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RESEARCH

Cytotoxic lymphocytes induced by engineered human dendritic cells mediate potent anti-leukemia activity

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Abstract

Effective treatment of acute myeloid leukemia (AML) remains an urgent unmet need. Adoptive transfer of cytotoxic T cells (CTLs) against leukemia-associated antigen (LAA) has strong potential to improve AML treatment. However, the clinical translation of this therapeutic modality is hindered by the difficulty of obtaining large quantities of LAA-specific CTLs. Stimulating naïve T cells using monocyte-derived dendritic cells (MoDCs) loaded with LAA is commonly used for the generation of CTLs. This approach has drawbacks as MoDCs loaded with desired antigen need to be developed repeatedly with multiple steps and have limited growth potential. We have established immortalized human dendritic cells (DC) lines (termed ihv-DCs). Here, we report the successful generation of CTLs by culturing AML patient-derived T cells with our off-the-shelf ihv-DCs that carry HLA-A2-restricted human telomerase reverse transcriptase (hTERT), a known LAA. These CTLs exert a potent cytotoxic activity against leukemia cell lines and primary AML blasts in vitro. Importantly, using a highly clinically relevant PDX model where CTLs (derived from clinical donors) were adoptively transferred into NSG mice bearing patient-derived AML cells (that were partial or full HLA match with the donors), we showed that the CTLs effectively reduced leukemia growth in vivo. Our results are highly translational and provide proof of concept using the novel DC methodology to improve the strategy of adoptive T cell transfer for AML treatment.

Keywords AML · Post-transplant relapse · Cytotoxic lymphocytes · Dendritic cell

Introduction

Acute myeloid leukemia (AML) is a devastating blood cancer with 5-year overall survival of only 29.5% in adults. Allogeneic hematopoietic stem cell transplantation (alloSCT) is the only potentially curative treatment in many clinical settings. However, leukemia relapse remains the primary cause of post-transplant death [1]. The clinical benefit of alloSCT greatly relies on the graft-versus-leukemia (GVL) effect, which is mediated mainly by donor T cells. However, donor T cells may also cause graft-versus-host disease (GVHD) [2–5]. One strategy to improve GVL without promoting GVHD is the adoptive transfer of cytotoxic T cells (CTLs) against leukemia-associated antigen (LAA).

Adoptive cell transfer therapy (ACT) is promising in cancer treatment [6, 7]. An increasing number of LAAs have been identified as targets for ACT against AML. These include human telomerase reverse transcriptase (hTERT), Wilms tumor antigen 1 (WT1), survivin, cathepsin G, and PR1, etc. [8]. They are highly expressed in leukemic blasts and leukemic stem cells, whereas minimal expression of these LAAs was observed in normal tissues. CTLs reactive to hTERT, WT1, PR1, and survivin, separate or combined, are highly capable of cytotoxic killing against leukemia cells in AML-bearing immunodeficient xenograft mouse models [9–13]. Importantly, persistent CTLs were found in the

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bone marrow, and no GVHD was observed [9]. Furthermore, results of multiple clinical trials applying adoptive transfer of LAA-reactive CTLs in treating AML patients are encouraging. Acceptable safety data, adequate LAA-specific T cell response, and effective anti-leukemia activity have been observed [14–17]. Therefore, ACT with LAA-specific donor CTLs has a strong potential to improve the GVL effect and treat leukemia. However, the clinical translation of this therapeutic modality is hindered by the difficulty of obtaining large quantities of CTLs. Stimulating naïve T cells using matured monocyte-derived dendritic cells (MoDCs) loaded with LAA is currently the most common approach to generate CTLs [18–20]. To create MoDCs, monocytes from fresh peripheral blood mononuclear cells (PBMCs) must be properly differentiated and activated in the presence of multiple cytokines [21, 22]. Compared with blood dendritic cells (DCs), MoDCs are less efficacious in polarizing the proliferating T lymphocytes [23, 24] which is likely due to the suboptimal conditions during the maturation of MoDCs [25, 26]. In addition, it is challenging to obtain adequate numbers of MoDCs as they have limited growth potential. Currently, most studies utilize autologous DCs pulsed with tumor lysate or specific peptides, thereby preventing broad implementation in large clinical settings due to patient specificity. Hence, long-lasting "off-the-shelf" donor-derived DCs that are pre-activated and pre-loaded with LAA would be the key to efficiently generate anti-leukemia CTLs and facilitate ACT in alloSCT patients with AML.

We have recently developed a novel DC methodology and established immortalized human primary blood DC lines by transducing PBMCs with the Tax gene derived from a non-oncogenic virus (termed ihv-DCs) [27]. The ihv-DCs are constitutively activated and able to potently prime naïve T cells. They can be genetically modified to deliver selected tumor antigens and subsequently stimulate the tumor antigen-specific CTLs in high efficiency. Importantly, ihv-DC-activated CTLs significantly inhibit tumor growth and metastasis in a mouse model of human lung cancer [27]. Here, we generate CTLs by stimulating donor PBMCs with ihv-DCs that express human leukocyte antigen (HLA)-A2- and the LAA hTERT. We investigate, in vitro and in vivo, the efficacy of CTL killing against AML.

Results

Generation of CTLs by stimulating PBMCs with ihv-DCs that are engineered to express hTERT

HLA-A2$^+$ ihv-DCs engineered to express hTERT (ihv-DC-hTERT) were used to induce CTLs. PBMCs from an HLA-A2$^+$ healthy donor were cocultured with ihv-DC-hTERT. We observed significant cell growth starting at 3 to 5 days and subsequent continuous expansion, achieving 2×10^8 (104-fold increase) at the end of 4 weeks of coculture (Fig. 1A and supplemental Figure 1A). To examine the accumulation of different cell types during coculture, we performed a serial flow cytometry analysis of the cells prior to and at different times post coculture. We found a predominant accumulation of CD8 T cells and NK cells. In contrast, no significant expansion of CD4 T cells, B cells or monocytes was observed (Fig. 1B, Supplemental Figure 1A, and Supplemental Figure 2). Consistent with our previous report [27], no ihv-DC were detected after 4 weeks of coculture. We then focused on the generated CD8 T cells (CTLs) and further assessed their phenotype and functional status. T cell differentiation was evaluated by flow cytometry based on the surface expression of CD45RA and CCR7, and was defined as naïve (T_N, CCR7$^+$CD45RA$^+$), central memory (T_{CM}, CCR7$^+$CD45RA$^-$), effector memory (T_{EM}, CCR7$^-$CD45RA$^-$) and terminally differentiated effector memory T cells (T_{EMRA}, CCR7$^-$CD45RA$^+$). There was a quick decline of T_N subpopulation, while T_{EM} rapidly increased during the culture and became the predominant subset by day 10 and plateaued thereafter (Fig. 1C and Supplemental Figure 1B). We also observed a remarkable up-regulation of intracellular transcription factor T-bet and eomesodermin (Eomes) 25 days after coculture, indicating an increased activation status. Importantly, upon coculture with ihv-DC-hTERT, the majority of CD8 T cells expressed Ki67 and showed high intracellular production of granzyme B and perforin (Fig. 1C), suggesting a potent proliferation and cytotoxic capacity. Collectively, these data demonstrate successful generation of CD8 T cells with a CTL phenotype by coculture with ihv-DC-hTERT. We term these cells CTL-hTERT.

