Developing Allogeneic Double-Negative T Cells as a Novel Off-the-Shelf Adoptive Cellular Therapy for Cancer

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Abstract

Purpose: To expand clinical-grade healthy donor-derived double-negative T cells (DNT) to a therapeutically relevant number and characterize their potential to be used as an “off-the-shelf” adoptive cellular therapy (ACT) against cancers.

Experimental Design: We developed methods to expand DNTs under GMP conditions and characterized their surface molecule expression pattern using flow cytometry–based high-throughput screening. We investigated the off-the-shelf potential of clinical-grade DNTs by assessing their cytotoxicity against various cancer types and their off-tumor toxicity in vitro and in xenograft models and determining the effect of cryopreservation under GMP conditions on cell viability and cytotoxicity. Further, we determined the susceptibility of DNTs to conventional allogeneic T cells in vitro and in vivo.

Results: Clinical-grade DNTs expanded 1,558 ± 795.5-fold in 17 days with >90% purity. Expanded DNTs showed potent in vitro cytotoxic activity against various cancer types in a donor-unrestricted manner. DNTs enhanced the survival of mice infused with a lethal dose of EBV-LCL and significantly reduced leukemia engraftment in xenograft models. Expanded DNTs cryopreserved using GMP-compliant reagents maintained viability and anticancer functions for at least 600 days. Live allogeneic DNTs did not induce cytotoxicity of alloreactive CD8+ T cells in vitro, and coinfusion of DNTs with peripheral blood mononuclear cells (PBMC) from a different donor into mice resulted in coengraftment of DNTs and PBMC-derived allogeneic conventional T cells in the absence of cytotoxicity toward DNTs, suggesting the lack of host-versus-graft reaction.

Conclusions: We have established a method to generate therapeutic numbers of clinical-grade DNTs that fulfill the requirements of an off-the-shelf ACT.

Introduction

The effectiveness of adoptive cellular therapy (ACT) using T cells to treat different hematologic and solid malignancies has been demonstrated in multiple clinical studies (1, 2). Advances in technologies such as genetically modifying immune cells to express a chimeric antigen receptor (CAR) or a transgenic-T-cell receptor and the use of artificial antigen-presenting cells have been implemented to improve the therapeutic potency of ACTs (3–5). Recently, CD19-CAR T-cell therapy achieved effective clinical responses in patients with B cell malignancies (1, 6) and has been FDA approved for clinical use for these diseases (7, 8). However, with increasing numbers of patients treated with ACT, limitations of current forms of ACT have become apparent, including sophisticated expansion methods resulting in uncertainty of producing therapeutically relevant numbers of T cells, time required for cell expansions, requirement of clinically approved facilities for cell expansion, inconsistency of manufactured cellular products, and high production costs (9–11).

Recently, the number of studies exploring the use of ACT as an off-the-shelf treatment has grown (8, 12–16). Off-the-shelf ACT focuses on generating large batches of cells from allogeneic donors and using them to treat a large array of patients. As this approach is not patient-specific, cellular products can be premanufactured to save time. Mass production also increases product consistency, availability, and reliability at a lower cost (8, 13). However, an effective clinically applicable off-the-shelf allogeneic T-cell therapy should meet the following criteria: (i) expandable to a therapeutically relevant number under clinically compliant condition; (ii) do not cause graft-versus-host disease (GVHD); (iii) are able to target an array of cancers in a donor-unrestricted manner; (iv) are not rejected by the recipient’s immune system; (v) can be stored under GMP conditions without hampering their function (10). There are reports on ACTs with a potential to be used as an off-the-shelf therapy, including NK-92 (14), primary NK cells (17), and γδ T cells (18), due to their HLA-unrestricted antitumor toxicity and low risk of off-tumor toxicity. However, to the best of our knowledge, there has not been a report to show an ACT that can avoid host-versus-graft (HvG) rejection while fulfilling other requirements of off-the-shelf therapy without any genetic modification.

Double-negative T cells (DNT) are mature T cells that comprise 3% to 5% of peripheral T cells and is defined by expression of CD3...
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**Translational Relevance**

Adoptive T-cell therapy is a promising treatment option for patients with cancer. However, with an increase in the clinical use of adoptive cellular therapy (ACT), its limitations, including high treatment costs and technical requirements, are becoming apparent and are restricting the wide clinical use of ACT. Off-the-shelf allogeneic ACT has several advantages including lower treatment cost, reliable supply of cellular products, and ease accessibility, but multiple requirements must be met before its clinical applications. In this study, we established a straightforward and easily applicable method to expand clinical-grade double-negative T cells from healthy donors that fulfills the requirements of an off-the-shelf ACT: a therapy that targets various cancer types without apparent off-tumor toxicity, does not induce host-versus-graft reaction, and is cryopreservable. To the best of our knowledge, this is the first report to show a T-cell therapy that can be used as an off-the-shelf therapy without any genetic modification.

in the absence of CD4, CD8, and iNKT cell markers (19–22). Recently, we demonstrated that healthy donor (HD) derived allogeneic DNTs target acute myeloid leukemia (AML) *in vitro* and in patient-derived xenograft models (20) and have synergistic anticancer activities with conventional chemotherapies (19). Importantly, allogeneic DNTs are not toxic toward normal cells and infusion of DNTs into mice does not cause xenogeneic GVHD (20). Although these characteristics support the use of allogeneic DNTs as an off-the-shelf product, other required features such as expansion capacity and storage of expanded cells under clinically compliant conditions, as well as their ability to avoid HvG reaction and *in vivo* persistence have not been addressed previously.

