Allogeneic Human Double Negative T Cells as a Novel Immunotherapy for Acute Myeloid Leukemia and Its Underlying Mechanisms

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Abstract

Purpose: To explore the potential of *ex vivo* expanded healthy donor–derived allogeneic CD4 and CD8 double-negative cells (DNT) as a novel cellular immunotherapy for leukemia patients.

Experimental Design: Clinical-grade DNTs from peripheral blood of healthy donors were expanded and their antileukemic activity and safety were examined using flow cytometry–based *in vitro* killing assays and xenograft models against AML patient blasts and healthy donor–derived hematopoietic cells. Mechanism of action was investigated using antibody-mediated blocking assays and recombinant protein treatment assays.

Results: Expanded DNTs from healthy donors target a majority (36/46) of primary AML cells, including 9 chemotherapy-resistant patient samples *in vitro*, and significantly reduce the leukemia load in patient-derived xenograft models in a DNT donor–unrestricted manner. Importantly, allogeneic DNTs do not attack normal hematopoietic cells or affect hematopoietic stem/progenitor cell engraftment and differentiation, or cause xenogeneic GVHD in recipients. Mechanistically, DNTs express high levels of NKG2D and DNAM-1 that bind to cognate ligands preferentially expressed on AML cells. Upon recognition of AML cells, DNTs rapidly release IFNγ, which further increases NKG2D and DNAM-1 ligands' expression on AML cells. IFNγ pretreatment enhances the susceptibility of AML cells to DNT-mediated cytotoxicity, including primary AML samples that are otherwise resistant to DNTs, and the effect of IFNγ treatment is abrogated by NKG2D and DNAM-1–blocking antibodies.

Conclusions: This study supports healthy donor–derived allogeneic DNTs as a therapy to treat patients with chemotherapy-resistant AML and also reveals interrelated roles of NKG2D, DNAM-1, and IFNγ in selective targeting of AML by DNTs. *Clin Cancer Res; 24(2); 370–82. ©2017 AACR.*

Introduction

Acute myeloid leukemia (AML) is the most common form of adult acute leukemia with 5-year survival rates of approximately 5% and 35% for elderly and younger patients, respectively (1–4). Despite decades of using chemotherapy to treat AML patients, a high relapse rate and refractoriness to chemotherapy are major challenges to patient survival (1–3). Allogeneic hematopoietic stem cell transplantation is a potential curative cellular therapy for chemotherapy-resistant leukemia (5–7), but limited donor availability, the risk of GVHD, and other transplantation-related toxicities restrict its wide application in elderly and debilitated patients (4, 7–10).

Recently, chimeric antigen receptor (CAR)-T-cell therapies have shown excellent clinical responses against lymphocytic leukemia (11–13) and promising results in preclinical AML studies (14–17). However, in clinical trials, CAR-T cells specific for CD33 and LeY antigen caused severe toxicity in some of the treated AML patients (18, 19). Also, genetic modification of infused cells increases the treatment cost and potential risk to patients (17). Multiple clinical trials using allogeneic natural killer (NK) cell therapy to treat AML patients demonstrated the safety and clinical benefits, but a noticeable number of patients either did not achieve complete remission or experienced disease relapse (20–25). Some studies highlighted the importance of precise HLA-KIR matching for improved efficacy of NK therapy (24, 25), but this can limit treatment availability to patients. Furthermore, long-term efficacy of NK-cell therapy has yet to be demonstrated. Therefore, the need for safer and more effective cellular therapies with a broader and easier clinical applicability to target chemotherapy-resistant AML and prevent disease relapse remains.

In this study, we explored a nonconventional T-cell subset named double-negative T cells (DNT) as a novel therapy to target AML, including leukemic cells that are resistant to the conventional chemotherapy. DNTs are mature peripheral T lymphocytes expressing CD3–T-cell receptor (TCR) complex but not CD4,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Chemotherapy resistance represents a significant barrier to acute myeloid leukemia (AML) therapy. Different forms of cellular therapy have been developed to overcome this barrier, but low efficacy and associated toxicity have hampered their wide use in clinic. Here, we demonstrate that double-negative T cells (DNT) from healthy individuals can be expanded *ex vivo* to therapeutic levels under GMP conditions and be cryopreserved. Expanded human DNTs target a large array of primary AML cells, including chemotherapy-resistant patient samples *in vitro*, and significantly reduce the leukemia load in patient-derived xenograft models in a DNT donor–unrestricted manner. Importantly, allogeneic DNTs neither attack normal human cells nor cause xenogeneic GVHD. Collectively, healthy donor–derived allogeneic DNTs provide a potential new off-the-shelf cellular therapy that is safe and effective to treat patients with chemotherapy-resistant AML. A first-in-human phase I clinical trial (NCT03027102) using allogeneic DNTs to treat patients with high-risk AML has been initiated.

CD8, nor invariant NK T-cell markers. They represent 1% to 3% of peripheral blood mononuclear cells (PBMC; ref. 26). The function of human DNTs was largely unknown due to their low frequency *in vivo* and a lack of an effective method to expand them to sufficient numbers for *in vivo* studies. We have shown previously that DNTs expanded from AML patients were cytotoxic to autologous AML cells *in vitro* (26). However, attempts to expand autologous DNTs for treating AML patients have failed perhaps due to the defect of DNTs in patients. Whether allogeneic DNTs expanded from healthy individuals can effectively target AML cells *in vitro* and *in vivo* while sparing nonmalignant cells and tissues of recipients, and the mechanisms involved, has not been explored previously.

Here, we demonstrate, for the first time, that therapeutic quality and quantity of DNTs can be expanded *ex vivo* from healthy donors (HD) and that these cells can selectively target a large array of primary AML samples, including those from chemotherapy-resistant patients without observed toxicity toward normal cells and tissues. Furthermore, we identified a positive feedback loop of NKG2D, DNAM-1, and IFNγ, which helps to explain the ability of DNTs to selectively recognize and target AML but not normal cells. Collectively, our findings open a new window of cellular therapy using DNTs expanded from healthy volunteers as a potential off-the-shelf product to treat patients with high-risk AML, and perhaps other cancers.

Materials and Methods

DNTs and leukemic cell lines

DNTs were expanded *ex vivo* as described previously (26). Briefly, DNTs enriched by depleting $CD4^+$ and $CD8^+$ cells from PBMCs using $CD4^-$ and $CD8^-$ depletion cocktail (Stemcell Technologies) were cultured in anti-CD3 antibody-coated plates (OKT3; 5 μg/mL) for 3 days in RPMI1640 supplemented with 10% FBS and 250 IU/mL of IL2 (Proleukin, Novartis Pharmaceuticals); soluble anti-CD3 (0.1 μg/mL) was added on day 7, 10, and 14. On days 3, 7, and 10, fresh media and IL2 were added. DNTs were harvested 10 to 20 days postexpansion for subsequent

experiments. The leukemic cell lines AML3/OCI (AML3), KG1a, and MV4-11 were obtained from ATCC.

