

Development of the T-ALLiPSC-based Therapeutic Cancer Vaccines for T-cell acute Lymphoblastic Leukemia

Zhu Li

Shenzhen University

Xuemei Chen

Shenzhen Luohu Hospital Group Luohu People's Hospital

Luning Liu

Shenzhen Luohu Hospital Group Luohu People's Hospital

Meiling Zhou

Shenzhen Luohu Hospital Group Luohu People's Hospital

Guangqian Zhou

Shenzhen University

Tao Liu (✉ tao2020@sohu.com)

Shenzhen Luohu People's Hospital <https://orcid.org/0000-0002-1199-0076>

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Abstract

Purpose

The T-cell acute lymphoblastic leukemia (T-ALL) is a kind of hematological malignancy in children. Despite the significant improvement in the cure rate of T-ALL upon treatment with chemotherapy regimens, steroids, and allotransplantation there are relapses. This study focuses on the tumor-specific therapeutic vaccines derived from the induced pluripotent stem cells (iPSC) to address the issue of T-ALL recurrence.

Methods

Patient-derived tumor cells were reprogrammed into the iPSCs and the RNA-seq data of the T-ALL-iPSCs and H-iPSCs were analyzed. *In vitro*, the whole cell lysate antigens of iPSCs were prepared to induce the dendritic cells (DC) maturation, which in turn stimulated the tumor-specific T cells to kill the T-ALL tumor cells (Jurkat, CCRF-CEM, MOLT-4).

Results

Both T-ALL-iPSCs and H-iPSCs were highly related to the tumor-related genes. The transcriptome analysis showed the T-ALL-iPSCs to be similar to the T-ALL tumor cells. The cytotoxic T lymphocyte (CTL) stimulated by the DC-loaded T-ALL-iPSC-derived antigens showed specific cytotoxicity against the T-ALL cells *in vitro*.

Conclusions

The T-ALL-iPSC-based therapeutic cancer vaccine can elicit a specific anti-tumor effect on T-ALL.

Introduction

Childhood acute lymphoblastic leukemia (ALL) is a genetically heterogeneous cancer accounting for 10–15% of the T-cell ALL (T-ALL) cases. The T-ALL event-free survival rate (EFS) is as high as 80–90% (Kato and Manabe 2018). It has a higher risk of relapse than the B-lineage ALL with a poor outcome after relapse (Patrick and Vora 2015). Here, the potential of using the tumor-associated proteins in the induced pluripotent stem cells (iPSC) was explored as the basis of a T-ALL therapeutic vaccine.

A therapeutic cancer vaccine is based on the principle of inducing a specific anti-tumor immune response by introducing a tumor-related antigen. Generally, the antigens of the cancer vaccines constitute the tumor cells, tumor antigen-related proteins and peptides synthesized *in vitro*, and antigen-related genes (Rosenberg et al. 2004; Xiang et al. 2021). Studies performed a century ago showed that immunizing mice with embryonic tissues triggered the rejection of the transplanted tumors (Brewer et al. 2009). Furthermore, the embryonic stem cells (ESC)-vaccinated mice received obvious anti-tumor immunity, protecting them from lung cancer formation and development (Dong et al. 2010). Emerging

data have demonstrated ESCs to share common transcriptome features and antigens with the tumor cells, however, ethical concerns limit their usage in the cancer vaccines.

Since the discovery of iPSCs (Takahashi et al. 2007; Takahashi and Yamanaka 2006), the autologous iPSCs have been found to share almost the same gene expression and surface markers as ESCs (Bock et al. 2011; Mallon et al. 2014). In addition, the tumorigenicity and immunogenicity of the autologous iPSCs indicate their potential efficacy in cancer vaccination (Kooreman et al. 2018; Ouyang et al. 2021). This highlights that iPSCs can provide more accurate and representative patients' tumor immunogen than the non-autologous ESCs (Kooreman et al. 2018).

Latest studies have shown that (Kooreman et al. 2018; Ouyang et al. 2021) iPSCs inactivation can be harnessed as a cancer vaccine inducing extensive anti-tumor immune responses. Through RNA sequencing (RNA-seq), the researchers found that iPSCs from both humans and mice express tumor-associated antigens, indicating that all of these iPSCs could be utilized as an anti-cancer vaccine. Injecting the iPSC-based vaccine can effectively prevent the growth of breast cancer, mesothelioma, and melanoma in tumor-bearing mice. The iPSC-based vaccine injection can also effectively kill the tumor tissue *in vivo*. These results indicated that the iPSC-based vaccine can induce an effective immune response in the mice, and exert widespread killing effects on the different types of tumors. Compared to the current immunotherapy strategy, the iPSC vaccine can reactivate the immune system and target cancer without any side effects, proving a novel method for developing a clinical cancer vaccine.

This study first reprogrammed the tumor cells from the T-ALL patients into iPSCs, preparing the whole cell lysate antigens of iPSCs for obtaining a specific T-ALL-iPSC-based vaccine. Subsequently, it was presented to the T cells through the dendritic cells (DC), stimulating specific immune reactions against T-ALL.

Materials And Methods

Cell culture

The human T-ALL cell lines (Jurkat, CCRF-CEM, MOLT-4) and NSCLC cell line NCI-H1299 were cultured in the RPMI 1640 medium supplemented with 10% FBS. The human cervix adenocarcinoma, HeLa cells were cultured in Eagle's minimum essential medium supplemented with 10% FBS and 1% NEAA (10 mM). The breast cancer cell line, MCF-7 cells were cultured in the DMEM supplemented with 10% FBS.