In this study, we developed a method to generate therapeutic numbers of clinical-grade DNTs with minimal manipulations and extensively characterized their cell-surface marker expression. Expanded DNTs have cytotoxic function against various cancer types in a donor-independent manner, can be cryopreserved under GMP conditions, and are resistant to immune rejection by allogeneic T cells *in vitro* and *in vivo*. Collectively, our data support the use of DNTs as an off-the-shelf ACT for cancers.

**Materials and Methods**

**Ex vivo expansion of DNTs under GMP conditions**

DNT expansions were done as previously described (20) under GMP conditions with some modifications. Briefly, CD4 and CD8 T-cell–depleted peripheral blood mononuclear cells (PBMC) were cultured on anti-CD3 antibody-coated plates (GMP grade OKT3, Miltenyi Biotec) for 3 days in AIM-V (Thermo Fisher) or GT-551 (Takara Bio) media with 250 IU/mL of IL2 (Proleukin, Novartis Pharmaceuticals); soluble anti-CD3 antibody and IL2 were added to the cultures. The purity of DNTs was assessed on days 0 and 10 of expansion as well as after harvesting before use for subsequent experiments. DNT purity was measured by staining cells with fluorochrome-conjugated anti-human CD3, CD4, CD8, and CD56 antibodies and flow cytometry analysis. For validation runs, DNTs were expanded at the Philip S. Orsino Cell Therapy Facility at Princess Margaret Cancer Centre or at Sunnybrook Research Institute GMP facility. To test for sterility, *Mycoplasma*, and endotoxin, expanded DNT products were sent to Mount Sinai Hospital, WuXi AppTec, and Princess Margaret Cancer Centre, respectively.

**Flow cytometry–based in vitro killing assay**

For nonadherent cancer cells, DNTs were cocultured with target cells for 2 to 4 hours, and then stained with anti-human CD3 (HIT3a), CD33 (WM53), CD45 (H130), and CD34(561) antibodies, Annexin V, and 7AAD (all from BioLegend), and analyzed using flow cytometry. Specific killing was calculated by

\[
\frac{\% \text{Annexin V}^- \text{with DNT} - \% \text{Annexin V}^- \text{without DNT}}{100 - \% \text{Annexin V}^- \text{without DNT}} \times 100
\]

For adherent cancer cells, cell lines were labeled with DiO (Invitrogen) and cocultured with DNTs for 14 hours. All cells were collected after incubation in 0.25% trypsin-EDTA solution and stained with TO-PRO-3 (Life Technologies). Cell suspensions were analyzed by flow cytometry to determine specific lysis of labeled target cells. Specific killing was calculated by

\[
\frac{(\% \text{DiO}^- \text{TO-PRO-3}^- \text{with DNT} - \% \text{DiO}^- \text{TO-PRO-3}^- \text{without DNT}) \times 100}{100 - \% \text{DiO}^- \text{TO-PRO-3}^- \text{without DNT}}
\]

**Antibodies and flow cytometry**

The following anti-human antibodies were used for cell staining: CD3-FITC or -PECy7, CD4-FITC or -PE, CD8-FITC or -PE, CD33-APC or -PECy5, CD56-PE, iNKT TCR (V24Jr18 TCR)-APC, and Annexin V-FITC or -Pacific Blue and were purchased from BioLegend. Data acquisition was performed using either a BD Accuri C6 Flow cytomter (BD Biosciences) or an Attune NXT cytometer (Thermo Fisher). Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.).

**High-throughput flow cytometry screening**

Ex *vivo*–expanded DNTs were prepared for flow cytometry–based high-throughput screening as described previously (23). Briefly, expanded DNTs were spun down and treated with Ex TruStain (BioLegend) in PBS containing 0.5% BSA for 10 minutes followed by staining with anti-CD3 PE-Cy7 antibody. Subsequently, cells were sent to Princess Margaret Genomics Centre, where cells were stained with antibodies against 385 different cell-surface molecules followed by staining with a viability dye, DAPI, prior to being analyzed by flow cytometry. Intracellular staining of CTLA-4 was performed using the protocol described in Supplementary Materials and Methods. Data were analyzed using FlowJo software (Tree Star, Inc.).