Flow cytometry–based *in vitro* killing assay

DNTs stained with PKH-26 (Sigma) were cocultured with target cells for 2 to 4 hours; cells were then stained with anti-human CD3 (HIT3a), CD33 (WM53), CD45 (HI30) FITC-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 blocking assays, DNTs were incubated with neutralizing antibodies for 0.5 to 1 hour prior to coincubation with target cells at 4:1 effector-to-target ratio for 2 hours or 2:1 effector-to-target ratio for 4 hours. Percent inhibition of killing was calculated by $\frac{\% \text{ Specific killing}_{\text{without Ab}} - \% \text{ Specific killing}_{\text{with Ab}}}{\% \text{ Specific killing}_{\text{without Ab}}} \times 100 \frac{(\% \text{ Specific killing}_{\text{without Ab}} - \% \text{ Specific killing}_{\text{with Ab}})}{\% \text{ Specific killing}_{\text{without Ab}}} \times d.$ For IFNγ pretreatment assays, DNTs or AML cells were pretreated with 50 ng/mL of recombinant human IFNγ (BioLegend) for 1 hour or overnight. Percent increase in killing was calculated by $\frac{\% \text{ Specific killing}_{\text{IFN}\gamma \text{ treated}} - \% \text{ Specific killing}_{\text{IFN}\gamma \text{ untreated}}}{\% \text{ Specific killing}_{\text{IFN}\gamma \text{ untreated}}} \times 100.$

Xenograft models

NOD.Cg-*Prkdc^scid Il2rg^{tm1Wjl}*/SzJ (NSG) mice (The Jackson Laboratory) were maintained at UHN animal facility. Eight- to 12-week-old females were irradiated (250 cGy) 24 hours prior to intrafemoral or tail vein injection of the $2-5 \times 10^6$ primary AML blasts. DNTs (2×10^7) were injected intravenously at the indicated time points. rIL2 (Proleukin) was administered (10^4 IU/mouse) intraperitoneally concordant with the DNT injections on days 1, 2, 4, and 7 and weekly thereafter until euthanized. Two to 4 weeks after the last DNT injection, mice were sacrificed and spleen and bone marrow cells were harvested, and the frequency of AML was analyzed by flow cytometry. For GVHD-related experiments, $5-20 \times 10^7$ DNT, PBS, or 5×10^6 PBMCs were intravenously injected into irradiated naïve NSG mice, and percent body weight change was calculated as $\frac{\text{body weight}_{\text{day 0}} - \text{body weight}_{\text{day x}}}{\text{body weight}_{\text{day 0}}} \times 100$. Mice with weight loss greater than 25% were euthanized via cervical dislocation. Fourteen days postinjection, liver, lung, and small intestine were obtained, fixed in 10% formalin for 24 to 48 hours, and then in 70% ethanol until samples were sent for hematoxylin and eosin histologic analysis. Histologic staining and picture acquisition was done by the Advanced Optical Microscopy Facility at UHN. For safety-related experiments, irradiated NSG mice were injected with 3×10^5 $CD34^+CD133^+$ hematopoietic stem/progenitor cells (HSPC) before treated with DNTs.

Statistical analysis

All graphs and statistical analysis 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.

Human samples and study approval

Human blood, bone marrow, and $CD34^+$ cells were collected from healthy adult donors and AML patients after obtaining written informed consent and used according to University Health Network (UHN) Research Ethics Board (05-0221-T) and NHLBI-approved protocols. PBMCs from HD or AML

patients were separated by Ficoll (GE Healthcare) density gradient. AML patient samples were viably frozen in the Princess Margaret Leukemia Bank and stored in liquid nitrogen until used. Animal studies were approved by the Institutional Animal Care Committee of the UHN (permit number: 741.22) and carried out in accordance with the Canadian Council on Animal Care Guidelines.

Results

Antileukemic function of HD-derived DNTs

To explore the potential of using allogeneic human DNTs to treat leukemia, we developed a protocol allowing for a large-scale GMP expansion of DNTs from HDs. So far, we have expanded DNTs from more than 60 HDs, of which 6 expansions were done
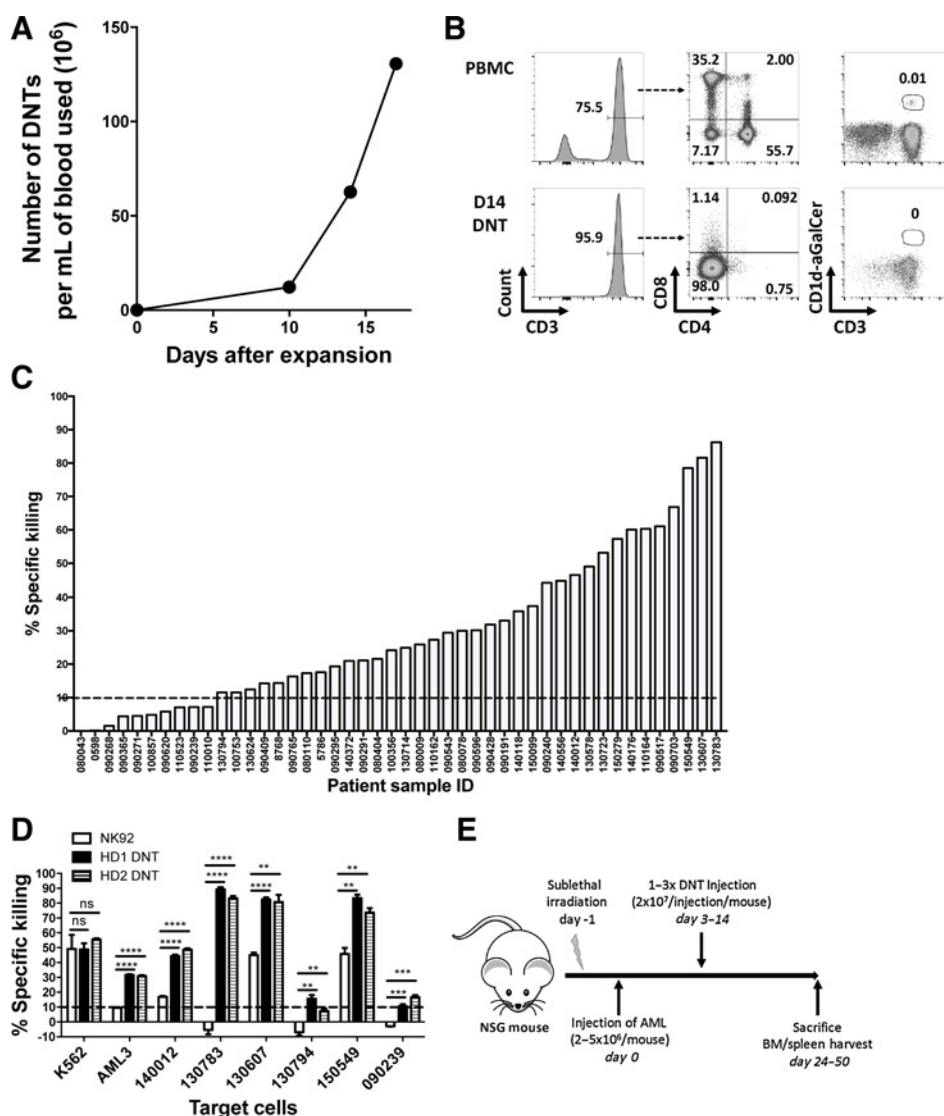