CTL-hTERT exerted potent cytotoxicity against hTERT-expressing tumor and primary AML cells in vitro

We performed cytotoxicity assays to test the killing ability of the CTL-hTERT. First, U2OS, an HLA-A2$^+$ osteoblast cell line, was selected as a model target. U2OS cells are known to be TERT negative [28, 29]. U2OS-hTERT cells that constitutively express hTERT were established by transducing hTERT into the parental U2OS cells. We observed strong killing of U2OS-hTERT cells by the CTLs, whereas the cytotoxic effect on U2OS parental cells was minimal (Fig. 2A). Notably, the killing was significantly diminished by adding the HLA-A2-blocking antibody, indicating a predominant HLA-A2 restricted cytotoxicity (Fig. 2A). Next, we conducted the cytotoxicity assay using THP-1, an acute monocytic leukemia cell line, as the target. THP-1 cells are HLA-A2$^+$. Consistent with prior observations [11], our study verified that almost all THP-1 cells expressed intracellular

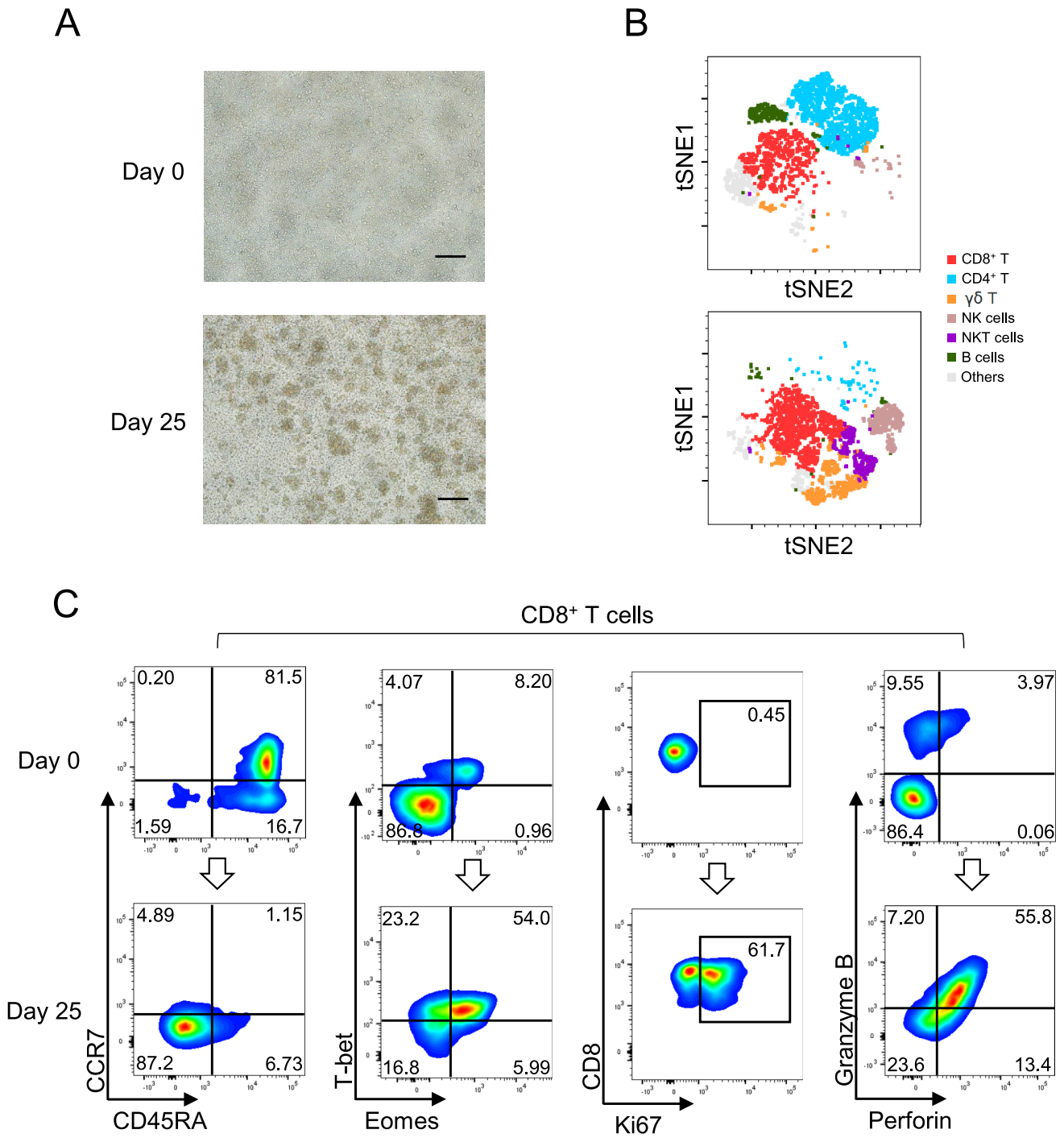

Fig. 1 Cell components and immunophenotyping analysis of the generated CTLs. **A**. Microscopic images of cell coculture with donor PBMCs and ihv-DC line at the beginning and day 25. Scale bar 100 μm. **B**. Cell component data by flow cytometry was analyzed through t-distributed stochastic neighbor embedding (tSNE) algorithm on day 0 and day 25. DN T cells are $CD4^{-}CD8^{-}$ double negative T cells; NK like cells are $CD56^{+}CD3^{+}$ cells. **C**. Immunophenotype of generated CTLs, showing the subsets (T_{CM}, central memory: $CCR7^{+}CD45RA^{-}$; T_{N}, naïve: $CCR7^{+}CD45RA^{+}$; T_{EMRA}, terminally differentiated effector memory: $CCR7^{-}CD45RA^{+}$; T_{EM}, effector memory $CCR7^{-}CD45RA^{-}$), proliferating ability and cytotoxic potentials on day 0 and day 25 after the coculture

hTERT (Fig. 2B). Similar to the killing of U2OS-hTERT cells, CTL-hTERT had significant cytotoxicity against THP-1 cells. Importantly, CTLs expanded using ihv-DC without hTERT transduction (ihv-DC-blank ctrl) showed much less killing of THP-1(Fig. 2C). We also observed that the HLA-A2-blocking antibody inhibited the killing effect

