

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*

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Supplementary data

Method

Patients and samples

We reviewed 28 biopsy samples from 24 patients diagnosed with ENKL at Juntendo University School of Medicine, Department of Hematology, between 2006 and 2017. Biopsy specimens obtained at our faculty underwent histopathologic diagnosis (READ, Koutoubiken, Tokyo, Japan) according to World Health Organization classification guidelines.

Multiple samples were obtained from three patients (two samples (7,8 and 18,19) from two patients, three samples (15,16 and 17) from one patient) because a new biopsy was performed at relapse. The clinical stage was evaluated according to the modified Ann Arbor classification using computed tomography (CT), fluorodeoxyglucose-positron emission tomography / CT, and bone marrow biopsy. Of 24 patients, 15 patients had advanced stage disease and 9 had localized disease. Of the 24 patients, 11 received SMILE or SMILE-like regimens: MILD (methotrexate, ifosfamide, L-asparaginase and dexamethazone) is a SMILE-like regimen that we invented as a pilot regimen before SMILE as a first L-asparaginase-containing regimen in advanced stage ENKL. Three patients received allograft bone marrow transplants and three patients underwent autograft peripheral blood stem cell transplantation. Three patients with localized ENKL received SMILE/SMILE-like regimens following concurrent radiotherapy and DeVIC (dexamethasone, etoposide, ifosfamide, and carboplatin) (RT-DeVIC) therapy as they had primary refractory disease. EBV DNA loads in peripheral blood were evaluated at the time of biopsy in 16 samples. One patient's survival duration could not be obtained because this patient was referred to another hospital after initial treatment. One patient was not able to receive chemotherapy because she was too frail. Clinical information was obtained from clinical charts and attending physicians. Use of 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. Tissue sections on glass slides were deparaffinized with xylene and ethanol, rehydrated, and subjected to antigen retrieval in EDTA buffer (pH 8.0) in a microwave oven at 95°C for 20 minutes. After cooling and rinsing with buffer, the slides were processed using a Dako autostainer (Dakocytomation, Kyoto, Japan). Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide for 5 minutes. Slides were incubated with anti-PD-L1 rabbit monoclonal antibodies at 4°C overnight.¹ The slides were incubated with an EnVision1 System horseradish-peroxidase – labeled anti-rabbit polymer (K4003; Dakocytomation) for 30 minutes. Diaminobenzidine exposure for 10 minutes was used to visualize PD-L1 (brown reaction product). Slides were counterstained with hematoxylin, dehydrated with ethanol, and mounted under coverslips. Sections of human placenta (trophoblast) served as positive controls. Tissue sections incubated with antibody diluent (Dakocytomation) without primary antibody served as negative controls.^{1, 2} To calculate ratios of PD-L1 expression of ENKL cells, we counted the number of PD-L1 positive ENKL cells per 100 macrophages, identifying macrophages by their characteristic features on light microscopy. ENKL cell : macrophage ratios were used to define 4 groups as 5-10% positive (+), 2-3% weakly positive (+/-), 1% slightly positive (-/+), and negative. Numbers of PD-1 - expressing (PD-1+) tumor-infiltrating lymphocytes (TILs) were assessed: Up to 5 representative high

power fields (HPF) were selected, PD-1+ TILs were counted, and the average number of PD-1+ TILs per HPF was calculated for each patient.^{1, 2}

Generation of LMP1/2-specific CTLs and establishment of T-iPSCs

LMP1/2-specific CTLs were generated using peripheral blood mononuclear cells (PBMCs) obtained from two human leukocyte antigen (HLA)-A*24:02-expressing healthy donors and one HLA-A*02:01-expressing ENKL patient (approved, Research Ethics Committee for the Faculty of Medicine, Juntendo University). To generate CTLs, PBMCs stimulated with autologous-peptide – pulsed DCs were cultured in the presence of 400U/ml of interleukin 4 (IL4) and 10ng/ml of interleukin 7 (IL7) (both Miltenyi Biotech, Bergish Gladbach, Germany). On day 9 of culture, effector T cells were restimulated with autologous-peptide – pulsed DCs. On day 16, T cells were harvested and the antigen specificity of LMP1/2-specific CTLs was determined by staining with HLA- A*02:01/LMP1₁₅₉₋₁₆₇, A*24:02/LMP2₁₃₁₋₁₃₉ tetramer, A*24:02/LMP2₄₁₉₋₄₂₇, or A*02:01/LMP2₃₅₆₋₃₆₄ (MBL, Nagoya, Japan). We slightly modified a published protocol.^{3, 4} LMP1 or LMP2-specific CTLs were single-cell cloned by limiting dilution after tetramer-PE coupled / Anti-PE- MicroBeads magnetic cell separation (Miltenyi Biotech).^{3, 4} Selected clones were transduced with Sendai virus vectors to establish T-iPSCs. Transduced cells were transferred onto iMatrix-511 (Nippi, Tokyo, Japan) coated plates and cultured in NS-A2 (GreenDay Corporation, Ibaraki, Japan) in the presence of 100U/ml of interleukin 2 (IL2), which was gradually replaced with StemFit AK03N (Ajinomoto Healthy Supply, Tokyo, Japan). After T-iPSC establishment, all iPSC lines were maintained in culture with CTS Essential 8TM medium (Thermo Fisher Scientific, Waltham, MA, USA).

