iPSC-derived Rejuvenated T-cell Therapy for Extranodal NK/T-cell Lymphoma, Nasal Type

MIKI ANDO*1 2), JUN ANDO*1), TADAHIRO HONDA*1), MIDORI ISHII*1), HIROMITSU NAKAUCHI*2 3), NORIO KOMATSU*1)

*1)Department of Hematology, Juntendo University Faculty 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)Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, CA, USA

Extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKL), is an aggressive lymphoma that rapidly disseminates to various sites, resulting in a very poor prognosis. Antigen-specific cytotoxic T lymphocyte (CTL) therapy to target and kill tumor cells induces durable remissions in selected malignancies such as melanomas. As ENKL is invariably infected by Epstein-Barr virus (EBV), this lymphoma is a good target of CTL therapy. However, for most tumors, clinical utility is limited because CTL are continuously exposed to viral or tumor antigens and become exhausted. Exploiting fully rejuvenated induced pluripotent stem cell (iPSC)-derived antigen-specific CTL would be a powerful approach. After we reported the robust antitumor effect and survival advantage of rejuvenated CTL against EBV-infected tumor, we started a preclinical study utilizing this novel immunotherapy to EBV-associated lymphoma. We believe that iPSC-derived rejuvenated CTL therapy targeting EBV will provide a promising salvage therapy for ENKL.

Key words: iPSC-derived CTL therapy, rejuvenated CTL, rejT, Extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKL), Epstein-Barr virus (EBV)

Introduction

Epstein-Barr virus (EBV) was the first human virus to be directly implicated in oncogenesis. EBV primarily infects human oropharynx epithelial cells, and subsequently replicates and spreads to B cells, resulting in latent infections. Some EBV infection evolves into EBV-associated lymphoma, which generally exhibits poor prognosis. All EBV-positive tumors are associated with the virus's latent cycle, and 3 types of EBV latency are characterized, depending on the patterns of EBV gene expression. Burkitt lymphoma shows type I latency and only Epstein-Barr nuclear antigen (EBNA)-1 is expressed. Hodgkin lymphoma, extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKL), and diffuse large B cell lymphoma show Type II latency and express latent membrane protein (LMP) 1, LMP2, and EBNA-1. Type III latency is associated with lymphoproliferative diseases developing in immunosuppressive situations such as post-transplant lymphoproliferative diseases and methotrexate–related lymphoproliferative disease, and is characterized by expression of all EBV latent proteins.

ENKL is a highly aggressive disease. It occurs mainly in the nasal cavity with extensive necrosis and angioinvasion. Expressing high concentrations of multidrug-resistance P-glycoprotein, ENKL cells resist anthracycline-based standard
We found that L-asparaginase selectively induces apoptosis in ENKL \(^6\) and developed the L-asparaginase-containing regimen SMILE (dexamethasone [“steroids”], methotrexate, ifosfamide, L-asparaginase, etoposide) for advanced-stage ENKL. SMILE significantly prolongs survival in advanced ENKL and is now the most commonly employed initial therapy \(^7\).

However, even the SMILE regimen or SMILE-like regimens still fail in 20% to 40% of cases and the 5-year overall survival rate stands at 47\% \(^8\). Substantial numbers of patients still suffer relapse. No effective salvage therapy exists and outcome is always fatal.

Cytotoxic T lymphocytes (CTL) play an important role in immune-system responses to viral infection and malignancies, reacting with malignant or virus-infected cells. Adoptive T-cell therapy, the administration of a large number of activated antigen-specific CTL expanded \textit{ex vivo}, can specifically target viral or tumor antigens. This therapy can mediate cancer regression and induce durable remissions in melanoma and selected other cancers \(^9\)-\(^{12}\). ENKL by definition is infected by EBV and invariably expresses LMP1 and LMP2. As T cells specific for LMP1 and LMP2 antigen do not exist in large numbers in the peripheral blood of ENKL patients and often are anergic in the tumor microenvironment \(^{13}\)-\(^{18}\), this lymphoma should be a good target of adoptive T-cell therapy directed to LMP1 and LMP2 antigens \(^{13}\)-\(^{18}\). However, such therapy is incompletely successful in most patients with this lymphoma in advanced stages, seemingly because CTL continuously exposed to their target antigens become exhausted \(^19\).