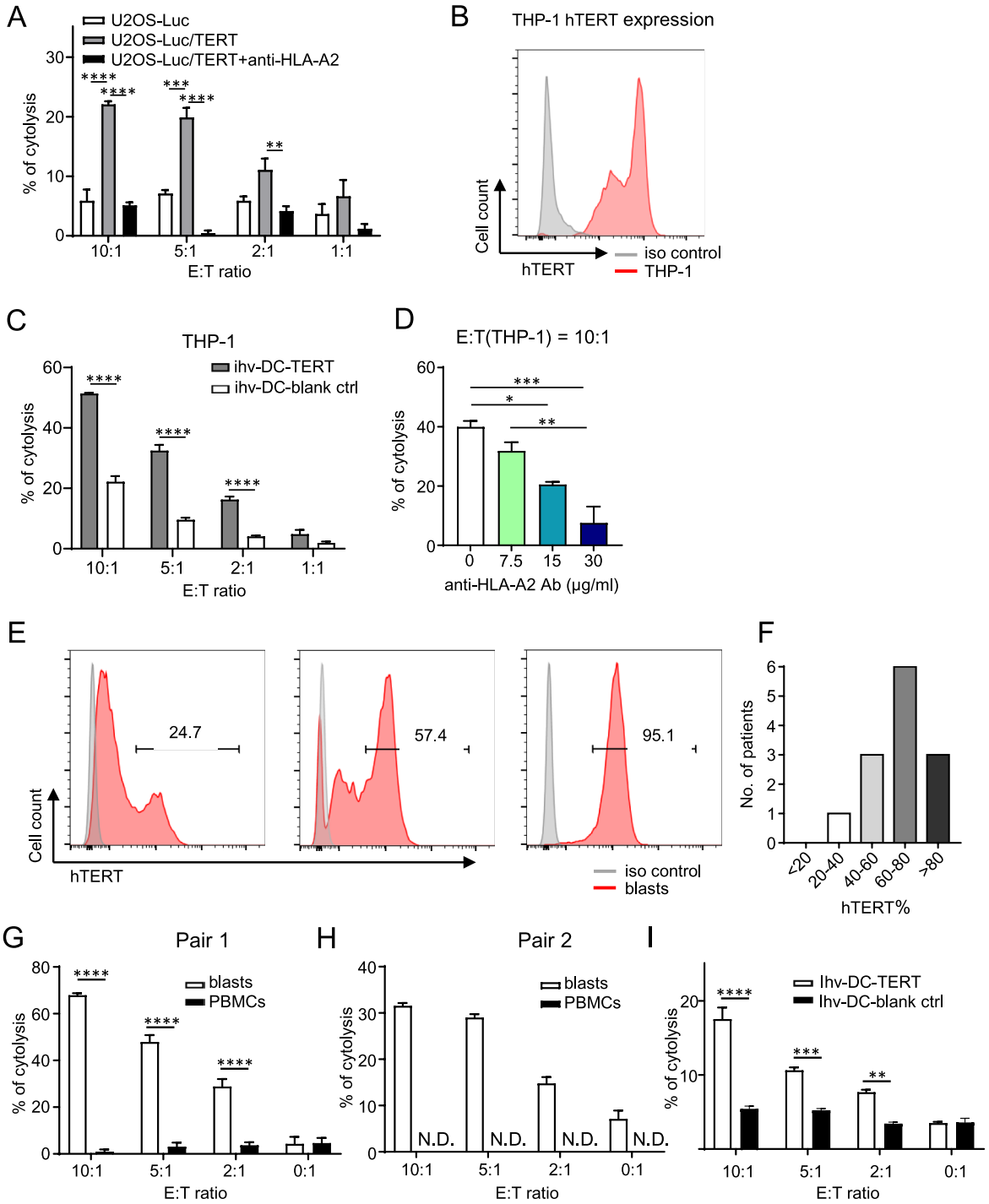

Fig. 2 In vitro cytotoxicity tests of generated CTLs. **A**. Cytotoxicity assay using U2OS-luc or U2OS-luc/TERT as target cells, with ihv-DC primed and CD8 enriched CTLs as killer cells. HLA-A2 blocking antibody (30 μg/ml) was added to interfere the killing process (n = 3). **B**. Representative flow cytometry histogram showing the hTERT expression of THP-1 cells. **C**. Cytotoxicity assay using THP-1 as target cells, CTLs expanded by ihv-DC with hTERT (ihv-DC-HERT) vs. without hTERT (ihv-DC-blank ctrl) were as killer cells (n = 3). **D**. CTLs killing THP-1 cells in the presence of increasing amounts of anti-HLA-A2 antibodies (n = 3). **E**. Representative flow cytometry histograms showing the hTERT expression on AML blasts from 3 patients at initial diagnosis. **F**. Summary of hTERT expression on blasts from 11 AML patients. **G, H**. Two sets of cytotoxicity assays using primary AML blasts (at first diagnosis) and PBMCs (during complete remission) from 2 AML patients as target cells, with ihv-DC primed and CD8 enriched CTLs from 2 healthy donors as killer cells. N.D. represents not detected. **I**. Cytotoxicity assay using primary AML cells as target cells, CTLs generated by ihv-DC with (ihv-DC-TERT) vs. ihv-DC without hTERT (ihv-DC-blank ctrl) as killer cells. Each cytotoxicity assay was repeated three times, and the values presented are from one representative experiment, the data shown are technical replicates. Data represent mean ± SEM. P-value achieved byone-way ANOVA or Kruskal–Wallis test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.001$

in a dose-dependent manner (Fig. 2D). These data demonstrate a potent hTERT-specific and HLA-A2-restricted killing capacity of the CTL-hTERT.

To translate the strategy of CTL-hTERT to clinical applications for AML therapy, we further examined their cytotoxicity against primary AML cells. Blood samples from 11 HLA-A2$^+$ AML patients were examined for intracellular expression of hTERT by flow cytometry. Although variable, blasts from all patients expressed significant hTERT (Fig. 2E, F). To minimize the contribution of allo-reaction, AML cells from one patient, who has 6/8 HLA matches (Supplemental Table 1) with the donor where the CTL was derived, was chosen as the target in the killing assay. PBMCs from this same patient that achieved complete remission (thus leukemia-free) were used as the negative control. CTL-hTERT displayed a robust cytotoxic activity to primary AML blasts, but little effect was shown on non-blast PBMCs (Fig. 2G). To verify this critical finding, we set up another cytotoxicity analysis using a second patient-donor pair with 5/8 HLA match (Supplemental Table 1). Consistent with our initial observations (Fig. 1C), CTL-hTERT derived from donor 2 were also predominantly T_{EM} subset and expressed a high level of Ki67, granzyme B and perforin (Supplemental Figure 3A, B). They were able to effectively kill the primary AML cells, whereas cytotoxicity on non-blast PBMCs was not detected (Fig. 2H). Furthermore, we performed an additional experiment generating CTLs by co-culturing donor PBMCs with ihv-DC-hTERT vs. ihv-DC-blank ctrl (no hTERT). The cytotoxicity against 5/8 HLA matched primary AML was examined. Consistent with the data in Fig. 2G, H, the CTL generated by ihv-DC-hTERT displayed a robust cytotoxic activity to primary AML, significantly less killing was observed in CTLs generated by ihv-DC-blank ctrl (Fig. 2I). These data demonstrated a potent hTERT-specific killing of the CTLs. Of note, there are some (albeit significantly less) killing by CTLs generated by blank ihv-DC especially at high E:T ratio (Fig. 2I), indicating an involvement of alloreactive response.