T-ALL reprogramming and preparation of the T-ALL-iPSC lysates

The peripheral blood mononuclear cells (PBMC) from the T-ALL patients were infected with the CytoTune-iPS 2.0 Sendai virus reprogramming Kit (Thermo Fisher). The obtained ESC-like clones were identified for pluripotency, using methods like alkaline phosphatase staining, flow cytometry, and immunofluorescence staining for the pluripotency markers, and teratoma formation. The T-ALL-iPSCs were cultured with mTeSR plus medium (STEMCELL Technologies).

To prepare cell lysates, the iPSCs derived from the healthy cells (H-iPSCs) (1×10^7 /ml) and T-ALL-iPSCs (1×10^7 /ml) were frozen in liquid nitrogen and disrupted by three freeze-thaw cycles. The cell lysates were then spun at 13000g for 20 min, and the supernatants were collected as iPSC antigens. The total protein concentration of the cell lysates was quantified using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China).

Generation of the different types of DC-CTLs

The DCs were prepared from the monocytes of the healthy donors. The PBMC were isolated by Lymphoprep (Stemcell, USA) density gradient centrifugation at 800g for 15 min at 20 °C. After 2 h of incubation, the adherent cells were cultured in an Alys-505 complete culture medium (Zhuhai Baso Cell Science & Technology Co. Ltd, China) supplemented with 100 ng/ml GM-CSF and 30 ng/ml IL-4 induction of DCs. The non-adherent cells were cultured in the Alys-505 complete culture medium containing 1000 U/ml IL-2 and 200 ng/ml CD3 monoclonal antibody for generating the CTLs.

On day 3, the immature DCs were treated with 30µg/ml of different antigens and 1000 U/ml TNF-α. On day 7, the CTLs were co-cultured with autologous DCs at a 5:1 ratio. There were four groups, T group (only CTLs), DC-T group (CTLs were co-cultured with DCs), DC^{H-iPSC}-T group (CTLs were co-cultured with H-iPSC lysates-stimulated DCs), and DC^{T-ALL-iPSC}-T group (CTLs were co-cultured with T-ALL-iPSC lysates-stimulated DCs). All the groups were cultured at 37°C in a humidified 5%CO₂ incubator and passaged every 3 days in an Alys-505 complete culture medium with 1000 U/ml IL-2.

Flow cytometry

The T cells were stained with FITC anti-human CD3, PerCp/Cyanine5.5 anti-human CD4, APC anti-human CD8a, PE anti-human CD25, PE anti-human PD-1, and PE anti-human CD57 (Biolegend, USA) for 20 min at 4°C and analyzed by the flow cytometry software.

***In vitro* cytotoxicity assay**

The CTLs from each group were examined for the cytotoxic activity toward the target tumor cells by performing a lactate dehydrogenase (LDH) release assay using the LDH cytotoxicity assay kit (Beyotime Institute of Biotechnology). The CTLs and tumor cells were co-cultured at the effector-to-target (E/T) ratios of 5:1, 10:1, 20:1 for 24 h. The specific lysis was calculated as follows: Percentage of cytotoxicity = $[(\text{experimental release} - \text{effector spontaneous release} - \text{target spontaneous release}) / ((\text{target maximum release} - \text{target spontaneous release}))] \times 100\%$.

Cytokine Release Assay

The T cells were co-cultured with or without DCs for 24 h. The supernatants from each group were collected for assessing IL-6, IL-13, IFN-γ, and TNF-α (Biolegend Human Th Cytokine Panel (12-plex) with V-bottom Plate V02, USA) using flow cytometry.

CTLs from each group were co-incubated with the target cells at an E:T ratio of 10:1. Following 24 h of incubation at 37°C, the supernatant was harvested and the concentration of the released IFN- γ was measured using the human IFN- γ Precoated ELISA Kit (Dakewe Bio-engineering Co. Ltd, Shenzhen, China).

Degranulation assay

The CTLs from each group were co-cultured with the target cells at an E:T ratio of 10:1. The PE anti-CD107a antibody (Biolegend, USA) was included during the culture. After 1 h, 2 μ M monensin (Biolegend, USA) was added to the co-cultured system and incubated for an additional 4 h, and then the cells were stained with the APC anti-human CD8a and analyzed using flow cytometry. All the CD8⁺ CD107a⁺ cells were regarded as the degranulated T cells.

Analysis of RNA-seq data

The human RNA-seq data for the cancer cell lines were downloaded from the Cell Model Passports Database (<https://cellmodelpassports.sanger.ac.uk/>). The cancer-related genes list was downloaded from the Network of Cancer Genes (http://ncg.kcl.ac.uk/cancer_genes.php). The list of over-expressed genes in T-ALL was screened from the Oncomine's Haferlach Leukemia Dataset (Haferlach et al. 2010). The heatmap was generated using GENE-E.

Statistical analysis

The GraphPad Prism 5 software was used for data analysis. All data are presented as the mean \pm the standard deviations. The one-way ANOVA with Bonferroni's post hoc test was used to analyze the differences between the groups. $P < 0.05$ indicates a statistical difference.

