectively) against ENKL cells in ascites (Figure 6C). These findings indicated that mutant ENKL cells did not appear after three rejT treatments and that the tumor cells had not developed resistance to LMP2-rejT therapy.

Discussion

We sought to verify that iPSC-derived rejT therapy specific to LMP1/LMP2 antigen would be an effective salvage therapy for refractory and relapsed ENKL. Although L-asparaginase is a key drug for ENKL[4] and L-asparaginase-containing chemotherapies improve prognosis in advanced ENKL patients,[5,6] tumor in about half of these patients resists L-asparaginase therapy or relapses after remission, resulting in a miserable clinical course. Adoptive T-cell therapy using peripheral blood-derived EBV-specific CTL is clearly effective for EBV-driven lymphoproliferative diseases after hematopoietic stem cell transplantation.[11-14] As these lymphomas develop in immunosuppressive situations and show type III latency, EBV-specific CTL adjuvant therapy can induce durable remissions. However, EBV-associated lymphomas showing type II latency, such as ENKL, Hodgkin lymphoma, and diffuse large B-cell lymphomas, are weakly immunogenic, with lymphoma cells that express only LMP1 and LMP2 antigens.[15] Therefore, EBV-CTL therapy targeting EBV-associated lymphomas with type II latency is more

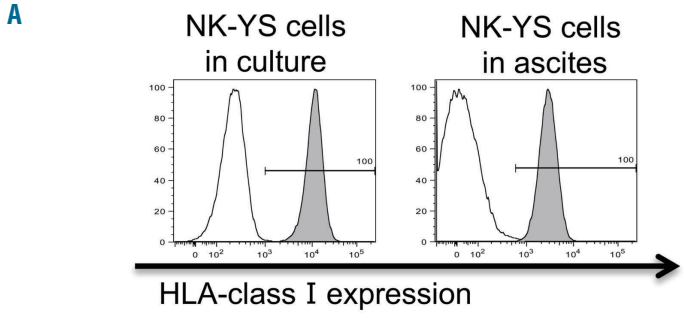

Figure 6. Phenotype of extranodal NK/T-cell lymphoma, nasal type cells in ascites of relapsed mice treated with LMP2-rejT. (A) Flow cytometric analysis of HLA class I (A, B, C) expression on NK-YS cells in ascites. (B) LMP2 sequence of post-rejT NK-YS cells in ascites. (C) *In vitro* ^{51}Cr release assay of EBV-rejT (effector), and HLA mismatched T cells (control effector) against NK-YS cells in culture and NK-YS cells in ascites (targets). Data are presented as mean ± SD. E:T ratio: effector : target ratio; ENKL: extranodal NK/T-cell lymphoma, nasal type; LMP: latent membrane protein; HLA: human leukocyte antigen; EBV: Epstein-Barr virus; SD: standard deviation.

challenging. Bollard and colleagues successfully demonstrated effective control EBV-associated lymphomas with either type II or type III latency using patients' own LMP1- and LMP2-specific T cells: among six NK/T cell lymphoma (EBV-infected) patients whose tumors had relapsed after standard chemotherapies and who had received LMP1 or LMP2-specific CTL, three remained in CR, with responses that were associated with percentages of effector and central memory LMP1-specific T cells in the infused population.[16] Functionally rejT differentiated from T-iPSC include younger phenotypes such as central memory and effector memory phenotypes and have longer telomeres and a stronger proliferation ability (100-fold to 1,000-fold after T-cell stimulation) than original peripheral-blood derived CTL.[18-20,30] Although the original CTL expressed PD-1 strongly, their redifferentiated LMP2-rejT descendants almost lacked PD-1 expression (Figure 3C). CTL generation from heavily treated patients is generally more difficult than from healthy donors because of T-cell exhaustion. Although CTL clones from an ENKL patient showed relatively strong cytotoxicity against EBV-infected autologous LCLs *in vitro* (Figure 3A), the proliferation ability of patient-derived CTL clones was lower than that of healthy donor-derived CTL clones, and much lower than that of rejT. LMP2-rejT had a distinct survival advantage in ENKL-bearing mice over original LMP2-CTL (Figure 4C). Histopathological examination revealed that LMP2-rejT completely eradicated ENKL and that LMP2-rejT persisted in the spleen of long-surviving ENKL-bearing mice, supporting our hypothesis that rejT contribute to ENKL eradication as long-lived memory T cells (Figure 5A). Furthermore, we actually confirmed the presence of central memory phenotype human T cells in the peripheral blood of a long-surviving ENKL-bearing LMP2-rejT treated mouse (Figure 5B). However, neither necrotic lesions nor features of activation of immune cells (which might lead to organ injury) were found in these long-surviving mice, suggesting that long-term persistence of rejT does not reduce safety in this *in vivo* model.