Antigen-specific CTL can be generated from iPSC that are in turn generated by reprogramming original CTL (Figure-1). Proliferative capacity is higher, memory phenotype is younger, and telomeres are longer in iPSC-derived CTL than in original CTL: The iPSC-derived CTL are functionally rejuvenated (rejuvenated CTL; rejT \(^{20}\)-\(^{22}\)). LMP2-specific rejT (LMP2-rejT) are robustly effective against EBV-infected tumors \textit{in vivo} and survive significantly longer than peripheral blood-derived original CTL \(^{21}\) \(^{23}\): LMP2-rejT successfully

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure-1.png}
\caption{Rejuvenation of antigen–specific CTL for adoptive T cell therapy}
\end{figure}

CTL lose their ability to proliferate when they are continuously exposed to their target antigens. Exhausted CTL are rejuvenated by converting CTL into T-iPSC. These T-iPSC can redifferentiate into CTL that retain ancestral antigen specificity and cytotoxicity. (Ando M, Nakauchi H: Experimental Hematology, 2017; 47: 2-12 \(^{22}\))

\textit{Declaration from corresponding author (MIKI ANDO): This paper is reorganization of “Long-term eradication of extranodal NK/T-cell lymphoma, nasal type, by induced pluripotent stem cell–derived Epstein-Barr virus–specific rejuvenated T cells in vivo. Haematologica, 2020; 105 (3): 796–807”}.
eradicated ENKL by existing long-term in vivo as memory T cells. Therefore, EBV-specific rejT offer promise as salvage therapy for ENKL in which SMILE regimen fails.

This review focuses on our preclinical study of iPSC-derived EBV-specific rejT therapy targeting ENKL. We also sketch the prospects for rejT therapy.

**Generation of EBV antigen-specific CTL from donor peripheral blood**

LMP1- and LMP2-specific CTL clones were established from EBV-specific CTL obtained from donors’ peripheral blood. To generate CTL, PBMCs stimulated with autologous peptide-pulsed dendritic cells were cultured in the presence of interleukins (IL) 4 and IL7. T cells were harvested, and the antigen specificity of LMP1- or LMP2-specific CTL was determined by staining with LMP1 or LMP2 tetramer. LMP1- or LMP2-specific CTL were single-cell cloned by limiting dilution after tetramer-PE coupled anti-PE-MicroBeads magnetic cell separation. T-iPSC were subsequently established from each CTL clone.

**Establishment of T-iPSC from EBV-CTL clones and T cell differentiation from T-iPSC**

LMP1- or LMP2-specific CTL clones were reprogrammed into iPSCs (T-iPSC) by transduction with 2 Sendai virus vectors that encode the 4 Yamanaka reprogramming factors (Oct3/4, SOX2, KLF4, and c-MYC) and SV40 large T antigen.

Germline-configuration T cell receptor (TCR) α and β loci are present in T-iPSC, which retain the same rearranged sequences of TCR α and β chain genes (TCRA, TCRA, and TCRB) as those in the original CTL. CDR3 sequences are the most diversifiable antigen-recognition site of the TCR, yet CDR3 sequences from T-iPSC and their original CTL clone were completely identical at both the TCRA and the TCRB loci. The result constituted evidence that antigen specificity encoded in the genomic DNA of original CTL is retained throughout cloning and reprogramming.

T-iPSC were subsequently differentiated into rejT. To differentiate human T-iPSC into hematopoietic cells, small clumps of T-iPSC were transferred onto C3H10T1/2 cells with co-culture in the presence of vascular endothelial growth factor. Hematopoietic cells collected from iPSC sac contents were transferred onto DL1/4-expressing C3H10T1/2 feeder cells and co-cultured in the presence of stem cell factor, FMS-related tyrosine kinase 3 ligand, and IL7. T-lineage cells were then harvested, stimulated to expand with phytohemagglutinin, mixed with irradiated PBMC, and cocultured in T cell medium in the presence of IL7 and IL15.

**Functional rejuvenation in rejT**

The rejT differentiated from T-iPSC keep the same antigen-specificity as that of the original CTL clone. Successfully generated EBV-specific rejT showed much stronger proliferative ability than that of the parental original EBV-specific CTL clone. Another factor in ENKL prognosis is the programmed death 1 (PD-1) pathway for immune escape. EBV-associated lymphoma cells often express the PD-1 ligand (PD-L1). The complete remission rate of ENKL is high after PD-1 blockade by pembrolizumab in ENKL patients in whom L-asparaginase therapy has failed.

Peripheral-blood derived original LMP2-CTL clone expressed high levels of PD-1, a marker of exhausted T cells, whereas the expression level of PD-1 in iPSC-derived LMP2-rejT fell much lower than that in its parental original LMP2-CTL. Functional rejuvenation in rejT was demonstrated by these findings.