Results

The identification of pluripotency of the T-ALL-iPSC

The pluripotency of the T-ALL-iPSC was identified by the alkaline phosphatase staining (Figure 1A) and the expression of pluripotent markers (Figure 1B, 1C, and 1D). Figure 1E shows the karyotype analysis of the T-ALL-iPSC. When the T-ALL-iPSC were transplanted to the immunodeficient mice, these cells could successfully form the teratomas with three germ layers (Figure 1F). Collectively, these data show that the T-ALL cells could be successfully reprogrammed to T-ALL-iPSCs.

The T-ALL-iPSCs have more similar T-ALL-related gene expression profiles than the HiPSCs

The iPSCs were reported to share the gene expression profiles with the cancer cells (Kooreman et al. 2018; Ouyang et al. 2021). Our analysis of the RNA-seq data yielded a similar result. The expression profiles of a selected cancer-related gene list to the cancer cell lines (Hela, MCF-7, NCI-H1299) were compared by performing the RNA-seq on the 6 HiPSC clones and 3 T-ALL-iPSC clones (Figure 2A). The results

indicated an important cancer gene expression overlap between the cancer cells and iPSCs. To investigate whether the autologous iPSCs have the potential to yield tumor-specific therapeutic vaccines, the transcriptome expression correlation matrix was used to analyze the similarity between the T-ALL-iPSC, H-iPSC, and T-ALL cell lines (Jurkat, CCRF-CEM, MOLT-4). The T-ALL-iPSC had a higher similarity with T-ALL cell lines than the H-iPSC (H-iPSC-1, H-iPSC-2) (Figure 2B). The upregulation of a subset of genes was identified in the T-ALL-iPSCs which were over-expressed in the T-ALL cell lines (Figure 2C). Collectively, these data show that the T-ALL-iPSCs were more similar to the T-ALL-related gene profiles than the H-iPSC, suggesting that the T-ALL-iPSCs are more suitable for yielding cancer vaccines than the H-iPSCs.

DC-CTL phenotype and cytokine secretion

The immature DCs were loaded with the cell lysates of iPSCs, and the ability of the *ex vivo*-generated mature DCs to stimulate the T cell function was evaluated (Figure 3). The proportion of the activated T cells (Figure 3A), exhausted T cells (Figure 3B), and senescent T cells (Figure 3C) were analyzed in the different groups. As shown in Figure 3A, the proportion of the CD3⁺CD25⁺ T cells and CD3⁺CD8⁺CD25⁺ T cells increased in the DC-T group, H-iPSC group, and T-ALL-iPSC group compared to the T group.

We measured the concentrations of the cytokines IL-6 (Figure 3D), IL-13 (Figure 3E), IFN- γ (Figure 3F), and TNF- α (Figure 3G) in the supernatants secreted by the CTLs. The CTLs stimulated by the DCs had a stronger function and cytokine secretion than the T-group, whereas there were no significant differences between the DC^{H-iPSC}-T and DC^{T-ALL-iPSC}-T groups.

The DC^{T-ALL-iPSC}-CTLs and DC^{H-iPSC}-CTLs had similar cytotoxicity against the non-T-ALL tumor cells

The cytotoxicity of CTLs against the non-T-ALL cells including Hela (Figure 4A), MCF-7 (Figure 4B), and NCI-H1299 (Figure 4C) was examined and the level of IFN- γ secreted by CTLs was determined by ELISA at an E:T ratio of 10:1 (Figure 4D–E). The percentage of CD8⁺ CD107a⁺ (degranulation assay) was measured by flow cytometry at an E:T ratio of 10:1 (Figure 4G-I). As shown in Figure 4, the DC stimulated CTL groups had higher cytotoxic activity than the non-DC stimulated CTL group, but no significant differences were found between the DC^{H-iPSC}-T and the DC^{T-ALL-iPSC}-T groups.

The DC^{T-ALL-iPSC}-CTLs exhibited specific cytotoxicity against the T-ALL cells *in vitro*

To assess whether the T-ALL-iPSCs were effective vaccines against the T-ALL cells and whether the stimulated T cells contained antigen-specific CTLs, the cytotoxic activity of the DC^{T-ALL-iPSC}-CTLs against T-ALL cells were analyzed *in vitro*. The cytotoxicity of the DC^{T-ALL-iPSC}-T group against the T-ALL cells including Jurkat (Figure 5A), CCRF-CEM (Figure 5B), and MOLT-4 (Figure 5C) was significantly higher compared to the DC^{H-iPSC}-T group. To further verify the cytotoxicity, the level of IFN- γ secreted by the CTLs co-cultured with the T-ALL cells at an E:T ratio of 10:1 was measured (Figure 5D–E). Finally, the percentage of the CD8⁺CD107a⁺ cells was detected when the CTL killed the T-ALL tumor cells (Figure

5G-I). Collectively, these data show that the DC^{T-ALL-iPSC}-CTLs exhibited specific cytotoxicity against the T-ALL cells.

Discussion

Both tumorigenesis, as well as tumor development, comprise highly proliferative and low-immune cells that escape the surveillance of the immune system. Therefore, targeting tumors by reactivating the immune system constitutes a cancer treatment. The novel therapy called the chimeric antigen receptor T (CAR-T) cell therapy for T-ALL is under investigation. The CAR-T cells targeting CD7, CD5, and CD1a have been developed to show therapeutic potential (Gomes-Silva et al. 2017; Mamonkin et al. 2015; Sanchez-Martinez et al. 2019). However, CAR-T cell therapy remains a challenge for T-lineage neoplasms owing to its finite ability in distinguishing between the normal and malignant T cells; hence, it requires further development and trials (Lato et al. 2021).