Collectively, we observed a potent in vitro hTERT-specific cytotoxic killing of tumor cell lines and primary AML cells by CTL-hTERT.

Adoptive transfer of CTL-hTERT reduced AML growth in vivo

To determine whether CTL-hTERT can eliminate AML in vivo, the CTLs were adoptively transferred into a patient-derived xenograft (PDX) model, where primary AML cells were injected into NSG mice. Two model systems using CTLs and AML derived from the same donor-patient pairs used in the in vitro assays (Fig. 2G, H) were tested. At 6 weeks post AML injection, when leukemia blasts were detectable in peripheral blood of the mice (Supplemental Figure 4B), purified CTLs (Supplemental Figure 4A) were injected via tail vein every 5 days for a total of 4 times. Mice were euthanized upon completion of treatments, organs including bone marrow, spleen, liver, and blood were collected and evaluated for AML blast burden by flow cytometry. As shown in Fig. 3, the adoptive transfer of CTLs significantly reduced the percentage as well as the absolute number of leukemia blasts in the bone marrow. This was observed in both model systems (Fig. 3). The same trend was found in the blood and spleen, although not all achieved statistical significance likely due to limited sample size (Fig. 3 and Supplemental Figure 4C). To validate these findings and further mimic clinical settings, we established a third model using CTLs and AML cells derived from a paired clinical donor and recipient patient in an HLA-matched unrelated alloSCT (Fig. 4A). Both the donor and the recipient patient are HLA-A2$^+$. CTLs were again made by culturing PBMCs of the donor with ihv-DC-TERT. Consistently, we observed significantly reduced tumor burden in the bone marrow and the blood of the PDX mice built by AML derived from the recipient patient (Fig. 4B). These important data demonstrate that CTL-hTERT effectively suppress AML growth in vivo.

Expression of TIGIT and PD-1 were up-regulated on CTLs after their adoptive transfer into the PDX model

Although a significant reduction of leukemia burden was achieved upon CTLs adoptive transfer, residual leukemia blasts were detected in all organs tested upon completion of treatment (Figs. 3, 4B, and Supplemental Figure 4C). Intriguingly, we found persistent CTLs in the PDX models at the endpoint of the observation (Fig. 5A and supplemental Figure 5). To evaluate the impact of leukemia on the transferred CTLs in vivo and determine why the persistent CTLs were unable to eradicate residual leukemia, we performed comprehensive phenotypic and functional studies to compare the features of CTLs prior to vs. after adoptive transfer into AML-bearing NSG mice. Considering the possibility that the status of CTLs might change due to the environmental shift from in vitro to in vivo. We compared our results to a non-AML control group, in which the NSG mice received CTLs only (without AML).

First, we evaluated the differentiation subsets of the CTLs. We observed that in blood, although the T_{EM} subset remained dominant, the frequency of T_{EMRA} subset was significantly increased in the CTLs adoptively transferred into the PDX model (with AML), compared with the CTLs pre-transfer or CTLs transferred into NSG mice (non-AML control). A similar trend was found in the bone marrow (Fig. 5B). Next, we assessed the CTL expression of TIGIT and PD-1 as previous studies, including ours, showed the

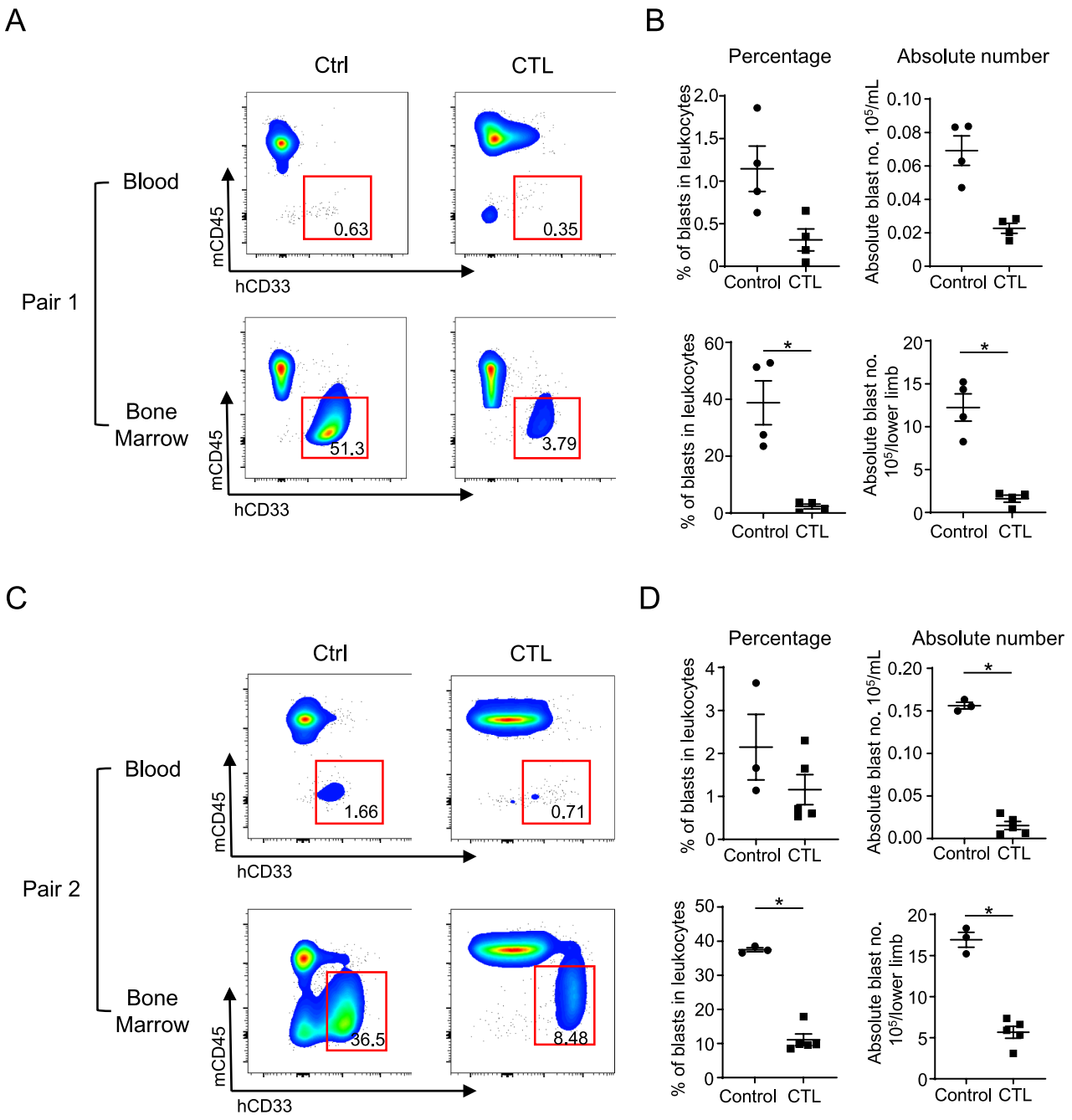

Fig. 3 In vivo cytotoxicity tests of generated CTLs with partial HLA-matched pairs. **A**. Representative flow cytometry graph showing the percentages of blasts from the blood and bone marrow of AML bearing mice receiving tail vein injection of vehicle (as control), or with ihv-DC primed and CD8 enriched CTLs (q.5.d×5, 5 million in 100μL plus 1,000 IU IL-2). **B**. Summary of the percentages and the absolute numbers of blasts in blood and bone marrow on day 20 after adoptive transfer. **C**, **D**. The second donor-patient pair for cytotoxicity assay in vivo, showing the same pattern of graphs as the first pair. Data are mean ± SEM of 3–5 mice per group. P-value achieved by unpaired student *t*-test. *, $P < 0.05$