**Cryopreservation of DNTs**

Day 14 to 20 *ex vivo*–expanded DNTs were resuspended in CyroStor2 (StemCell Tech) containing 2% clinical-grade DMSO (StemCell Tech), followed by adding an appropriate volume of CyroStor10 (StemCell Tech) containing 10% DMSO to make the final concentration of DMSO at 7.5% and cell concentration at 5 to 10 × 10^6 cells/mL. Cells were frozen in –80°C freezer and then transferred to liquid nitrogen the next day for longer storage. To thaw DNTs, cells taken from liquid nitrogen were thawed in a 37°C water bath for 1 minutes. Cells were then transfused to a 50 mL polystyrene tube and PBS was added at 20× the volume of cells. Cells were centrifuged at 300 × g for 10 minutes. Pellets were resuspended at 10^6 cells/mL with IL2 and anti-CD3 (OKT3) antibody containing AIM-V and rested overnight before use.
Figure 1.
Clinical-grade DNTs expanded under GMP conditions. 

A and B, Number of DNTs derived from each mL blood (A) and fold expansion (B) after 17 days culture are shown. Each symbol represents the result from 11 of 13 DNT cultures derived from 11 different donors. 

C, DNTs expanded as described in Materials and Methods were stained with immune cell subset markers (CD3, CD4, and CD8) to check the purity of cells. Result shown is a representative of DNTs expanded from 13 expansions. 

D–I, Results of flow cytometry–based surface molecule high-throughput screening on day 14–20 expanded DNTs from 3 donors are shown. Histograms show representative results for T-cell–associated markers, CD2, CD3, and CD5, and B-cell–associated markers, CD19 and CD20, to confirm the validity of the screening method (D). Graphs show expression of T-cell differentiation markers (E), chemokine receptors (F), cytotoxic (G), costimulatory (H), and coinhibitory (I) molecules on expanded DNTs from 3 donors. Each symbol represents DNTs from one donor. Numbers shown are the percentages of cells that expressed corresponding molecules on DNTs. Horizontal bars, mean ± SEM.
Mixed lymphocyte reaction

CFSE-labeled or unlabeled PBMCs obtained from HDs were cocultured with live or irradiated (3,000 cGy) expanded DNTs from autologous or allogeneic donor at 2:1 PBMC to DNT ratio for 4 to 6 days. Prior to the coculture, DNTs were spun down and washed once with PBS and cultured in AIM-V + 250 IU/mL IL2 without anti-CD3 antibody for 4 to 5 days to lower the level of anti-CD3 antibody bound on the surface of DNTs. CFSE-labeled cells were then stained with anti-CD4 and anti-CD8 antibodies and DAPI, and percent proliferating cells was determined by flow cytometry based on CFSE dilution. Percent increase in proliferation was calculated by

\[
\% \text{Proliferated with DNT} = \frac{\text{CFSE-labeled cells} - \text{CFSE-unlabeled cells}}{\text{CFSE-unlabeled cells}} \times 100
\]

To determine alloreactivity, CD8+ T cells from DNT-PBMC cocultures were isolated using a CD8-positive selection kit (StemCell Tech) and were used as effector cells against DNTs at 4:1 CD8:DNT ratio for 4 to 14 hours. The cells were then stained with Annexin V and anti-CD8 antibody and analyzed by flow cytometry.

Xenograft models

For all xenograft experiments, NOD.Cg-Prkdscid Ile2rgm1Wji/Sjcl (NSG) mice (Jackson Laboratories) maintained at the University Health Network (UHN) animal facility were used. To characterize persistence of DNTs, 8- to 12-week-old female mice were irradiated (250 cGy) 24 hours prior to a single injection of 5 μmol/L CFSE-labeled 2 × 10^6 DNTs. Cells from the bone marrow, spleen, liver, lungs, and peripheral blood were harvested on days 2, 7, 10, and 14, and the frequency of DNTs and CFSE dilution was determined by flow cytometry. To determine antitumor activity in vivo, irradiated NSG mice were injected with 2 × 10^6 MV4-11 or EBV-LCL cells through tail-vein injection. DNTs (2 × 10^6) were injected intravenously on days 3, 6, and 10 after cancer cell injection. MV4-11-infused mice were sacrificed 2 weeks after the last DNT injection, and the engraftment of MV4-11 in the bone marrow was determined using flow cytometry as described previously (20). EBV-LCL-infused mice were euthanized when their body weight decreased by 20%. To assess the tumor burden, MV4-11-bearing mice were infused with DNTs, as described above, or PBMCs as a positive control. The liver and lung tissues were harvested and fixed in 10% formalin overnight and sent to Pathology Research Program Laboratory (Toronto General Hospital) for hematoxylin and eosin (H&E) staining. The H&E-stained histology slides were blindly scored by a pathologist for tissue damage following a previously described scoring method (24) with some modifications. The modified scoring method used is described in Supplementary Materials and Methods. To determine the alloreactivity of DNTs, mice were infused with 2 to 3 × 10^6 HLA-A2+ PBMCs on day 0 and HLA-A2– DNTs on days 0, 3, and 6. Four weeks after infusion, cells from bone marrow, spleen, and lung were analyzed by flow cytometry to monitor the engraftment level of human T cells. In all experiments, rIL-2 (Proleukin) was administered (10^4 IU/mouse) intraperitoneally at the time of DNT infusion and weekly after the last DNT injection until euthanization.