**Cytotoxicity of LMP1- and LMP2-specific rejT against ENKL in vitro**

We compared the cytotoxicity of rejT against EBV positive tumor cells with that of original CTL using 51Cr release assays. LMP1-rejT generated from an ENKL patient showed stronger cytotoxicity against autologous EBV-infected lymphoblastoid cells (LCL) (90.3%, 90.0%, 77.8%, and 58.8%; effector : target [E:T] ratios of 40:1, 20:1, 10:1, and 5:1) than that of the original LMP1-CTL clone (77.8%, 61.9%, and 43.8%; E:T ratios of 20:1, 10:1, and 5:1) (Figure-2A). LMP2-rejT generated from a healthy donor demonstrated more robust cytotoxicity against autologous EBV-infected LCL (70.4% and 69.8% at E:T ratios of 40:1 and 20:1, respectively).
and 65.4%; E:T ratios of 20:1 and 10:1) than that of original LMP2-CTL clone (51.7% and 49.4%; E:T ratios of 20:1 and 10:1) (Figure-2B).

Our results demonstrated in vitro that EBV-rejT exhibited stronger cytotoxicity against EBV-infected tumors than the original CTL.

Anti-ENKL effect of rejT in vivo

To evaluate the antitumor effects of LMP2-CTL and LMP2-rejT against ENKL in vitro, female NOD/Shi-scid, IL-2Rγ KO Jic (NOG) mice aged 6wk were engrafted intraperitoneally with cells from the NK–YS ENKL cell line (1x10⁵ cells/mouse) that were labeled with retrovirus-derived firefly luciferase (FFluc). Tumor growth was monitored by detecting bioluminescence of FFluc-NK–YS. No treatment was given to mice in the control group (n=6). The treatment groups consisted of mice treated with original LMP2-CTL (5x10⁶ per dose weekly, 3 doses) (n=6) and mice treated with LMP2-rejT (5x10⁶ cells per dose weekly, 3 doses) (n=6); treatment began 4d after tumor inoculation. By day 21, bioluminescence signal strength had progressively increased in untreated mice. By contrast, tumor suppressive effects were observed in both treatment groups, more strongly apparent in mice treated with LMP2-specific rejT than in those receiving original CTL (23). (Figure-3A)

Long-term antitumor effect of LMP2-rejT in vivo

On long-term observation, original CTL did not prolong survival beyond that in the untreated mice group (p=0.09), whereas LMP2-rejT markedly prolonged survival (mean 239 days, 58-296 days) beyond that in the group treated with original CTL (p=0.03, mean 74.5 days, 58-140 days) (Figure-3B). It is noteworthy that 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. 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 (23). These results demonstrate the robust and sustained tumor suppressive effect of LMP2-rejT against ENKL in vivo.

Future perspectives for rejT therapy

Conventional adoptive immunotherapies that utilize peripheral blood-derived T cell therapy have been developed in the United States and are currently used in clinical practice. Chimeric antigen receptor T cell therapy directed against CD19 antigen, such as Tisagenlecleucel (Kymriah®) for relapsed and refractory acute B-lymphoblastic leukemia, has shown remarkable success (24-26).
Kymriah® was also approved in Japan by the Japanese Ministry of Health, Labour and Welfare on 26 March 2019. Use of Kymriah® is limited to specific institutes that meet global requirements, such as access to a good manufacturing practice-level cell processing center. Juntendo University Hospital meets all these requirements and we are starting to recruit refractory leukemia and lymphoma patients for this therapy. However, a major hurdle persists: donor-dependence of cell resources hinders timely application and stable cell supply. RejT technology may possibly overcome this problem. Once we establish T-iPSC from a CTL clone, we can supply rejuvenated T cells unlimitedly. Banking of T-iPSC as a source of therapeutic T cells enables us to supply rejT to patients. If we bank HLA-matched or HLA-edited allogeneic cell resources from healthy donors, “off-the-shelf” therapy truly will be feasible.

We believe that iPSC technology will enable us to supply limitless good quality cell resource that can be used anytime, anywhere, and for anyone, the ultimate “off-the-shelf” therapy.

Acknowledgements

We thank A.S. Knisely for critical reading of the manuscript. The project was supported by JSPS KAKENHI (Grant Number 16K09842). This work was carried out in part at the Intractable Disease Research Center, Juntendo University Graduate School of Medicine. The institutional regulation boards for human ethics at Juntendo University Faculty of Medicine and at the Institute of Medical Science, University of Tokyo, approved the experimental protocol.
References