involvement of these two T cell inhibitory pathways in AML pathogenesis [30–33]. TIGIT and PD-1 expression on CTLs in the non-AML control mice were comparable to CTLs that were pre-transfer. Strikingly, a significant increase of TIGIT and PD-1 expression was observed on CTLs from both blood and bone marrow after adoptive transfer into the PDX models bearing AML (Fig. 5C). These data suggested exhaustion among CTLs after their encounter with AML blasts. Consistent with this notion, the proliferation marker Ki67 was significantly decreased in the blood and

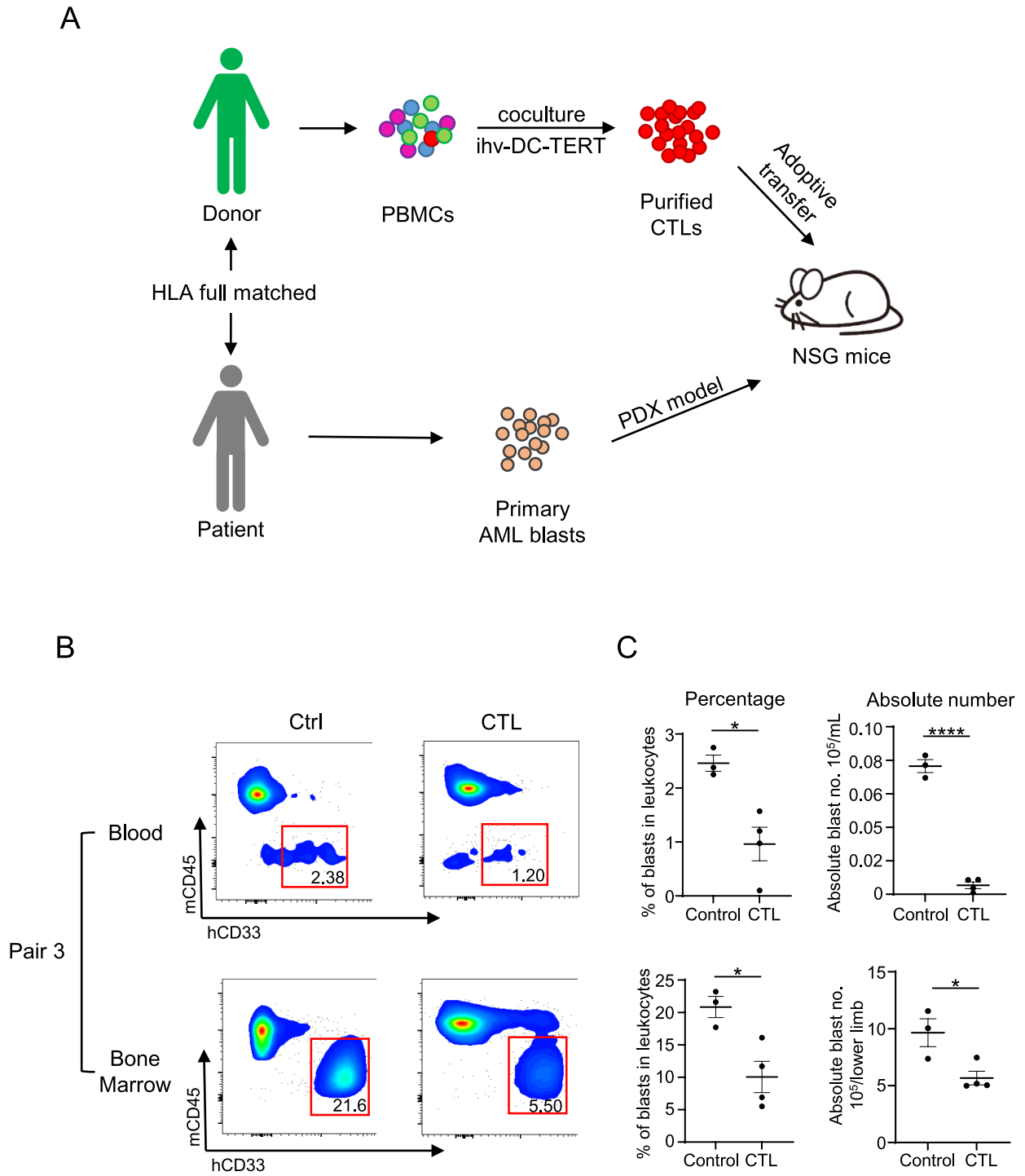

Fig. 4 In vivo cytotoxicity tests of generated CTLs with total HLA-matched pair. **A**. A schema showing the designed process of experiment. **B**. Representative flow cytometry graph showing the percentages of blasts from the blood and bone marrow of AML bearing mice receiving tail vein injection of vehicle (as control), or with ihv-DC primed and CD8 enriched CTLs (q.5.d×5, 5 million in 100μL plus 1,000 IU IL-2). **C**. Summary of the percentages and the absolute numbers of blasts in blood and bone marrow on day 20 after adoptive transfer. Data are mean ± SEM of 3–5 mice per group. P-value achieved by unpaired student *t*-test. *, $P < 0.05$. ****, $P < 0.001$

bone marrow of the PDX models, whereas it remained high in non-AML control (Fig. 5C). In addition, the expression of granzyme B in CTLs was significantly lower in the bone marrow of PDX models compared with that in the non-AML control (Fig. 5C). Furthermore, another T cell exhaustion-related marker, the transcription factor Eomes, was found