Statistical analysis

All graphs and statistical analyses were generated using GraphPad Prism 5. Student t test and linear regression test were used. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 indicate significance between experimental and control values. Error bars represent ± SEM or SD as indicated.

Human samples and study approval

Human blood was collected from healthy adult donors after obtaining written informed consent and used according to UHN Research Ethics Board (05-0221-T). Animal studies were approved by the Institutional Animal Care Committee of UHN (AUP: 741.22) and carried out in accordance with the Canadian Council on Animal Care Guidelines.

Results

Ex vivo expansion and characterization of clinical-grade DNTs

Expansion of a cellular product under GMP conditions is required before its clinical application. Our previous studies were conducted with DNTs expanded using research grade reagents containing xenogeneic components (20, 21). Use of animal-derived supplements restricts the use of final products for patient treatment due to risks associated with the xenogeneic additives (25). To expand DNTs under GMP conditions, clinical-grade reagents were used and the yield, purity, and function of DNTs expanded using different types of animal serum-free media were compared (Supplementary Fig. S1). Subsequently, several parameters were optimized including cell concentration, IL2 concentration, and the schedule of cell splitting. With an optimized expansion method, as described in Methods and Materials, DNTs were expanded from 11 donors in 13 cultures. By 17 days of expansion, 1.11 ± 0.63 × 10^6 DNTs were generated from each milliliter of peripheral blood (PB; Fig. 1A) with an average fold expansion, 1.11 ± 0.63 × 10^6 DNTs and an average purity of 77.3%, respectively. Lower levels of other cytotoxic molecules, (Fig. 1H), and coinhibitory molecules (Fig. 1I) were examined. To better understand immune responses mediated by DNTs, expression of cytotoxic molecules (Fig. 1G), costimulatory molecules, (Fig. 1H), and coinhibitory molecules (Fig. 11) were examined. Among the cytotoxic molecules, 2 previously identified molecules involved in DNT-mediated antileukemia activity, NK2D and DNAM-1, were expressed at high levels (83.3% and 77.3%, respectively). Lower levels of other cytotoxic molecules, Nkp30 (13.4%), KIR2DS4 (15.2%), and membrane-bound...
TRAIL (16.3%) were detected, but DNTs were negative for Fasl, NKp44, NKp46, and KIR3DS1. DNTs expressed costimulatory molecules CD30 (49.5%), GITR (22.5%), CD27 (15.3%), and CD28 (25.2%), but expression of OX40, CD40, 4-1BB, and HVEM was very low or absent. Unlike most ex vivo–expanded effector T cells, expanded DNTs were low for coinhibitory molecules, ICOS, CITL-4, and PD-1, and PD-1 ligands, suggesting that DNTs may be resilient to T-cell exhaustion or cancer immune-escape mechanisms. However, high expression of TIM-3 (65.7%), LAIR1 (95%), and NK2A/CD94 (58.9% and 42.6%) were also detected, suggesting a potential inhibitory activity of these molecules on DNT-mediated anticancer activity.

Expanded DNTs target various types of cancers in vitro and in vivo in a non-donor-restricted manner. For an off-the-shelf ACT, cells manufactured from a single donor should be able to target cancers from multiple patients in a donor-unrestricted manner. To determine the function of clinical-grade DNTs, the cytotoxicity of expanded cells toward various cancer cell lines derived from myeloma, T-cell leukemia, Burkitt lymphoma, AML, EBV-LCL, large cell lung cancer, and lung adenocarcinoma was examined in vitro. DNTs exhibited broad anticancer cytotoxicity toward all of the cancer targets tested (Fig. 2A). Furthermore, clinical-grade DNTs from a single donor effectively targeted multiple cancer targets, OCI/AML3 and
Figure 2.
(Continued.) E, Mice were sacrificed 2 weeks after last DNT infusion and AML engraftment level in BM was determined. Result shown is representative of 4 separate experiments. Each dot represents result from one mouse and horizontal bars represent the mean values ± SEM of each group. F, In vitro killing assay conducted against primary AML patient sample containing leukemic blasts and normal cells. Left flow panels show the gating strategy used to distinguish leukemic from normal cells. Histogram shows the absence of off-tumor toxicity mediated by DNTs while inducing potent cytotoxicity toward cancerous cells. Experiments were done in triplicates. Result shown is representative of 4 independent experiments done with different patient samples.