So far, researchers have utilized ESCs as a vaccine for activating the immune system against cancer, but it has not shown much effectiveness and safety (Li et al. 2009; Yaddanapudi et al. 2012). The clinical applications of ESCs have been obstructed mainly by ethical issues, tumorigenicity, and alloimmunization (Yaddanapudi et al. 2012). Therefore, searching for a new cancer vaccine to overcome these problems is necessary. The iPSC vaccines are undoubtedly a good tumor vaccine candidate to replace ESCs. Joseph et al. used the irradiated autologous iPSCs to produce iPSC vaccines inhibiting the recurrence of a variety of tumors and reducing the burden of metastatic tumors (Kooreman et al. 2018). Tailoring iPSCs by introducing other driver mutations as a vaccine can prevent the initiation and progression of pancreatic cancer (Lu et al. 2020) and lung cancer (Zhang et al. 2020). Although the irradiated iPSC-based vaccines are effective at inducing immunity against multiple cancer types in mice, it is still fraught with risks such as tumorigenicity (Lee et al. 2013) and immunogenicity (de Almeida et al. 2014) which need to be overcome (Yaddanapudi et al. 2018).

The iPSCs have been successfully derived from a variety of human cancers (Amabile et al. 2015; Kotini et al. 2015; Lee et al. 2015; Suva et al. 2014). The differentiation of the leukemia-specific iPSCs is an effective strategy for studying the early stages of the normal and abnormal hematopoietic cells (Li et al. 2021). Kumano's laboratory has successfully constructed the iPSCs from the CML patients and then differentiated the iPSCs into blood cells sensitive to imatinib (Kumano et al. 2012). Besides, when the AML-iPSCs are differentiated into the blood cells, these cells regain the gene expression pattern of leukemia (Chao et al. 2017). Muñoz-López and others also tried to generate iPSCs from the B-cell ALL patients (Munoz-Lopez et al. 2016). Compared to the normal cell-derived iPSCs, the tumor-derived iPSCs may possess gene expression and epigenetic characteristics similar to that of the original tumor cells. Consistently, our results support the hypothesis that for T-ALL, the T-ALL-iPSC-based vaccines are more suitable than the H-iPSC-based vaccines.

Joseph C Wu and colleagues demonstrated iPSCs alone to be incapable of stimulating an effective immune response, and also stated that only the combination of iPSCs and adjuvant CpG can cause anti-

tumor effects(Kooreman et al. 2018). Our findings specified that the iPSC lysates could serve as complete antigens for stimulating the immature DCs and stimulating the killing effect of the T-ALL-iPSCs vaccine on the tumor cells. The DCs are currently known as the strongest professional antigen-presenting cells activating the resting T cells(Katz et al. 2014). They are reportedly pulsed with the tumor cells or tumor cell lysates to obtain the vaccines and providing a variety of specific tumor antigens without prior knowledge of the targets(Robbins et al. 2011). Recent studies have reported that the fusion of the tumor cells with DCs can induce various anti-tumor immune responses(Markov et al. 2019). The only non-antiviral cancer vaccine approved by the FDA is Sipuleucel-T (Provenge), a TAA pulsed autologous dendritic cell -based prostate cancer vaccine(Cheever and Higano 2011). The flow cytometry experiments showed that DC can enhance the function of the T cells, but produces no significant difference between the DC^{H-iPSC-T} and the DC^{T-ALL-iPSC} groups. This indicated that DCs had the same antigen presentation effect on the H-iPSC and T-ALL-iPSC.

This study clarified the inhibitory effect of T-ALL-iPSCs on the tumors and found that the DC^{H-iPSC-T} and DC^{T-ALL-iPSC-T} groups had similar cell -killing effects on the non-T-ALL tumor cells. Akin to the study by Nigel et al., the iPSC-based vaccine generated broad tumor immunity against various types of cancer. The iPSC vaccine generates a large number of tumor antigens for the immune system(Kooreman et al. 2018). But co-culturing with the T-ALL cell lines including Jurkat, CCRF-CEM, and MOLT-4 showed that the DC^{T-ALL-iPSC-T} cells significantly increased the cytotoxicity compared to the DC^{H-iPSC-T} cells. These results indicated that the T-ALL-iPSC have a larger pool of T-ALL-related epitopes, and DC^{T-ALL-iPSC} can induce the T cells to produce a specific killing effect against the T-ALL cell lines.

The patient-derived iPSCs were used as an antigen source for the anticancer vaccines because they can prime the immune system to respond to multiple patient-specific tumor antigens(Goyvaerts and Breckpot 2018). Hence, the patient's T-ALL cells were successfully reprogrammed into the T-ALL-iPSCs. When the H-iPSCs from the healthy people were compared to the T-ALL-iPSCs there were more similar gene expression profiles with the T-ALL cell lines, and the T-ALL-iPSC vaccine was found to stimulate more powerful toxic effects on the T-ALL cells. Therefore, T-ALL-iPSCs as a therapeutic tumor vaccine induces a better anti-tumor effect on the T-ALL. These beneficial properties make the iPSCs vaccine a potential choice for personalized adjuvant immunotherapy soon after the primary cancer treatment. Future studies should verify these effects through various *in vivo* experiments as they will establish the foundation for solid tumor cell derived-specific iPSC vaccine. Hence, exploring the clinical benefit of this new strategy would be valuable for these patients.

Declarations

Authors' contributions ZL performed the main experiments. XC, LL and MZ assisted with all experiments. GZ and TL designed the project. TL wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest The authors declare no competing financial interest.