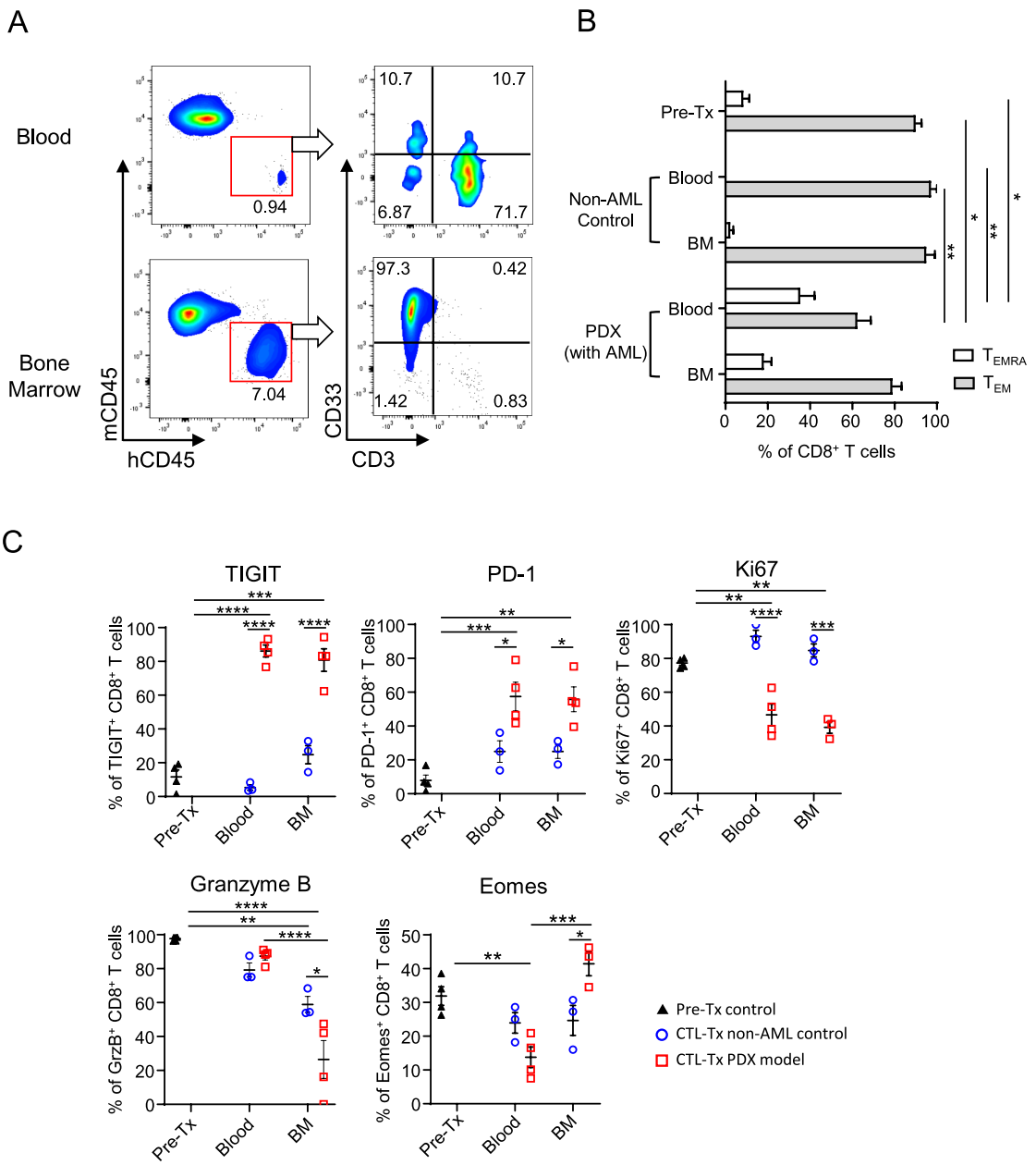

Fig. 5 Comparisons of immunophenotype changes after the adoptive transfer of CTLs in total HLA-matched pair. **A**. Representative flow data showing the evidence of the T cells remained in the blood and bone marrow of PDX models on day 20 after the CTL treatment. **B**. Compared to the T cells in vitro before adoptive transfer (Pre-Tx), the subpopulations of the remaining CTLs were changed in the peripheral blood and the bone marrow of the mice after 20 days of treatment. **C**. Immunophenotypes including TIGIT, PD-1, transcription factor Ki67 and Eomes, and cytotoxic potentials of CTLs in blood and bone marrow on day 20 after adoptive transfer. P-values achieved by one-way ANOVA or Kruskal–Wallis test. Data are mean ± SEM of 3–5 mice per group. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.001$

elevated in the CTLs from the bone marrow of the PDX models compared to non-AML control (Fig. 5C). Interestingly, the Granzyme B and Eomes in blood showed minimal change, suggesting disparate impact of AML on CTLs in different micro-environments.

The above findings were made in the paired HLA fully matched patient-donor PDX model system (Fig. 4). The up-regulation of T cell inhibitory receptors and reduced function were also observed in our HLA partial matched PDX models of both pair 1 and pair 2 (Supplemental Figure 6). In addition, we assessed the expression of immune checkpoint ligands on AML blast and observed a trend of up-regulation of PD-L1 on AML in mice after adoptive transfer of CTLs (Supplemental Figure 7). Collectively, our data suggested that the in vivo interaction between

AML and CTLs may cause T cell exhaustion resulting in residual leukemia.

Discussion

In our current study, using a novel DC methodology in which human DCs were immortalized by transducing with Tax from a non-oncogenic virus, human T cell leukemia virus type 2 (HTLV-2), and engineered to express hTERT, we successfully generated hTERT-specific CTLs from PBMCs of healthy donors. The ihv-DCs induced rapid expansion of CTLs that are highly functional. We observed a potent cytotoxic activity of the CTLs to tumor cell lines and primary AML blasts in vitro. Importantly, using a highly clinically relevant PDX model where CTLs (derived from donors PBMCs) were adoptively transferred into NSG mice bearing patient-derived AML cells (that were partial or full HLA match with the donors), we showed that the CTLs effectively reduced leukemia growth in vivo. Our data are highly translational and reveals a strong potential to improve the strategy of adoptive T cell transfer for treatment of AML relapse post alloSCT.

It has been well established, in both pre-clinical and clinical settings, that tumor-associated antigen (TAA)-specific CTLs are capable of recognizing and eradicating tumors. Adoptive transfer of CTLs represents a practical therapeutic approach for cancer treatment [6, 34]. T cells engineered with chimeric T cell receptors (CAR-T) has offered new hope for patients with refractory B cell lymphocytic leukemia and lymphoma [35, 36]. However, the development of CAR-T for AML has been challenging due to severe side effects, including extended cytopenia, likely due to targeting CD33 or CD123 expressed on myeloid progenitors [37, 38]. Adoptive transfer of non-engineered CTLs, also called tumor-infiltrating lymphocytes (TILs), is another well-developed methodology. TILs are isolated from the tumor mass, expanded and selected ex vivo before infusing back into patients [39–41]. Although promising, the process is invasive, time-consuming, and has low yield as the number of TILs isolated is often limited. In addition, the function of TILs is frequently impaired due to suppressive tumor microenvironment. CTLs against LAA is an attractive therapeutic approach for AML, however, generating adequate CTLs, both quantitatively and qualitatively, has been challenging. This is largely due to lack of optimal method to obtain sustainable and functional DCs, which are essential to induce CTLs [42]. Our novel ihv-DCs overcomes this obstacle in that 1) the ihv-DCs are immortalized and can be easily expanded in vitro and become "off-the-shelf" with proper HLA matching; 2) the ihv-DCs are constitutively activated to express co-stimulatory molecules and to produce cytokines that support T cell proliferation; 3) the ihv-DCs can be engineered to express any designated TAAs. Using ihv-DCs engineered with hTERT, an important LAA, we are now able to successfully generate large numbers of CTLs against leukemia. Upon coculturing PBMCs with ihv-DC-hTERT, CTLs were quickly induced and expanded more than 100-fold in 3 weeks. Importantly, these CTLs are highly functional and efficiently kill AML blasts both in vitro and in vivo. Therefore, our study provides a practical approach for generating CTLs that are adequate for clinical applications.