Figure 1.
Antileukemic activity of allogeneic DNTs against primary AML blasts. **A,** Expansion profile of DNTs expanded under GMP conditions from peripheral blood of healthy donor over 17 days. Result is representative of three separate experiments. **B,** PBMCs (top) and DNTs (bottom) expanded as described in Materials and Methods were stained with immune cell subset markers: CD3, CD4, CD8 for T cells, and α-galactosylceramide loaded-CD1d for invariant NKT cells. Result shown is representative of 11 separate expansions for DNTs and 3 separate experiments for PBMC and NKT cells. **C,** Susceptibility of primary AML blasts obtained from 46 patients to DNT-mediated cytotoxicity was determined using a 2-hour flow cytometry–based killing assay at 4:1 DNT-to-AML ratio. Effector DNTs were labeled with PKH-26, and AML blasts were defined as PKH-26⁻CD3⁻CD45^{low}/CD33$^+$/CD34$^+$ population. The level of target cell death was determined by Annexin V and 7AAD staining. Percent specific killing was determined according to the calculation in the Materials and Methods section. Dashed lines indicate 10% specific killing, where AML samples with % specific killing lower than 10% were considered as nonsensitive targets. Each number represents an ID for each patient. DNTs expanded from 11 different HDs were used. **D,** Specific killing mediated by DNTs from 2 HDs or NK92 against K562, AML3/OCI, and 6 primary AML samples was determined as described above. Experiments were done in triplicate using six different HD DNTs in three separate experiments. **E,** Schematic diagram of PDX model used. Sublethally irradiated (225 cGy) NSG mice were injected with 2.5–5 × 10⁶ primary AML blasts followed by single or triple infusion of 2 × 10⁷ DNTs or PBS between days 3 and 14 of tumor inoculation. Bone marrow or spleen harvested on day 24 to 50 after blast injection and AML engraftment analyzed by flow cytometry. (Continued on the following page.)
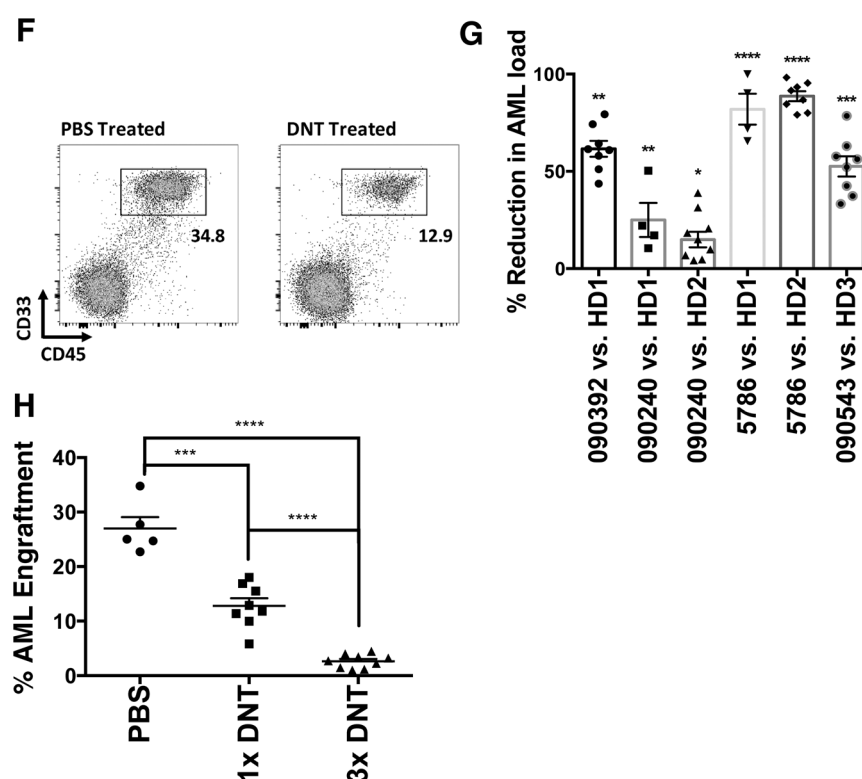
Figure 1.
(*Continued.*) **F,** Representative dot plot of human AML cells in mouse bone marrow. Numbers represent the frequency of AML cells. **G,** Four AML patient samples (090392, 090240, 5786, and 090543) engrafted NSG mice were treated with a single infusion of DNTs expanded from three different donors (HD1, HD2, and HD3). AML sample IDs and HD identification numbers are indicated at the bottom. Each symbol represents % reduction in AML load in each DNT-treated mouse ($n = 41$) compared with the average AML load in PBS-treated group ($n = 20$). Horizontal bars, mean ± SEM. **H,** NSG mice engrafted with primary blasts were injected with PBS ($n = 5$), a single injection of DNTs on day 3 ($n = 8$), or three doses of DNTs on day 3, 7, and 11 after blast injection ($n = 9$). AML engraftment in bone marrow was analyzed on day 32. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

under GMP conditions. On average, $1.41 \pm 0.51 \times 10^8$ DNTs were obtained from each milliliter of peripheral blood after 17 to 20 days *ex vivo* expansion with >90% purity (Fig. 1A). The *ex vivo* expanded DNTs expressed CD3 and αβ- or δγ- TCR but not CD4, CD8, or iNKT cell markers (Fig. 1B) and exhibited a central/effector memory T-cell phenotype (Supplementary Fig. S1). As αβ-TCR$^+$ and δγ-TCR$^+$ DNT subsets display comparable antileukemic function (26), for the simplicity of clinical applications, no further selection for specific TCRs was performed for the subsequent studies when more than 90% of cells in expanded products were DNTs.