Ethical approval The study conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) for scientific purposes. This study was also approved by the Ethics Committee of Shenzhen Luohu People's Hospital (LLBGS-2021-042, Shenzhen, China).

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Figures

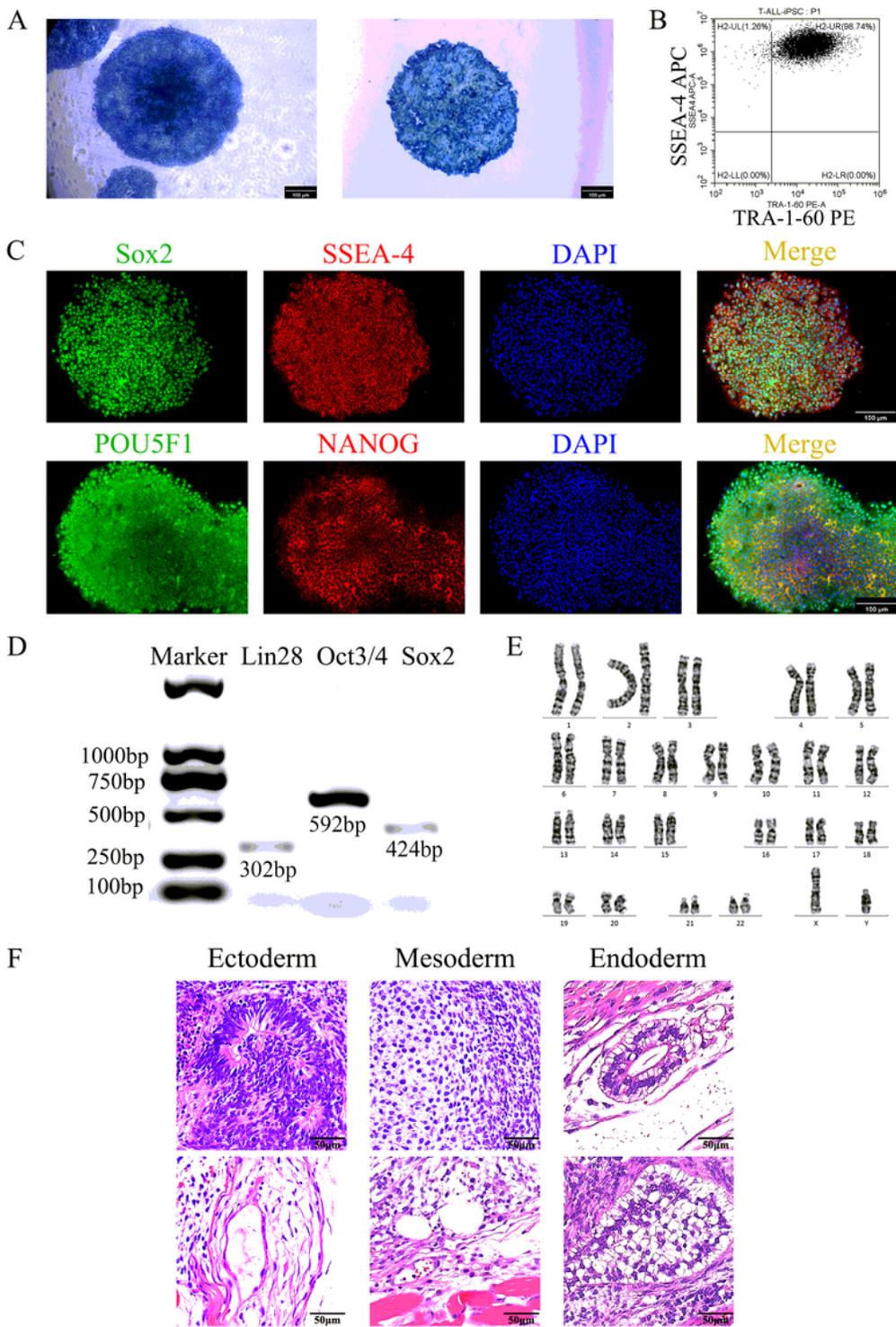


Figure 1

Pluripotency identification of the T-ALL-iPSCs. The pluripotency of the T-ALL-iPSC was identified by the alkaline phosphatase staining (A) and the expression of the pluripotent markers (B–D). Also, the karyotype analysis of the T-ALL-iPSCs was shown (E). When the T-ALL-iPSCs were transplanted into the immunodeficient mice, these cells could successfully form teratomas with three germ layers (F).