Our current result using no CTLs (with IL-2 only) as control demonstrates the overall treatment efficacy of hTERT-CTLs (Fig. 4), while comparing the anti-leukemia effect of non-hTERT-CTLs vs. hTERT-CTLs would help to dissect how much of the efficacy is attributable to hTERT-specific activity and how much is due to non-hTERT related component (e.g. alloreactive response). We conducted studies assessing in vitro cytotoxic activity against AML by CTLs generated from ihv-DC-TERT vs. blank ihv-DC (without TERT). We observed significantly less killing by CTLs generated by blank ihv-DC, demonstrating a potent hTERT-specific cytotoxicity (Fig. 2I). That being said, there are some killing by CTLs generated by blank ihv-DC especially at high E:T ratio, indicating an involvement of alloreactive response. Therefore, a critical step translating the strategy of our CTLs to clinic for treatment of AML relapse post alloSCT is to determine an optimal dose of CTLs that maximizes anti-leukemia effect while limits alloreactivity (thus minimizes GVHD). Careful dose titration of the hTERT-CTLs using CTLs generated by blank ihv-DC as control in our patient-specific in vivo preclinical model will help to move this process forward.

Overexpression of hTERT has been observed in many human malignancies, including AML [43, 44]. In contrast, expression in normal cells or tissues is minimal [8]. Previous studies showed that hTERT peptide-loaded MoDCs were able to generate CTL clones with strong cytotoxicity against leukemia [45]. In addition, the adoptive transfer of hTERT-specific TCR-engineered T cells significantly reduced the leukemia burden in the PDX mouse model receiving human AML cells [11]. Furthermore, primary DCs electroporated with mRNA-encoding hTERT stimulated hTERT-specific T-cell responses, which improved recurrence-free survival in AML patients post chemotherapy [46]. Therefore, hTERT is well characterized as immunogenic and considered as a strong LAA [47–50]. Consistently, our study also demonstrated effective anti-leukemia cytolysis by hTERT-specific CTLs that were generated from PBMCs using the ihv-DC method. Interestingly, although hTERT is highly expressed in normal human blood cells that are mitotically active, we observed minimal CTL cytotoxicity against hTERT positive PBMCs (Fig. 2G, H). Similar results have been reported in other studies [51, 52]. It is possible that the hTERT

presented by MHC molecules on normal cells was far below the threshold for CTL recognition [53]. In addition, multiple studies have reported that memory T cells were unable to induce GVHD [54, 55]. In this study, almost all CTLs cocultured with ihv-DC differentiated to effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) subsets on day 25 (Fig. 1 and supplemental Figure 1B and 3B). Indeed, we did not observe signs of GVHD in the xenograft mouse model until the end of the experiments on day 20. Besides hTERT, other LAAs, such as WT1, mucin 1, preferentially expressed antigen of melanoma (PRAME) and survivin, have been applied as targets in adoptive CTL therapy for AML [10, 56, 57]. Durable responses in several clinical trials have been reported. It is conceivable that combining CTLs against multiple LAAs may improve AML killing. Our DC methodology provides a strong platform to test this strategy as ihv-DCs can be engineered to express multiple LAAs. Importantly, we also found that the engineered ihv-DCs were capable of priming naïve T cells to generate HLA-A2-restricted, Melanoma-associated antigen 3 (MAGEA3)-specific CTLs [27] and HLA-A1101-restricted K-RAS neoantigen-specific CTLs in a separate project (data not shown). Therefore, the approach of generating effective CTLs by engineered ihv-DCs is not limited to one HLA or TAA.

Along with the generation of cytotoxic T cells, we also observed the expansion of NK cells and γδ T cells upon culturing PBMCs with ihvDC-hTERT. We observed up-regulation of intracellular transcription factor T-bet and Eomes 25 days after the co-culturing. In addition, the expression of Ki67, GranzymeB and Perforin were increased (Supplemental Figure 1C and Supplemental Figure 2). In addition, these NK cells showed potent cytotoxicity against K562 cell lines in vitro (Supplemental Figure 3C). This could be attributed to the IL-2 and IL-15 secretion by ihv-DCs as described in our previous published study [27]. The allogeneic NK cell-mediated GVL effect is also well characterized in the alloSCT settings in AML due to the KIR-mismatch with HLA class I of the recipient. Emerging data also showed anti-tumor effect of γδ T cells. Therefore, adding NK cells and/or γδ T cells to the CTLs holds strong potential to improve treatment of leukemia relapse post alloSCT. Our DC methodology provides a versatile tool to generate both types of cytotoxic lymphocytes. Further investigation is needed to facilitate this therapeutic strategy.

In the in vivo PDX study, we observed significant up-regulation of PD-1 and TIGIT on CTLs after their adoptive transfer into the AML-bearing NSG mice. These CTLs are less functional manifested by lower proliferation and killing capacity. They also expressed more Eomes, which is highly related to exhaustion phenotype. These findings suggest the development of exhaustion by CTLs upon interaction with AML in the PDX model. This result is consistent with an exhaustion-based mechanism for how residual AML cells evade CTL cytotoxicity. Therefore, combining CTLs and checkpoint inhibition may optimize this treatment strategy.

Methods

Human samples

Peripheral blood from AML patients was collected at the Penn State Cancer Institute of Penn State University College of Medicine, Hershey, PA. The study was approved by the Institutional Review Board (IRB) of Penn State University College of Medicine (STUDY00005272). Full informed consent was obtained from all patients. PBMCs were isolated using Ficoll density-gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), and cryopreserved. The cryopreserved cells were thawed and briefly cultured in 37 °C and 5% CO_2 incubator with RPMI-1640 medium (Corning, Corning, NY, USA) supplemented with 5% heat-inactivated human serum from male AB plasma (MiliporeSigma, St. Louis, MO, USA) before coculture with ihv-DCs.

Cell lines

The generation of ihv-DC cell line presenting hTERT (ihv-DC-hTERT) peptide is described previously [27]. The ihv-DCs were grown and expanded with RPMI-1640 medium supplemented with 5% heat-inactivated human serum (MiliporeSigma) in the presence of recombinant IL-2 (100 units/mL, R&D Systems, Minneapolis, MN, USA). U2OS cell line as well as U2OS transduced with TERT (U2OS/TERT) were modified to express luciferase and were also reported previously (27). THP-1 cell line was provided by Chunhua Song, Penn State University College of Medicine, and the cell concentration was maintained below 1×10^6 cells/mL. U2OS, U2OS/TERT, and THP-1 cell lines were grown in RPMI-1640 medium with 10% heat-inactivated FBS.