G and H, NSG mice inoculated with the AML cell line MV4-11 were treated with PBS, human DNTs, or PBMCs. Twenty-eight days after injection of AML, mice were euthanized and the liver and lung tissues were formalin-fixed and stained with H&E. G, Representative H&E-stained slides of liver (400 × magnification) and lung (200 × magnification) from each group are shown. White arrows indicate bile ducts, green arrows indicate bronchioles, and blue arrows indicate the vessels. PV, portal vein; alv, alveoli. H, Tissue damage of H&E-stained lung (left) and liver (right) slides were blindly scored by a pathologist. Each dot represents one mouse, and horizontal bar represents the mean ± SEM. Data shown are representative of 4 separate experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, using unpaired two-tailed Student t test.
parameters were optimized to cryopreserve ex vivo. The therapy to areas that cannot manufacture cells, altogether increasing therapy for patient infusion, and provides a way to distribute cell consistency of cell products, offers immediate availability of cell viability and function is overlooked (29). Effective cryopreservation clinically compliant condition with negligible effect on cell viability for a successful off-the-shelf allogeneic ACT.

Conditions are effective at targeting a broad range of cancer demonstrating that HD-derived DNTs expanded under GMP PBMC-treated group (Fig. 2H). Taken together, these data for a successful off-the-shelf allogeneic ACT.

Histology samples were blindly scored by a pathologist and the were seen. The degree of tissue damage in the liver and lung were obtained on days 2, 7, 10, and 14 after infusion. DNTs were determined by systemically injecting CFSE-labeled in vivo–expanded human DNTs into naive sublethally irradiated NSG mice. Cells from PB, spleen, bone marrow (BM), liver, and lung were obtained on days 2, 7, 10, and 14 after infusion. DNTs were determined by systemically injecting CFSE-labeled PBMCs. Consistent with in vitro findings, a significant antitumor activity of DNTs was observed in xenograft models, but none of the cancer-bearing DNT-treated mice exhibited signs of xenogenic GVHD (Fig. 2G and H). Liver tissue from PBMC-treated mice showed moderate portal lymphocyte infiltration and severe bile duct damage, whereas DNT-treated mice showed mild portal lymphocyte infiltration without bile duct, vascular, or other injuries (Fig. 2G). In lungs, PBMC-treated mice showed severe inflammation around vessels and bronchioles, endothelialitis, and septal inflammation around alveoli. In contrast, DNT-treated mice showed no inflammation around vessels and bronchioles, and no endothelialitis or septal inflammation around alveoli were seen. The degree of tissue damage in the liver and lung histology samples were blindly scored by a pathologist and the DNT-treated group scored significantly lower than that from the PBMC-treated group (Fig. 2H). Taken together, these data demonstrate that HD-derived DNTs expanded under GMP conditions are effective at targeting a broad range of cancer types in vitro and in xenograft models in a donor-unrestricted fashion without off-tumor toxicity, which are necessary features for a successful off-the-shelf allogeneic ACT.

**Expanded DNTs can be cryopreserved under GMP conditions**

Often the importance of cryopreserving expanded cells under a clinically compliant condition with negligible effect on cell viability and function is overlooked (29). Effective cryopreservation methods allow for storage of manufactured cells, increases the consistency of cell products, offers immediate availability of cell therapy for patient infusion, and provides a way to distribute cell therapy to areas that cannot manufacture cells, altogether increasing the flexibility and accessibility of ACT. To this end, multiple parameters were optimized to cryopreserve ex vivo–expanded DNTs in a GMP-compliant animal serum-free media, including DMSO concentration, methods to add and remove DMSO, restimulation of thawed DNTs, and cell concentration for freezing. Optimally, cryopreserved DNTs retained their viability (Fig. 3A) and antileukemic activity in vitro (Fig. 3B) and in a xenograft model (Fig. 3C), albeit with ~20% cell loss from the freeze–thaw procedure. To use cryopreserved DNTs as a ready-to-go product, their shelf-life as frozen cells is important. To investigate this, the viability and cytotoxicity of DNTs frozen for 617, 534, 276, 129, or 8 days were determined. All DNTs remained viable and retained their antileukemic function (Fig. 3D). Collectively, DNTs expanded under clinically acceptable conditions can be cryopreserved in GMP-compliant media for at least 600 days without compromising their function, providing a way to use allogeneic DNTs as a “ready-to-go” treatment for patients with cancer.