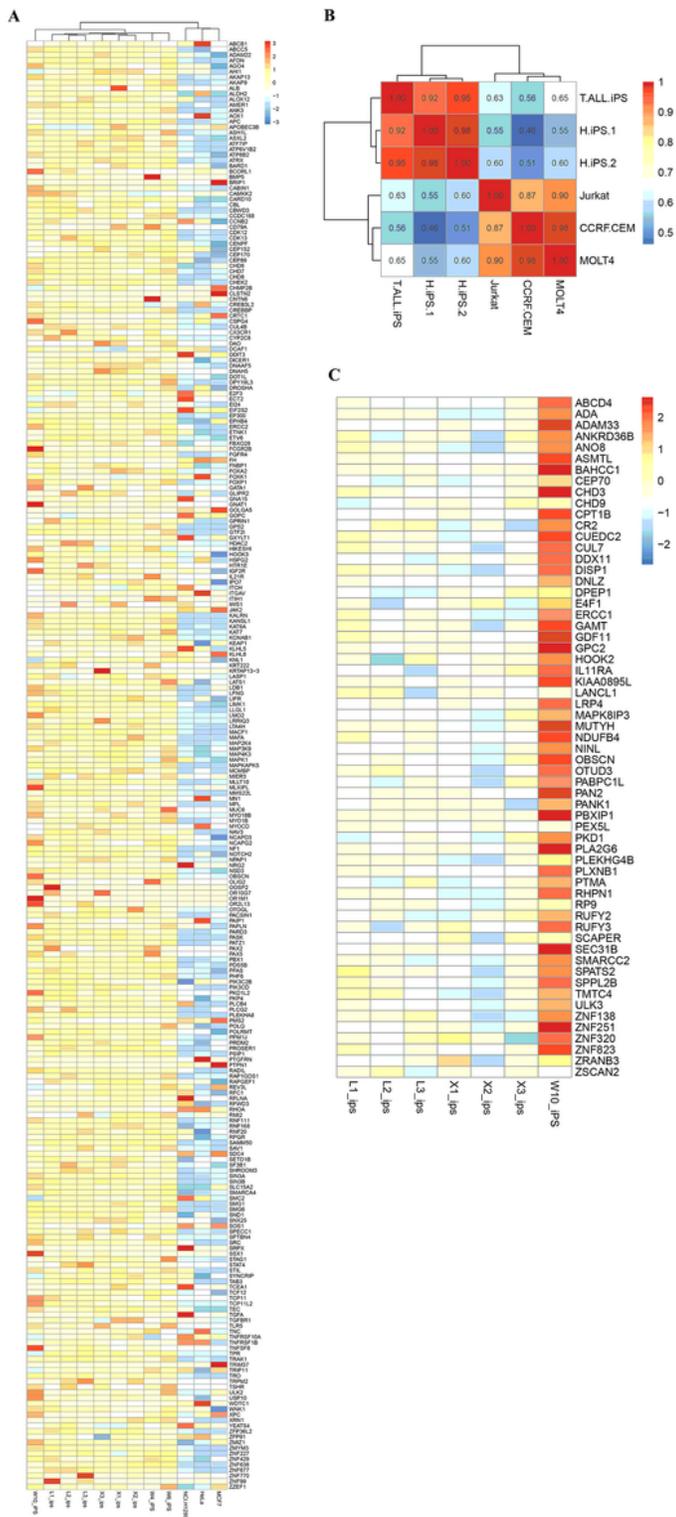


Figure 2

Analysis of the RNA sequencing data from the H-iPSCs, T-ALL-iPSCs, non-T-ALL tumor cells, and T-ALL tumor cells. (A) The RNA seq data of 9 H-iPSC clones generated from the healthy donors and T-ALL patients were compared with the tumor cells to analyze a selected list of cancer-related genes. The healthy donor 1 (L1-iPS, L2-iPS, L3-iPS); healthy donor 2 (X1-iPS, X2-iPS, X3-iPS); T-ALL patient (W4-iPS, W6-iPS, W10-iPS); NSCLC cell (NCI-H1299); cervix adenocarcinoma cell (Hela); breast cancer cell (MCF-7).

(B) The cell lines and iPSCs were subjected to a transcriptome expression correlation matrix, based on 15,687 filtered genes, which were generated. (C) Based on more than 1,000 over-expressed genes in the T-ALL patients, we were able to identify the presence of 61 genes that were highly expressed in the T-ALL-iPSC.