Expanded DNTs effectively killed 36 of 46 primary AML patient samples (patient information shown in Supplementary Table S1; Fig. 1C) in a dose-dependent manner (Supplementary Fig. S2), albeit with heterogeneity in the level of cytotoxicity to each patient sample. Higher effector-to-target ratio (Supplementary Fig. S2A) or longer incubation time (Supplementary Fig. S2B) showed increased killing. The threshold of 10% was used to distinguish susceptible and resistant AML samples, as samples with specific killing greater than 10% showed a dose-dependent cytotoxicity, whereas those with lower than 10% killing did not (Supplementary Fig. S2C). No correlation was observed between the susceptibility to DNTs and the patient's age at diagnosis, WBC count, or MRC cytogenetic risk groups (Supplementary Fig. S3A–S3C). However, significantly lower cytolysis was observed in

samples obtained from patients with lower percentages of AML cells in the bone marrow, in male patients, and patients with AML secondary to prior myelodysplastic syndrome (Supplementary Fig. S3D–S3F). On the other hand, samples obtained from M5 FAB classified AML patients showed a higher level of susceptibility to DNT-mediated cytotoxicity than the rest of the patient samples tested (Supplementary Fig. S3G).

To determine the relative potency of DNTs in comparison with other cytotoxic cells, DNTs from two HDs and NK92, an NK-cell line used in a clinical trial for AML patient treatment (NCT00900809; ref. 27), were used as effectors against eight leukemia targets. Although K562, a natural NK-cell target, was effectively killed at a comparable level by both DNTs and NK92, all six primary AML blasts and an AML3/OCI cell line were more susceptible to DNT- than NK92-mediated cytotoxicity (Fig. 1D). Notably, DNTs effectively killed OCI-AML-3 and three primary AML samples (130783, 130794, and 090239) that were resistant to NK92 (Fig. 1D). Similarly, DNTs were more effective at lysing leukemic targets than *ex vivo* expanded primary CD8$^+$ T cells (Supplementary Fig. S4A) and primary NK cells (Supplementary Fig. S4B).

The ability to generate large numbers of human DNTs allowed us, for the first time, to study their antileukemic activity *in vivo*. Using a patient-derived xenograft (PDX) model (Fig. 1E), we found that a single allogeneic DNT infusion significantly reduced

the frequency of human $CD45^+CD33^+$ leukemic cells in the bone marrow of NOD scid gamma (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ, NSG) mice inoculated with primary AML samples obtained from 4 different patients (Fig. 1F and G). Two additional DNT treatments further reduced leukemia load (Fig. 1H). Overall, 69 mice inoculated with primary AML blasts from 8 different patients, of which 42 received one dose of DNTs and 27 received three doses, had a 52.43% ± 30.31% and 74.35% ± 23.94% reduction in leukemia load, respectively, compared with the PBS-treated group. As inoculation of the patient AML samples that we have tested so far did not cause death in recipient mice even when engraftment reaches >80%, we were not able to study the effect of DNTs on recipient survival. Collectively, these results demonstrate that allogeneic DNTs can effectively target a broad range of primary AML cells both *in vitro* and *in vivo* in a dose-dependent manner.

Allogeneic DNTs can effectively target chemotherapy-resistant AML *in vitro* and *in vivo*

As chemotherapy resistance is the major cause of low survival rates in AML patients, we studied the effect of DNTs on chemotherapy-resistant AML cells. We found that 69.2% (9/13) of chemotherapy-resistant AML cells, obtained from chemotherapy-refractory or relapsing patients, were effectively killed by DNTs *in vitro* (Fig. 2A) and that their level of sensitivity to DNT-mediated cytotoxicity was comparable with those obtained from chemotherapy-susceptible patients (Fig. 2B). Consistent with this, significant reductions in the leukemia load were observed in mice inoculated with chemotherapy-resistant primary AML cells from 4 different patients after DNT cell treatment (Fig. 2C). Together, these results indicate that allogeneic DNTs are effective at targeting the majority of chemotherapy-resistant AML cells *in vitro* and in PDX models, supporting the use of these cells to eliminate AML cells that are not cleared by standard chemotherapy.

Infusion of DNTs does not cause GVHD or kill normal allogeneic PBMCs and $CD34^+$ HSPCs

Allogeneic HSCT induces curative graft-versus-leukemia effects (10), but is associated with morbidity and mortality due to donor-derived immune cells attacking normal host cells and tissue (8, 9). To determine the potential toxicity of allogeneic DNTs toward normal cells, the susceptibility of normal PBMCs and $CD34^+$ HSPCs to allogeneic DNT-mediated cytotoxicity was compared with that of primary AML patient samples and AML cell lines with similar maturation status ($CD33^+CD34^-$ for PBMCs and $CD33^-CD34^+$ for HSPCs). DNTs displayed potent cytotoxicity against primary AML samples and AML cell lines but had virtually no cytotoxicity toward normal allogeneic PBMCs (Fig. 3A), peripheral blood $CD33^+$ myeloid cells (Supplementary Fig. S5), or HSPCs (Fig. 3B).

To study whether DNTs have toxicities against normal tissues *in vivo*, DNTs or bulk human PBMCs were intravenously infused into NSG mice and monitored for associated morbidities. As expected from prior literature, infusion of human PBMCs caused severe xenogeneic acute GVHD (28) as evidenced by weight loss (Fig. 3C). Histologic analysis of PBMC-transfused mice revealed acute GVHD pathology in multiple organs, including portal inflammation in the liver, mononuclear infiltrates extending into the alveolar septa, endotheliitis in the

lungs, and lamina propria expansion with some architectural distortion in the small intestine (Fig. 3D). However, despite tissue infiltration by DNTs, neither weight loss (Fig. 3C) nor tissue damage was observed in the liver, lungs, and intestines (Fig. 3D) even when equal or 4-fold higher numbers of DNTs compared with PBMCs were infused.

To further assess potential detrimental effects of allogeneic DNTs on normal HSPC engraftment and differentiation, NSG mice were engrafted with normal $CD34^+$ HSPCs and subsequently treated with DNTs from two HDs that were allogeneic to the HSPC donors. Similar to reports by others (29, 30), we consistently observed a high level of chimerism from the HSPC donors within the spleen and bone marrow (~70%–80%), and approximately 15% in the peripheral blood of engrafted mice. Also, no differences in the frequency or differentiation of hematopoietic cells derived from transplanted HSPC cells between DNT-treated and PBS-treated mice were observed (Fig. 3E and F), including the $CD34^+$ HSPC population in bone marrow. These findings show that DNTs do not target allogeneic HSPCs and their progeny, nor interfere with differentiation of HSPCs into hematopoietic lineages. Together, these results demonstrate that *ex vivo* expanded allogeneic DNTs have potent antileukemic effects but are noncytotoxic to normal tissues and hematopoietic cells in xenograft models.

NKG2D and DNAM-1 contribute to DNT-mediated donor-unrestricted and leukemia-specific cytotoxic activity

Along with no associated toxicity, being able to use allogeneic cellular therapy without donor restriction will significantly increase its clinical applications. We found that DNTs from a single donor targeted blasts from different AML patients (Fig. 4A) and DNTs obtained from different donors showed comparable cytotoxicity with the same AML target (Fig. 4B), suggesting that the efficacy of DNT therapy does not depend on the donor origin and that the susceptibility of AML cells to DNTs is mainly determined by intrinsic characteristics of leukemic cells.