**Live DNTs do not activate allogeneic immune response in vitro and can persist in vivo**

The ability of adoptively transferred cells to persist in recipients affects the outcome of ACT (30, 31), and its importance is more apparent in allogeneic settings as infused cells can be recognized and rejected by a patient’s immune system (32). Thus, the persistence, proliferative capacity, and migration patterns of DNTs in vivo were determined by systemically injecting CFSE-labeled ex vivo–expanded human DNTs into naive sublethally irradiated NSG mice. Cells from PB, spleen, bone marrow (BM), liver, and lung were obtained on days 2, 7, 10, and 14 after infusion. DNTs were determined by systemically injecting CFSE-labeled PBMCs from one donor (HD1) were stimulated with live or irradiated ex vivo–expanded DNTs from the same donor (HD1) or a different donor (HD2) to determine the immunogenicity of allogeneic DNTs to conventional T cells. Stimulation with irradiated allogeneic DNTs increased proliferation of CD4+ and CD8+ T cells by 27.7% ± 0.12% and 37.7% ± 0.91%, respectively, which was significantly higher than that by irradiated autologous DNTs (13.7% ± 0.94% for CD4+ and 10.8% ± 0.12% for CD8+ T cells; Fig. 4E). Interestingly, significantly lower degree of proliferation of CD4+ (6.04% ± 0.69%) and CD8+ T cells (6.32% ± 0.55%) was induced by live allogeneic DNTs, which were comparable to the levels of proliferation induced by live autologous DNTs (6.61% ± 0.38% for CD4+ and 6.79% ± 0.21% for CD8+ T cells; Fig. 4E). To further determine whether CD8+ T cells cocultured with autologous or allogeneic DNTs, the CD8+ T cells were isolated 4 to 6 days after coculture and used as effector cells against autologous or allogeneic DNTs as illustrated in Fig. 4D. As expected, CD8+ T cells stimulated with live or irradiated autologous DNTs did not induce any cytotoxicity to

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allogeneic DNTs. Importantly, whereas CD8\(^+\) T cells stimulated by irradiated allogeneic DNTs elicited potent cytotoxicity against allogeneic DNTs in a dose-dependent manner, no cytotoxicity of CD8\(^+\) T cells against allogeneic DNTs was detected when the CD8\(^+\) T cells were stimulated with live allogeneic DNTs (Fig. 4F).

Collectively, these results demonstrate that while DNTs carry alloantigens, live allogeneic DNTs do not induce significant levels of alloreactivity of conventional T cells in vitro. To further confirm that viable DNTs are resistant to allogeneic immune response in vivo, naive NSG mice were infused with PBMCs from an HLA-A2\(^+\) donor followed by 0 or 3 injections of HLA-A2\(^+\) DNTs from another donor. Four weeks after infusion, cells from the spleen, BM, and lungs were isolated and engraftment of human T cells was determined. We found that in 4 of 5 treated mice, a significant level of HLA-A2\(^+\) DNTs coengrafted with HLA-A2\(^+\) CD4\(^+\) and CD8\(^+\) T cells in the same tissues (Fig. 4G), indicating that DNTs persisted in the recipients for at least 4 weeks in the presence of allogeneic conventional T cells. To further investigate the alloreactivity of engrafted CD8\(^+\) T cells, HLA-A2\(^+\) CD8\(^+\) T cells were subsequently isolated from DNT- and PBMC-treated mice and used as effectors against DNTs from the same donor origin as used for the xenograft experiment. No significant decrease in DNT cell viability was seen in the presence of isolated HLA-A2\(^+\) CD8\(^+\) T cells (Supplementary Fig. S3), further supporting that allogeneic CD8\(^+\) T cells did not cause elimination of DNTs in a xenograft model. Collectively, these data suggest that ex vivo–expanded DNTs are resistant to allogeneic immune cell–mediated rejection and warrant further testing the potential of allogeneic DNTs as an off-the-shelf ACT that is resistant to HvG rejection in a clinical setting.

**Discussion**

Although the efficacy and safety of a novel therapy are the priorities in the development of a new treatment, feasibility of translation into patient treatment should not be overlooked. Current forms of ACT take the "personalized" approach, generating cellular product for individual patients "on-demand" using a patients' own immune cells and have achieved remarkable results in some patients but factors limiting their broad
Figure 4.
DNTs can persist in vitro and in vivo in the presence of allogeneic CD4⁺ and CD8⁺ T cells. A–C, 2 × 10⁷ ex vivo-expanded human DNTs were labeled with 5 μmol/L CFSE and intravenously injected into sublethally irradiated (250 cGy) NSG mice (n = 12). On days indicated, cells from blood, spleen, BM, liver, and lung were stained with antihuman CD45 and CD3 antibodies, and DNTs were detected by flow cytometry (A, n = 5 per day). CFSE median fluorescence intensity (MFI) of DNTs on days 0, 2, 7, 10, and 14 after injection was measured by flow cytometry. Histogram (B) and relative reduction of CFSE MFI with respect to day 0 CFSE MFI (C) are shown. The results shown are obtained from 3 mice per time point and are representative of 2 separate experiments using DNTs from 2 different HDs.