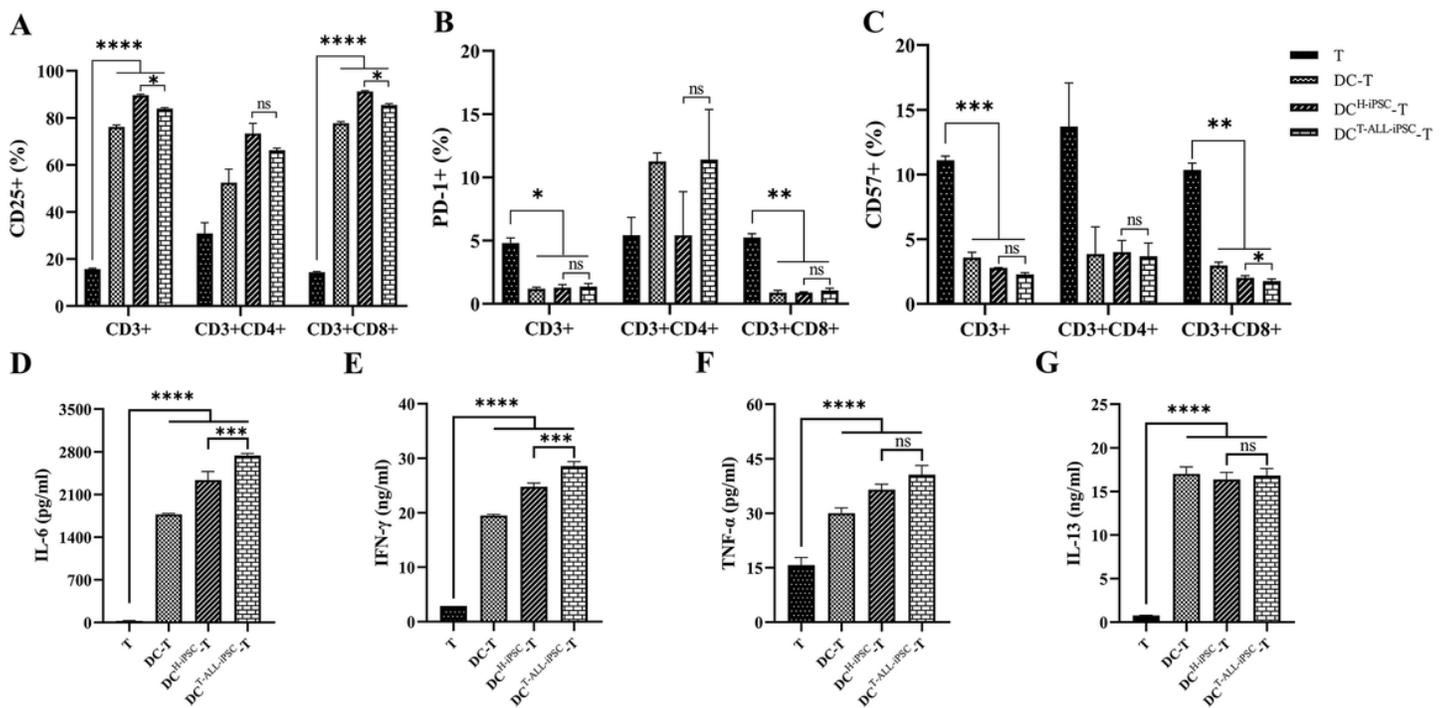


Figure 3

The phenotype and cytokine secretion after CTL stimulation by the antigen-loaded DCs. The proportion of the activated T cells (A), exhausted T cells (B), and senescent T cells (C) was measured by flow cytometry. IL-2 (D), IL-6 (E), IL-13 (F), IFN-γ (G), and TNF-α (H) secreted by the CTL cells were detected. Data were represented as mean ± SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 .

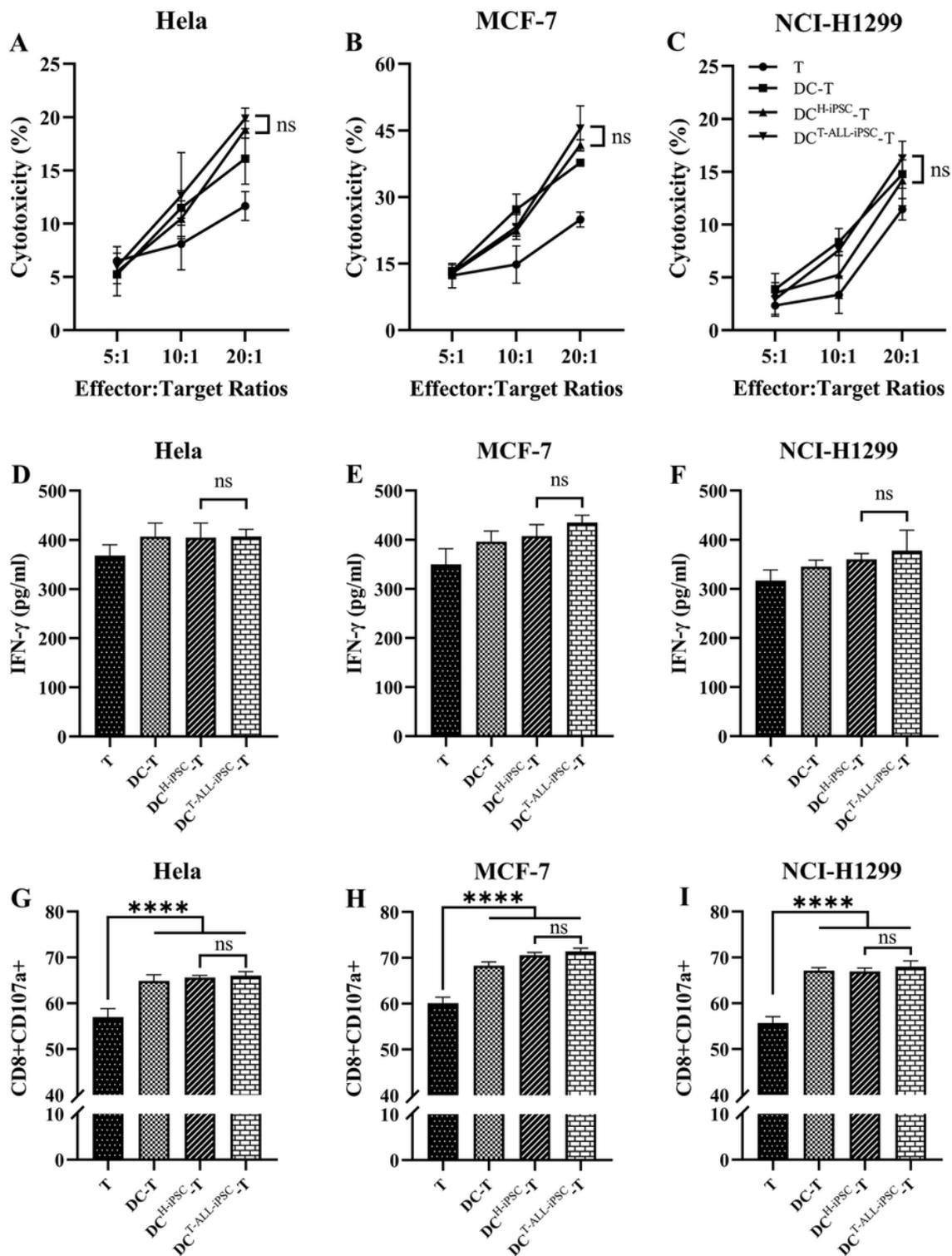


Figure 4

The cytotoxic activity of the CTL stimulated by the antigen-loaded DC on the non-T-ALL cells in vitro. The cytotoxicity (A), secretion of IFN- γ (D), and proportion of CD8+CD107a cells (G) analysis of the CTLs against the HeLa cells were detected. The cytotoxicity (B), secretion of IFN- γ (E), and proportion of the CD8+CD107a cells (H) analysis of CTLs against the MCF-7 cells were detected. The cytotoxicity (C),