To dissect the mechanisms by which DNTs selectively recognize AML cells over normal cells in a DNT donor–unrestricted manner, we first studied the involvement of TCR. We found that the addition of αβ- and γδ-TCR blocking antibodies significantly reduced the level of cytotoxicity toward Jurkat cells, a T-cell lymphoma cell line, but did not affect DNT-mediated cytotoxicity against AML cells (Fig. 4C), supporting a TCR-independent recognition of AML cells by DNTs.

Next, we focused on innate receptor–ligand molecules involved in anticancer immunity. High expression of activating receptors NKG2D and DNAM-1 was observed on DNTs (Fig. 4D), and significantly higher expression of NKG2D ligands (ULBP1 and ULBP3) and DNAM-1 ligands (CD112 and CD155) was found on primary AML cells over normal PBMCs (Fig. 4E). Blocking NKG2D, DNAM-1, or both significantly reduced the ability of DNTs to kill primary AML cells and AML3/OCI cell line (Fig. 4F). In contrast, blocking other activating receptors, NKp30, NKp44, and NKp46, used by other immune cells to recognize cancer cells (31, 32), showed no effect on DNT–AML interactions (Supplementary Fig. S6). Collectively, these results demonstrate that DNTs can preferentially recognize and kill AML cells but not normal cells in a donor-independent manner, partially, through NKG2D and DNAM-1.

A

B

P = 0.4890

C

Figure 2.
DNTs can effectively target chemotherapy-resistant primary AML blasts. **A,** Cytotoxicity of DNTs expanded from HDs against primary chemotherapy-resistant AML cells obtained from 13 patients was determined using the *in vitro* flow cytometry–based killing assay as described in Fig. 1A. **B,** The level of *in vitro* susceptibility of chemotherapy-susceptible ($n = 20$) and -resistant ($n = 13$) primary AML samples to DNT-mediated cytotoxicity was compared. AML samples from chemotherapy-susceptible and -resistant patients show a similar level of average sensitivity to antileukemic activity mediated by DNTs. **C,** NSG mice engrafted with primary AML cells from three chemotherapy-resistant patients were treated with three injections of DNT cells ($n = 6$) or PBS ($n = 7$). AML cells in the bone marrow were detected 23 days after blast injection. Result shown is representative of five separate experiments done with different AML patient samples. In total, 71 mice were engrafted with primary AML samples, and of those, 27 were treated with PBS and 44 were treated with DNTs. Each dot represents result from one mouse and horizontal bars represent the mean values, and the error bars represent SEM of each group. ****, $P < 0.0001$, using unpaired, two-tailed Student *t* test.
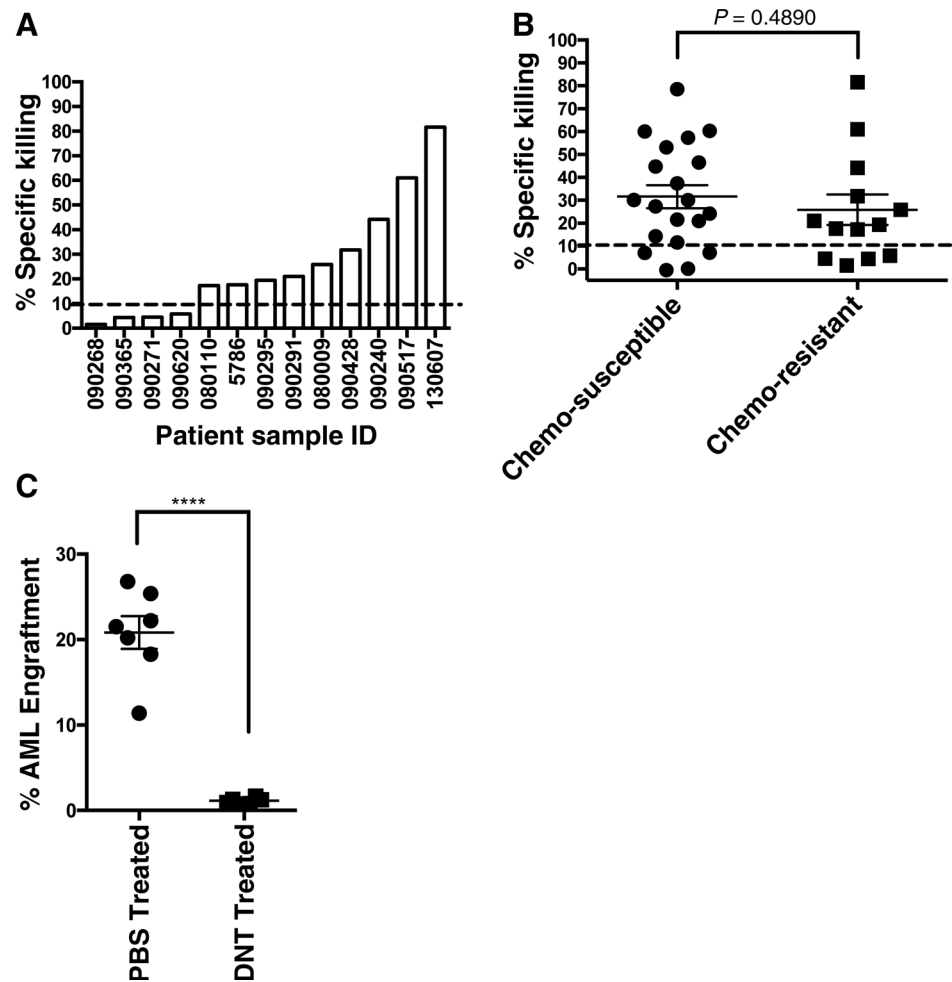
DNTs produce IFNγ upon encountering AML cells, which augments their cytotoxicity toward AML cells but not to normal PBMCs

Ex vivo expanded DNTs express a high level of intracellular IFNγ (Fig. 5A), but minimal IFNγ levels were detected in the supernatant from cocultures of allogeneic DNTs with normal PBMCs (0.50 ± 0.054 ng/mL) and DNT-resistant primary AML cells (0.28 ± 0.10 ng/mL). Interestingly, significantly higher levels of IFNγ (3.29 ± 0.58 ng/mL; Fig. 5B) were released when DNTs were cocultured with DNT-susceptible AML cells, which corresponded with the degree of cytotoxicity (Supplementary Fig. S7). The level of IFNγ release was significantly reduced in the presence of NKG2D and DNAM-1–blocking antibodies, further supporting the involvement of these molecules in the recognition of AML cells by DNTs (Supplementary Fig. S8). The addition of an IFNγ-neutralizing antibody significantly reduced AML cell death induced by DNTs (Fig. 5C), whereas addition of exogenous recombinant IFNγ (rIFNγ) resulted in a higher level of DNT-mediated cytotoxicity without direct toxicity toward AML cells (Fig. 5D-i and 5D-ii).