D–G, Mixed lymphocyte reaction (MLR) was conducted using HD1 PBMCs and HD2-expanded DNTs to determine the immunogenicity of expanded DNTs to allogeneic T cells. D, Schematic diagram shows the MLR conducted. E, CFSE-labeled or unlabeled HD1 PBMCs were cocultured with live or irradiated expanded HD1 or HD2 DNT for 4 to 6 days. At the end of the MLR, the percentage of increase in proliferating cells compared with the unstimulated control was determined as described in Materials and Methods. Left histogram shows the representative CFSE dilution, gated on CD8⁺ T cells. Experiments were done in triplicates, and the bar graph on the right shows the average of the triplicates. The results are representative of 2 separate experiments using different HDs for autologous and 5 separate experiments using 4 different HDs pairs for allogeneic DNTs. (Continued on the following page.)
application have also become apparent from these studies (33, 34). Therefore, off-the-shelf cell therapy that will allow more patient accessibility is considered as the next generation of ACT (8, 15). Unlike some other ACTs involving complicated procedures, the DNT expansion method developed here does not involve high technicality, feeder cells, cocktail of cytokines, or any genetic modifications. With the only added exogenous reagents being anti-CD3 antibody and IL2, this method reliably generated therapeutically relevant numbers of DNTs in 2 to 3 weeks with high purity.

An essential property of an off-the-shelf cellular product is its ability to target a broad range of cancers in a donor-independent manner. We have demonstrated that clinical-grade DNTs target an array of hematologic and solid cancers in vitro (Fig. 2A) and EBV-LCL and AML in xenograft models (Fig. 2D and E, respectively). In addition to cancer types presented in this study, Xu and
colleagues showed that DNTs could target pancreatic carcinoma in a xenograft model (35), and we observed significantly inhibited non–small cell lung cancer progression after DNT treatment in xenograft models (manuscript submitted, ref. 36). Notably, DNTs from a single donor could kill cancer cells of different origins (Fig. 2B), and the level of cytotoxicity against the same cancer target was comparable between DNTs derived from different donors (Fig. 2C) without observed off-tumor toxicity (Fig. 2F and G). Further, cryopreservation of expanded DNTs under clinically compliant conditions did not compromise the function of DNTs (Fig. 3). Collectively, these findings highlight the possibility of cryopreserving a large batch of expanded DNTs as a ready-to-go treatment for multiple patients.

One of the major concerns of using allogeneic T cells as a cellular therapy is the risk of GvHD (10). Jacoby and colleagues reported that infusion of allogeneic CD19-CAR T cells induced severe GvHD in the recipient mice due to the recognition of host allo-antigens by the endogenous TCR (37). To address this issue, several groups knocked out the endogenous TCR from CD19-CAR T cells (12) or transgenic TCR-transduced T cells (38) and successfully prevented the onset of GvHD and maintained the anticancer activity. However, such approaches are limited to ACT against known tumor antigens as the use of exogenously transduced receptors against cancer-associated antigens is required to replace the lost antitumor specificity of the endogenous TCR, and impose another hurdle in cell production. We and others have previously shown that infusion of allogeneic mouse DNTs or xenogeneic human DNTs does not cause GvHD, unlike conventional CD4+ and CD8+ T cells (16, 20, 39, 40). In line with previous findings, DNTs specifically targeted leukemic cells while sparing normal cells from the same patient sample in the same cytotoxicity assay (Fig. 2F), and cancer-bearing mice treated with expanded human DNTs showed significant reduction in leukemia load without signs of toxicities on normal mouse tissues (Fig. 2G). Taken together, these studies demonstrate that infusion of non-genetically modified allogeneic DNTs is unlikely to cause GvHD.

The persistence of infused immune cells has been shown to be correlated with treatment outcomes (41–43). Persistence of infused T cells is decided by intrinsic and extrinsic factors. As a cell-intrinsic factor, the activation status of infused cells can affect their persistence. We found that when DNTs were injected alone, they migrated and persisted in various tissues including the liver, lung, blood, BM, and spleen of NSG mice up to 14 days (Fig. 4A). Based on surface molecule profiling data, DNTs exhibited an effector memory phenotype (Fig. 1E) that is associated with a more robust immune response and shorter persistence compared with central memory T cells (42, 43). Although the persistence of infused allogeneic DNTs in patients is currently under study in our phase I clinical trial, modifications to the cell expansion method may help to generate DNTs with a central memory phenotype if longer persistence is desired. Alternatively, such an issue can be overcome by using the off-the-shelf property of DNTs and reinfusing patients with cryopreserved DNTs as needed.

The major extrinsic factor affecting cell persistence is the rejection of infused cells by the host-immune system. Many studies on allogeneic ACT focuses on avoiding GvHD, but less so on HvG rejection. In one study conducted by Torakai and colleagues, HLA expression on allogeneic CAR T-cells was knocked out by genetic manipulation, which successfully evaded allogeneic CD8+ T-cell–mediated cytotoxicity (44). However, the feasibility and safety of this approach in a clinical setting need to be validated. Interestingly, we found that stimulation of conventional T cells with live allogeneic DNTs does not elicit cytotoxic toward the allogeneic DNTs (Fig. 4E). Further, coinfusion of allogeneic DNTs with PBMCs from a different donor resulted in their coengraftment in NSG mice (Fig. 4C), and coengrafted allogeneic CD8+ T cells showed no alloreactivity against DNTs (Supplementary Fig. S3). To our knowledge, this is the first form of allogeneic ACT that is resistant to HvG reaction without genetic modification or exogenous immunosuppressant. Nevertheless, given that irradiated allogeneic DNTs were potent stimulators of allosreactive T cells, DNT viability for patient infusion needs to be carefully monitored to prevent the onset of HvG reaction.