As EBV-associated lymphomas express PD-L1 more strongly than lymphomas without EBV infection,[23-26] we examined PD-L1 expression in ENKL cells. Our statistical analysis demonstrated that PD-L1 expression was clearly related to very poor prognosis in ENKL patients (Figure 2A). Therefore, we anticipated that blockade of PD-1 and PD-L1 engagement would reinforce the efficacy of treatments using EBV-specific CTL expressing PD-1 and possibly EBV-rejT not expressing PD-1 for PD-L1 - expressing ENKL. Contrary to our expectations, we could not observe clear treatment enhancement by anti-PD-1 Ab with either original EBV-CTL or EBV-rejT (Figure 4 B-C), suggesting that PD-1 blockade is not necessarily required in treatment of ENKL with EBV-specific T cells. Anti-PD-1 Ab reportedly had no measurable effect on chimeric antigen receptor (CAR) T-cell expansion, persistence, or circulating cytokine levels when it was administered in combination with CAR T cells and lymphodepletion.[31] In our study, EBV-rejT alone effectively ablated ENKL in tumor-bearing mice, with such strong anti-tumor effects that any additive beneficial effects of anti-PD-1 Ab were unclear. Of relevance is that toxicities of anti-PD-1 Ab may have impaired survival: disruption of the PD-1/PD-L1 pathway can lead to imbalances in immunologic tolerance, resulting in unchecked autoimmune-like/inflammatory side effects.[32] By contrast, viral-specific antigen-specific CTL therapy is minimally toxic and does not harm healthy tissues.[16] We postulate that also EBV-rejT therapy is free from severe adverse events and suggest that it will be highly effective against ENKL when L-asparaginase therapy has failed.

EBV-rejT still exerted strong cytotoxic effects against tumor cells in ascites from mice with relapse after EBV-rejT therapy. These tumor cells maintained HLA class I expression and harbored no LMP2 mutations (Figure 6). This suggests that EBV-rejT may not fully penetrate all areas of tissue metastasis. We did not administer cytokines such as IL-2, IL-7 and IL-15 to mice to avoid an artificial increase of the activity of ENKL cells *in vivo*, because even without cytokines ENKL cells rapidly proliferated in mice and the tumor signal progressively increased. Using cytokines, the expansion of rejT might be much stronger and the incidence of relapse might decrease. However, it is encouraging that even without cytokines, rejT were well activated by recognizing ENKL cells and showed strong cytotoxic activity against ENKL cells.

Our results collectively suggest that to treat relapsed and refractory ENKL using LMP1- and LMP2-specific rejT will be very useful, as large numbers of functionally rejuvenated LMP1- and LMP2-specific CTL can reliably be obtained from T-iPSC. The greatest advantage of rejT therapy is that once T-iPSC are established from an EBV-CTL clone, therapeutic T cells can be generated from T-iPSC in unlimited numbers. If patient tumor cells strongly express PD-L1, the associated poor prognosis should prompt caregivers to generate patient-specific EBV-rejT targeting LMP1 and LMP2 while SMILE therapy is administered. EBV-rejT can supply a reliable salvage therapy for refractory and relapsed ENKL in which L-asparaginase therapy has failed. To establish banks of T-iPSC directed against a variety of viral antigens and HLA types may ultimately provide effective "off-the-shelf" T-cell adoptive immunotherapy treatments against EKNL. The principle demonstrated here can be extended to other virus-induced tumors and neoantigens.

Acknowledgments

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