Pretreatment of DNTs with rIFNγ did not significantly affect their function (Fig. 5D-v), but incubation of AML cells with rIFNγ rendered them more susceptible to DNT-induced cytotoxicity (18.4% vs. 31.9% for untreated versus rIFNγ pretreated; Fig.

5D-iii and 5D-iv), demonstrating that IFNγ sensitizes the AML cells rather than augmenting the DNTs' cytotoxic activity. Consistent with this, the level of DNT-mediated cytotoxicity significantly increased in 10 of 20 primary AML samples after rIFNγ pretreatment, including 4 of 6 otherwise DNT-resistant AML samples (Fig. 5E) and DNT-resistant AML cells were sensitized to a greater degree (Fig. 5F). Importantly, rIFNγ pretreatment did not affect the susceptibility of normal allogeneic PBMCs to DNTs (Fig. 5E and F).

IFNγ upregulates NKG2D and DNAM-1 ligand expression on AML cells

To understand the mechanism of how IFNγ sensitizes AML targets (Fig. 5D–F), we first tested the effect of IFNγ on NKG2D and DNAM-1 ligand expression given that these pathways contribute to DNT-mediated anti-AML activity. Although it has been reported that IFNγ downregulates expression of NKG2D ligands on solid tumours (33, 34), rIFNγ pretreatment upregulated the expression of NKG2D ligands ULBP1, ULBP2/5/6, ULBP3, and MICA/B, as well as DNAM-1 ligands CD112 and CD155, on AML cells (Fig. 6A). In contrast, IFNγ treatment did not affect the expression levels of NKG2D and DNAM-1 ligands on normal PBMCs (Supplementary Fig. S9), which is in agreement with IFNγ treatment lacking its effect on the susceptibility
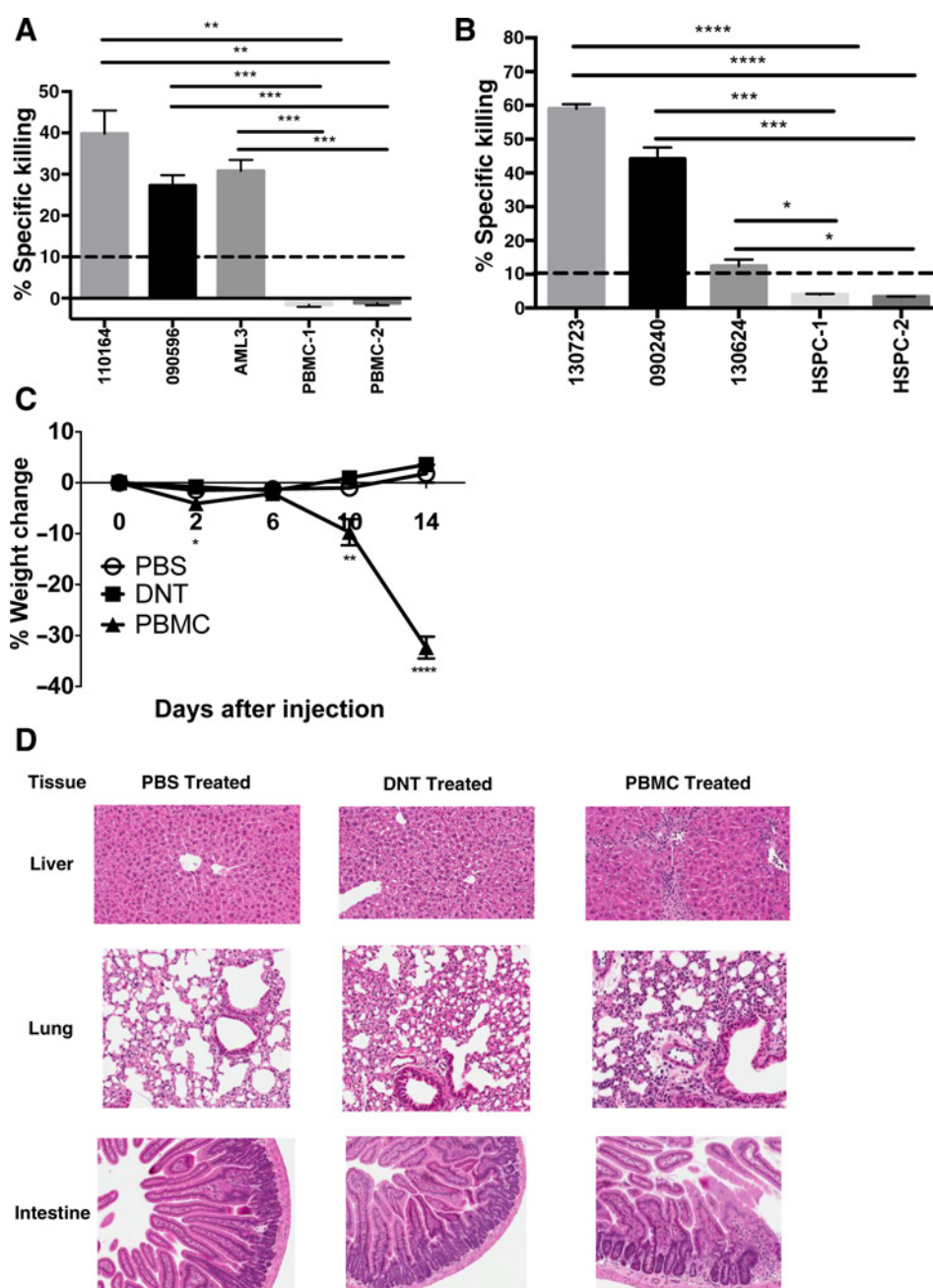
Figure 3.
Allogeneic DNTs do not kill normal cells *in vitro* or *in vivo*. **A** and **B,** Cytotoxicity of allogeneic DNTs expanded from 3 HDs against CD33$^+$CD34$^-$ AML: AML3-OCI, 2 primary AML patient blasts (110164 and 090596), and normal allogeneic PBMCs (PBMC-1 and PBMC-2) from 2 HDs (**A**) or 3 CD33$^-$CD34$^+$ AMLs (130723, 090240, and 130624) and HSPCs (HSPC-1 and HSPC-2) from 2 HDs (**B**) was determined *in vitro* as described in Fig. 1B. Experiments were done in triplicates in three separate experiments for PBMCs and two separate experiments for HSPCs using DNTs from three HDs. **C** and **D,** NSG mice were intravenously injected with PBS, 2×10^7 *ex vivo* expanded DNTs, or 5×10^6 human PBMCs obtained from 4 different HDs ($n = 5$ per group). **C,** Mouse body weight was measured on days indicated, and % weight loss was calculated as described in Materials and Methods. **D,** On day 14, liver, lung, and small intestine were examined histologically via hematoxylin and eosin staining ($\times$20 magnification for liver and lung, $\times$10 magnification for small intestine; $n = 3$). Data shown are representative of four independent experiments done using DNTs and PBMCs from 4 different HDs. (*Continued on the following page.*)