The mechanisms by which live allogeneic DNTs evade rejection by conventional T cells remain elusive. We have shown previously that mouse DNTs could kill allosreactive CD8+ T cells that were activated by the same alloantigens that activated DNTs (45–47). Furthermore, a higher DNTs versus CD8+ T-cell ratio correlates with a less severe GvHD in allogeneic hematopoietic stem cell transplantation patients (48–50). We are currently investigating whether ex vivo-expanded DNTs can suppress cytotoxicity of alloreactive conventional T cells and consequently protect themselves from HvG rejection. Collectively, these data support that, unlike conventional T cells, infusion of viable allogeneic DNTs is less likely to result in rejection as the result of HvG reaction, which will be validated in the ongoing clinical trial in patients.

NK cells have the potential to be used as an off-the-shelf therapy without genetic modification due to HLA-unrestricted antitumor function and limited GvHD causing activities (16, 17). NK-92, a cell line derived from a patient NK cell lymphoma, has been shown to be safe and feasible as an off-the-shelf ACT in clinical studies (14, 51, 52). However, only one study reported that of 15 treated patients, 2 had mixed responses and 1 had stable disease. Limited antitumor activity may be due to short persistence as NK-92 were detectable only for ~48 hours after infusion (52), possibly due to irradiation of the cells prior to patient infusion to avoid potential in vivo tumorigenesis as they are immortalized cells. It is also possible that the infused NK92 were rejected due to HvG rejection (53), which has not yet been studied. In a clinical study conducted by Romee and colleagues, infusion of cytokine-induced memory-like allogeneic primary NK cells showed a more promising clinical response, where 4 of 9 patients with AML achieved complete remission, in the absence of dose-limiting toxicity (54). However, donor-derived NK cells were not detectable by 2 to 3 weeks after infusion, suggesting that the host-immune system recovered and rejected donor-derived allogeneic NK cells or infused NK cells have a limited life expectancy. We found that significant numbers of DNTs were detected in mice 4 weeks after coinfusion with allogeneic PBMCs in the absence of lymphodepletion, suggesting a better in vivo persistence of allogeneic DNTs than NK cells (Fig. 4G).

In our previous study (20), we have directly compared the cytotoxic activity of DNTs with NK-92 and expanded primary NK cells. We showed that whereas similar toxicity was seen against a known NK cell target, K562, superior killing was mediated by DNTs against all 7 AML targets tested, including 4 samples resistant to NK-92–mediated cytotoxicity. Similarly, DNTs derived from HDs showed superior killing against AML cell lines than those of primary activated NK cells. This suggests that DNTs function through a different mechanism than that of NK cells, and
DNTs may be able to target cancers resistant to NK-92. Further, resistance of DNTs to Hvg reaction may result in a longer persistence of DNTs in patients and therefore a more prolonged effect. Whether DNTs have a better or similar efficacy compared with cytokine-induced memory-like allogeneic primary NK cells requires further investigation.

In summary, we have established a method to expand clinical-grade DNTs from HDs without genetic modification or extensive manipulations. To our knowledge, DNTs are the first T-cell ACT that fulfills all the requirements of an off-the-shelf allogeneic cell therapy without genetic alteration. The expanded DNTs can be cryopreserved, persist in an allogeneic environment in the absence of immunosuppression and are effective in targeting various cancers without off-tumor toxicity. These properties allow for the use of allogeneic DNTs as an off-the-shelf ACT for patients with different cancer types as a standalone therapy or in combination with other conventional therapies. We aim to validate the observed phenomena in our ongoing phase I clinical trial to treat high-risk AML patient using allogeneic DNTs (NCT03027102). Further, the role of surface molecules expressed on DNTs as identified from high-throughput flow cytometry–based screening, such as Tim-3, CD94/NKG2A, LAIR-1, CCR3, and CXCR3, needs to be elucidated. Using off-the-shelf potential of DNTs and taking advantage of recent successes of CAR, we are transducing DNTs with CARs to develop universal CAR-DNT cell therapies that do not require additional gene modification to avoid GvHD or Hvg reaction to provide an ACT that is accessible and clinically feasible for treating patients with cancer.

Disclosure of Potential Conflicts of Interest
O. Adeyi is an employee of Dynacare. L. Zhang is a consultant/advisory board member for WYZE Biotech Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.B. Lee, H. Kang, L. Fang, C. D’Souza, O. Adeyi, L. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.B. Lee, H. Kang, L. Fang, O. Adeyi, L. Zhang
Writing, review, and/or revision of the manuscript: J.B. Lee, C. D’Souza, O. Adeyi, L. Zhang
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Developing Alloimmune Double-Negative T Cells as a Novel Off-the-Shelf Adoptive Cellular Therapy for Cancer

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