secretion of IFN- γ (F), and proportion of the CD8+CD107a cells (I) analysis of CTLs against the NCI-H1299 cells were detected. Data were represented as mean \pm SEM from three independent experiments.

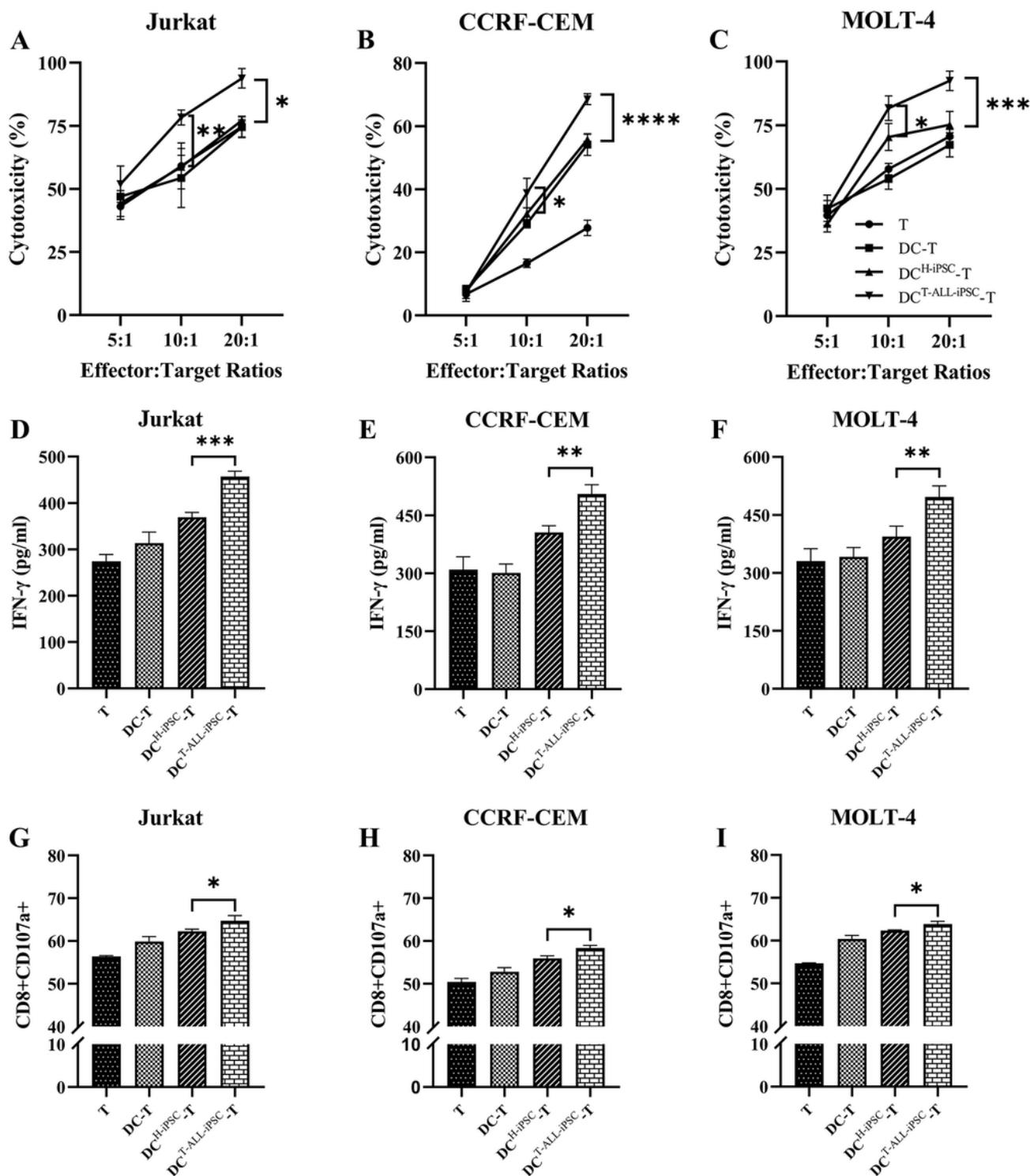


Figure 5

CTLs from the T-ALL-iPSC group show specific cytotoxicity against the T-ALL cells in vitro. The cytotoxicity (A), secretion of IFN- γ (D), and proportion of the CD8+CD107a cells (G) analysis of CTLs against the Jurkat cells were detected. The cytotoxicity (B), secretion of IFN- γ (E), and proportion of the

CD8+CD107a cells (H) analysis of CTLs against the CCRF-CEM cells were detected. The cytotoxicity (C), secretion of IFN- γ (F), and proportion of CD8+CD107a cells (I) analysis of CTLs against the MOLT-4 cells were detected. Data were represented as mean \pm SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001