of PBMCs to DNTs (Fig. 5F and G). Furthermore, the effect of rIFNγ on AML susceptibility to DNTs, as shown in Fig. 5F, was neutralized by the blocking of NKG2D and DNAM-1 (Fig. 6B), confirming that IFNγ can exert its role through NKG2D and DNAM-1 pathways. Furthermore, the level of DNT-mediated cytotoxicity was inhibited by NKG2D and DNAM-1 antibodies at a significantly greater level for IFNγ-pretreated AML targets than untreated ones (22.69% ± 1.86% vs. 13.65% ± 0.68%; Fig. 6C). These data indicate that IFNγ increases the sensitivity of AML cells, but not normal PBMCs, to DNT-mediated cytotoxicity in part by upregulating NKG2D and DNAM-1 ligand expression on leukemic cells. It also revealed,

for the first time, uniquely interconnected roles of NKG2D, DNAM-1, and IFNγ in AML cells, which form a positive feed-back loop to facilitate DNT cell recognition and elimination of AML cells.

Discussion

Previously, we showed that DNTs could be expanded from peripheral blood of a small number of AML patients during chemotherapy-induced complete remission and were able to kill autologous AML cells *in vitro* (26). Because of the low yield and purity of DNTs obtained from AML patients, it was
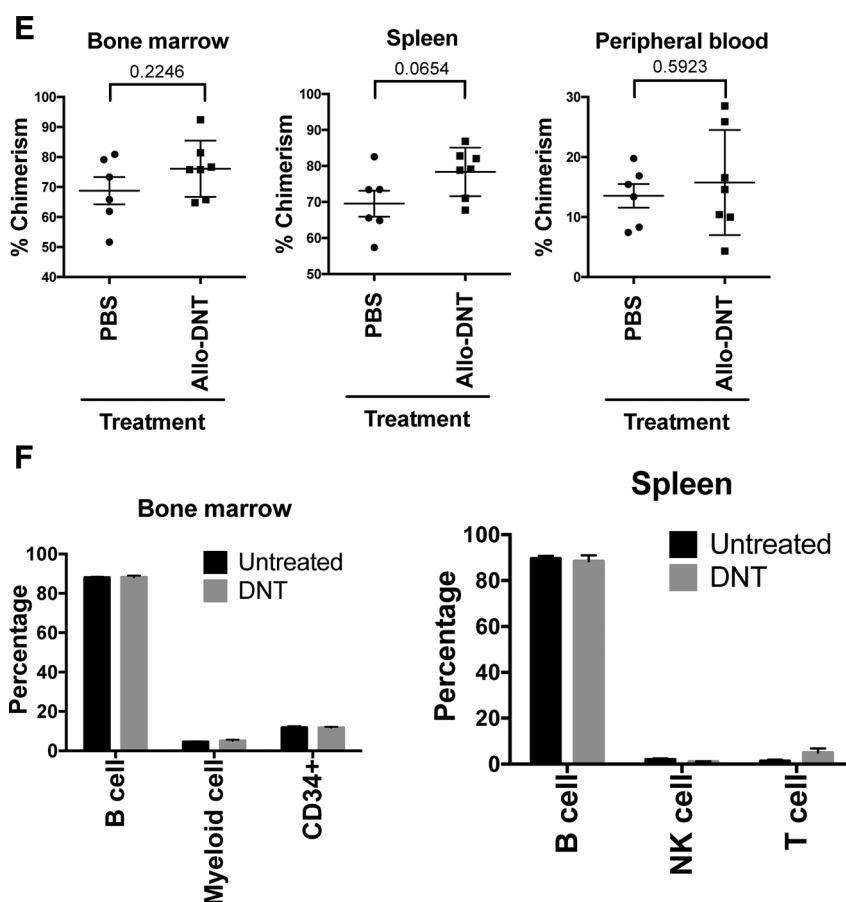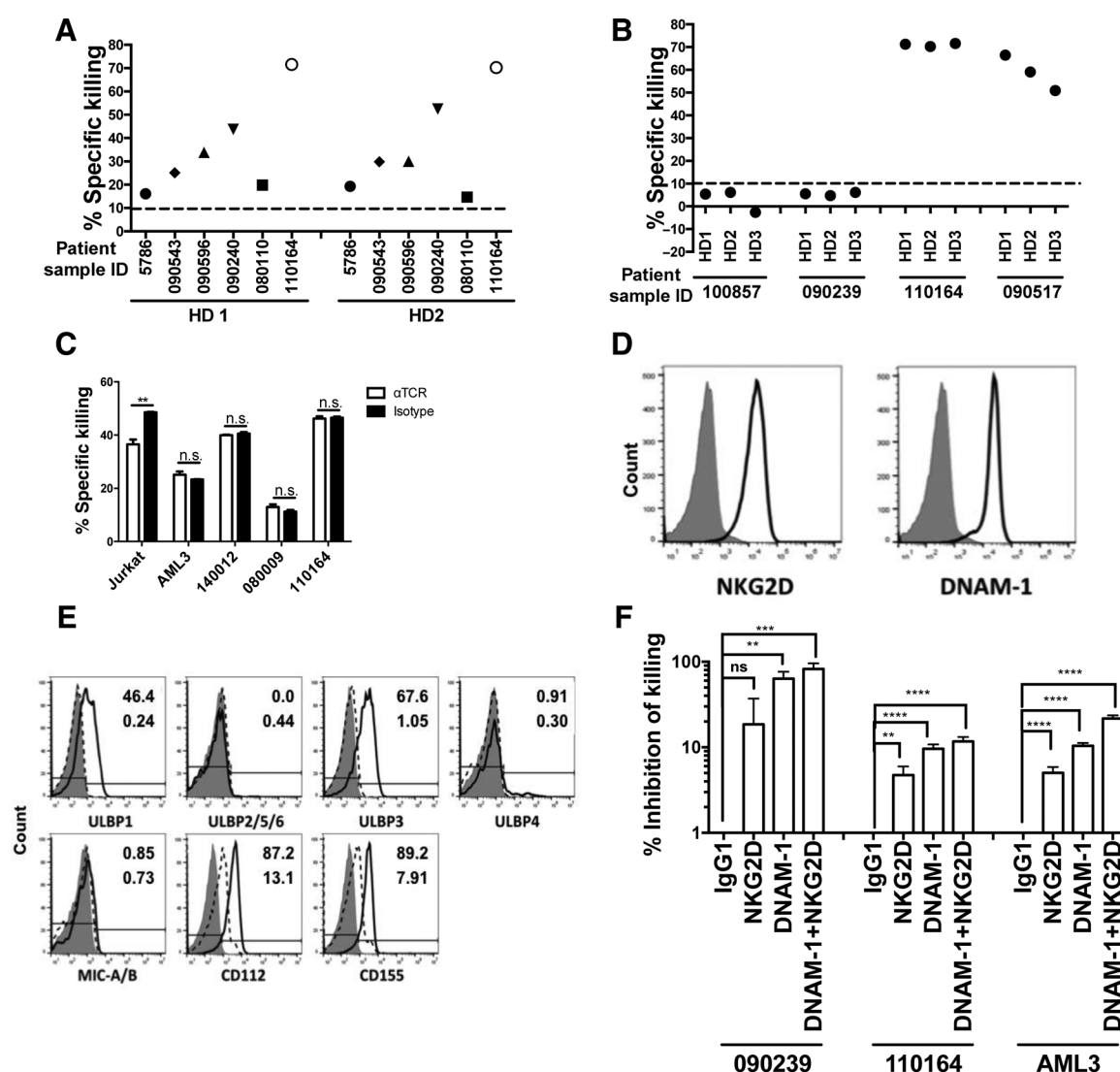
Figure 3.
(*Continued.*) **E** and **F,** CD133⁺CD34⁺ human HSPCs were infused into NSG mice (3 × 10⁵ cells/mouse, *n* = 13). Six to 8 weeks after HSPC injection, mice were treated with *ex vivo* expanded allogeneic DNTs (*n* = 7) or PBS (*n* = 6). The percentage of human leukocytes (**E**) and its subsets (**F**) in bone marrow, spleen, and peripheral blood was determined 8 weeks after DNT treatment. Each dot represents % chimerism in one mouse, and horizontal bars represent the mean ± SEM of each group. The graphs shown are a representative of three independent experiments done with HSPCs from 2 HDs and allogeneic DNTs expanded from 4 HDs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, using unpaired, two-tailed Student *t* test.