T-Cell Differentiation from T-iPSCs

To differentiate human iPSCs into hematopoietic cells, small clumps of iPSCs were transferred onto C3H10T1/2 cells with co-culture in EB medium (Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO) supplemented with γ -irradiated 15% fetal bovine serum [FBS] (HyClone, GE Healthcare UK, Little Chalfont, England) and a cocktail of insulin, transferrin, selenium solution, 100x 10 mg/ml human insulin, 5.5 mg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine (Thermo Fisher Scientific), 0.45 mM α -monothioglycerol (Sigma), and 50 mg/ml ascorbic acid (Takeda Pharmaceutical, Tokyo, Japan) in the presence of vascular endothelial growth factor (Miltenyi Biotech).

Hematopoietic cells collected from iPSC sac contents were transferred onto DL1/4-expressing C3H10T1/2 feeder cells, where the former underwent T-lineage differentiation during coculture in α MEM medium (Thermo Fisher Scientific) supplemented with γ -irradiated 20% FBS (HyClone, GE Healthcare UK) in the presence of stem cell factor, Fms-related tyrosine kinase 3 ligand and IL7 (all Miltenyi Biotech). T-lineage cells were then harvested, stimulated with 5 mg/ml phytohemagglutinin (PHA-L) (Sigma-Aldrich), mixed with irradiated PBMCs, and co-cultured in T cell medium in the presence of IL7 and interleukin 15 (Miltenyi Biotech). The antigen specificity of LMP1/2-specific reTjTs was determined by staining with LMP1/2 tetramer. Both C3H10T1/2 feeder cells and DL1/4-expressing C3H10T1/2 feeder cells were validated for clinical use.

Antibodies and Flow Cytometry

Monoclonal antibodies directed to surface antigens, including V500 mouse anti-human CD45 (BD Biosciences, San Jose, CA) and APC/Cy7 mouse anti-human CD3, Pacific Blue mouse anti-human CD8a, APC mouse anti-human CD4, Pacific Blue mouse anti-human CD45RA, PE mouse anti-human CD62L, APC/Cy7 mouse anti-human CD95, APC mouse anti-human CD27, PerCP/Cy5.5 mouse anti-human HLA-A,B,C, mouse anti-human CD274 (PD-L1), mouse anti-human CD273 (PD-L2), and mouse anti-human CD279 (PD-1) (all BioLegend, San Diego, CA) were used. Cell suspensions were stained with

a cocktail of fluorescently conjugated monoclonal antibodies quantified by flow cytometry (FACS ARIA II, BD Bioscience, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Cell Lines

ENKL cells (NK-YS line, kindly provided by Dr. Junjiroh Tsuchiyama, Okayama University Medical School, Okayama, Japan, and SNK-6 line, kindly provided by Dr. Norio Shimizu, Tokyo Medical and Dental University, Tokyo, Japan) were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 100 U/ml of IL2 and in NS-A2 (GreenDay) supplemented with 100 U/ml of IL2, respectively.^{5, 6}

Antitumor activity in in vivo model

All *in vivo* studies were approved by the Animal Research Committees of The Institute of Medical Science, The University of Tokyo, and of Juntendo University School of Medicine. To evaluate the antitumor effects of LMP2-CTLs and LMP2-rejTs 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 green fluorescent protein (GFP) expression by flow cytometry. Six-week-old female NOD/Shi-scid, IL-2R γ KO 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). Mice were injected intraperitoneally with d-luciferin (150 mg/kg) (OZ Biosciences, San Diego, CA) and light output was analyzed with Xenogen Living Image Software Version 2.50 (both Xenogen). The intensity of the signal was measured as total photon/s/cm²/sr (p/s/cm²/sr) as described.⁴ Once a progressive increase of bioluminescence occurred, usually 4 days after tumor inoculation, mice were treated intraperitoneally with 3 once-weekly doses of 5×10^6 LMP2-rejTs \pm 50

µg of anti-PD-1 Ab or with 5×10^6 original LMP2-CTLs \pm 50 µg of anti-PD-1 Ab (In VivoMAb anti-h PD-1, BioXCell, West Lebanon, NH).

PCR and sequencing

Genomic DNA was made using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan). EBV strain typing of the NK-YS cells in ascites was performed by polymerase chain reaction (PCR) using *LMP2*-specific primers 5'-TATGAATCCAGTATGCCTGC-3' and 5'-CGCAGTAAGCACTGTCACCG-3' as described⁷ to detect LMP2 epitopes that are associated with HLA-A*24:02. Purified DNA for sequencing was obtained by eluting the PCR product from the gel using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). For PCR, samples were heated to 94°C for 3 minutes, 98°C for 10 seconds, 60°C for 15 seconds, 68°C for 50 seconds, for 30 cycles, followed by 72°C for 7 minutes. DNA sequencing was performed by FASMAC (Kanagawa, Japan).

Statistics

All data are presented as mean \pm SD or SEM as stated in the figure legends. Results obtained with continuous values were analyzed by ANOVA or unpaired Student's *t* test (two-tailed) as stated in the text. Categorical values were compared by Fisher's exact test. Survival curves were compared using Kaplan-Meier analysis with log-rank testing. All statistical analyses were performed using Excel (Microsoft, Redmond, WA), SPSS (IBM, Armonk, NY) and Prism (GraphPad Software, San Diego, CA) programs. Values of $p < 0.05$ were considered significant.

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