HLA genotyping

HLA-A, B, C, and DRB1 were typed at the high-resolution level by next-generation sequencing (ALLType kit, One Lambda, West Hills, CA) or the combination of sequence-specific oligonucleotide probe (LabType kit, One Lambda) and Sanger sequence-based typing kit (SBTexcellerator kit, GenDx, Utrecht, Netherlands).

Generation of hTERT-specific CTLs by ihv-DC-hTERT

PBMCs from the HLA-$A2^+$ healthy donors were mixed with ihv-DC-hTERT at the ratio of 100:1 and cocultured in

RPMI-1640 containing 5% heat-inactivated human serum. The recombinant IL-2 (100–200 units/mL) was added per day, starting from the second day of culture. Flow cytometry was performed to monitor the proliferation of the lymphocytes. For cell surface staining, samples were taken from the medium and stained with Fixable Viability Dye eFluor 506 (eBioscience, San Diego, CA, USA) for 20 min at 4 °C. Then, cells were stained with fluorescence conjugated monoclonal antibodies (mAbs) for 20 min at 4 °C. Transcription factor staining was performed using the transcription factor buffer set (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions. Specific mAb panels are listed in the supplemental Table 2. LSRFortessa flow cytometer (BD Biosciences) was used for data acquisition. Two to 3 weeks after coculturing, the ihv-DC-hTERT activated lymphocytes were analyzed by flow cytometry. The CTLs were purified by magnetic cell isolation (STEMCELL Technologies, Vancouver, BC, Canada) using kits according to manufacture instructions. In brief, NK cells were first depleted with human anti-CD56 beads, and then CTLs were isolated by anti-CD8 positive selection. Purified cells were washed with PBS plus 2% FBS before subsequent cytotoxicity assay.

Cytotoxicity assay in vitro

For U2OS and U2OS/TERT cell lines as targets, cancer cells were placed in 48-well plates for at least 2 h to ensure complete attachment. Then, the purified CTLs were placed onto the cancer cells with indicated E:T ratios. Cells were cocultured in 37 °C and 5%CO_2 incubator for 4 h. After twice gentle wash with PBS buffer, luciferase activity assay was performed with the Promega luciferase assay system (Promega, Madison, WI, USA) according to manufacture instructions. The light intensity of the assay was detected via SpectraMac i3x luminescence reader (Molecular Devices, San Jose, USA). The cytotoxic activity was calculated based on the light intensity of target cells that treated with or without CTLs. For THP-1 cell line and primary AML blasts from the patient PBMCs, the target cells were labeled with 1 μM CellTrace Violet dye (ThermoFisher, Waltham, MA, USA). Purified CTLs were mixed with target cells at indicated E:T ratios and cocultured in 48 or 96-well plates for 4 h. To detect AML blasts from PBMCs, harvested cells were stained with cell surface fluorescence conjugated mAbs: anti-CD45-BV786, CD19-FITC, CD20-FITC, CD11b-AF700, CD56-PE-CF594, CD14-BV711 (BD Bioscience) and CD3-APC-Cy7 (ThermoFisher). Then, cell apoptosis analysis was performed on labeled THP-1 cells and surface stained patient PBMCs with Annexin V apoptosis detection Kit PE (ThermoFisher). The cytotoxic activity of CTLs was determined by comparing the Annexin V and 7AAD double negative target cells treated with or without CTLs.

Patient-derived xenograft (PDX) models

The animal study was approved by the Institutional Animal Care and Use Committee at Penn State University College of Medicine, according to the National Institutes of Health guidelines for animal use. NOD-Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/SzJ (NSG) female mice at 10 (24-26 g) weeks of age were purchased from The Jackson Laboratory (Sacramento, CA, USA). Patient-derived AML cells were washed and resuspended in Hanks balanced salt solution (HBSS, ThermoFisher). One million blasts in 20μL HBSS were transferred into anesthetized mice via intra-femoral injection. The establishment of the PDX model was confirmed by flow cytometry. In brief, 30μL peripheral blood was collected from the tail vein. Cells were stained with anti-mouse CD45-BV510, anti-human CD45-BV786 and CD33-APC and tested by flow cytometer. The PDX mice were randomized into two groups, CTL treatment and control. Two weeks after blasts cell implantation, purified CTLs were injected via the tail vein with 5 million purified CTLs supplemented with 1,000 units recombinant human IL-2 (in 200μL HBSS) every 5 days (total of 4 injections). The control group received the same amount of HBSS and recombinant IL-2. Twenty-five days after CTL treatment, mice were sacrificed and the peripheral blood, bone marrow from one lower limb, spleen were taken. Flow cytometry analysis was performed to examine the cell components and immune markers from the PDX model. Cell surface and intracellular staining methods were the same as described above. The absolute cell number was determined with CountBright Absolute Counting Beads (ThermoFisher) through flow cytometry. Detailed mAb staining panels are listed in the supplemental Table 2.

Statistics

FlowJo software, version 10.6.2 (Tree Star, Ashland, OR, USA) was used to analyze data collected by the flow cytometer. The tSNE analysis in Fig. 1 was performed using the built-in plugins of the FlowJo software. GraphPad Prism, version 7.00 (GraphPad Software, La Jolla, CA, USA) was used for statistical calculations and the generation of figures. Statistical parameters and significance are reported within the figures and figure legends. The normality of each continuous variable was evaluated by the Kolmogorov–Smirnov test. For normally distributed data, unpaired Student's *t*-test was performed to compare variables for two groups and one-way ANOVA for three or more groups; for data not distributed normally, the Mann–Whitney U test was used to compare variables for two groups and Kruskal Wallis test for three or more groups. All tests are two-sided and a $P < 0.05$ was considered statistically significant. Data represent mean ± SEM.

Study approval

The study was approved by the Institutional Review Board of Penn State University College of Medicine (STUDY00005272). Full written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The animal study was approved by the Institutional Animal Care and Use Committee at Penn State University College of Medicine (PROTO201800175), according to National Institutes of Health guidelines for animal use.

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Author contributions C.Z. designed the research studies, conducted the experiments, analyzed the results and wrote the manuscript. B.J. conducted the experiments and analyzed the results. Y.J. and T.D.S. designed the experiments, discussed the data and reviewed the manuscript. H.S, J.C, K.M, S.M, W.C.E. acquired samples, managed patients and discussed the data. H.C. designed the research studies, discussed the data and reviewed the manuscript; H.Z. conceived the concept, designed the experiments, oversaw the interpretation and presentation of the data, and wrote the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest Hua Cheng is the founder for ImmuCision Biotherapeutics, LLC, which develops DC technology for profit. All other authors declare no conflicts of interest.

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