not possible to study their function *in vivo* nor use them therapeutically. Herein, we developed a simple, cost-effective, highly reproducible method allowing for *ex vivo* expansion of DNTs from healthy individuals under GMP condition to therapeutic numbers with a high purity, in less than 20 days without feeder cells or genetic modifications. We demonstrated that the expanded DNTs selectively targeted a wide spectrum of primary AML samples both *in vitro* and in PDX models in a donor-unrestricted manner. Broad, yet cancer-specific cytotoxic activity of DNTs was also seen against cell lines derived from other forms of leukemia and lymphoma (Supplementary Fig. S10), such as Burkitt B-cell lymphoma (Daudi), T-cell lymphoma (Jurkat), histiocytic lymphoma (U937), chronic myeloid leukemia (K562), and myeloma (H929, 8226, and LDI), suggesting that our findings from this study may be translatable to other types of leukemia and lymphoma.

Importantly, although DNTs were cytotoxic to CD34⁺ AML cells *in vitro* and inhibited their engraftment in PDX models, they did not affect normal stem cell engraftment and differentiation. Contrary to the infusion of human CD4/CD8 T cells or PBMCs, infusion of human DNTs did not cause GVHD in recipients, demonstrating that DNTs selectively target leukemic cells while sparing normal cells and tissues. Moreover, we found that cryopreserved DNTs maintained comparable viability (Supplementary Fig. S11A) and antileukemia activity (Supplementary Fig. S11B) to those cultured without cryopreservation and thawing procedures. Given that *ex vivo* expanded

allogeneic DNTs from HDs have no observed toxicity, can target broad range of leukemia cells in a donor-unrestricted manner and are cryopreservable, these cells can potentially be used as a new "off-the-shelf" cellular therapy for treating leukemia. Also, given their superior expansion profile, potent cytotoxic function, and lack of allo-response by endogenous TCRs, DNTs would be a good vehicle for CAR or transgenic TCR technology.

Mechanistically, we found that DNTs preferentially recognize and target AML cells in a TCR-independent and HLA-unrestricted manner that is partially mediated through the innate receptors, NKG2D and DNAM-1. Natural cytotoxic receptors, such as NKp30, NKp44, and NKp46, and other activating receptors, such as NKG2D and DNAM-1, are expressed by NK cells and subsets of activated T cells; a role for these molecules in cancer immunity has been shown previously (31, 35–38). The expression of at least one of NKG2D (39) and DNAM-1 (35) ligands has been detected in the majority of AML patients in large cohort studies. We found that blocking of NKG2D and DNAM-1, but not natural cytotoxic receptors, reduced the level of AML cell apoptosis induced by DNTs, indicating the role of these molecules in AML–DNT interaction. Although others have shown the role of NKG2D and DNAM-1 pathways in targeting leukemic cells, we are the first to show their unique positive feedback interaction with IFNγ. Notably, blocking these pathways did not completely abrogate the killing activity of DNTs; thus, there are likely other molecules involved in the selective

Figure 4.
DNTs kill AML cells in NKG2D- DNAM-1–dependent, but TCR-independent manner. **A,** Killing assay performed using DNTs expanded from 2 different HDs against 6 primary AML samples (shown in different symbols), demonstrating that DNTs from a single HD can target an array of AML samples. Experiments were done in triplicates. Result shown is representative of two separate experiments. **B,** DNTs expanded from different HDs show similar levels of cytotoxicity against the same AML blasts. Killing assays were done by using DNTs expanded from 3 HDs as effectors against 4 primary AML samples (100857, 090239, 110164, and 090517). Experiments were done in triplicates, and a summary of pooled results from three separate experiments is shown. **C,** DNTs were preincubated with IgG2a isotype control or anti-TCRαβ and TCRγδ antibodies (10 μg/mL for each antibody) for 30 minutes before coculture with Jurkat, AML3/OCI cells, or primary AML cells (140012, 080009, and 110164) at 4-to-1 DNT-to-target ratio, and % specific killing was determined. Experiments were done in triplicates, and the graph represents the results of four independent experiments. **D,** *Ex vivo* expanded DNTs were stained with DNAM-1 and NKG2D antibodies. Filled histograms represent FMO controls. The graphs shown are representative of DNTs expanded from 3 different HDs. **E,** Primary AML patient blasts (solid line) and normal PBMCs from HDs (dotted line) were stained for NKG2D ligands ULBP-1, ULBP-2/5/6, ULBP-3, ULBP-4, and MIC-A/B, and DNAM-1 ligands CD155 and CD112. Filled histograms represent FMO controls. Numbers shown are % of cells that expressed corresponding ligands by AML blasts (top) or normal PBMCs (bottom). **F,** DNTs were preincubated with IgG1 isotype control, anti-NKG2D, DNAM-1, or NKG2D + DNAM-1 blocking antibodies for 1 hour before coculture with primary AML blasts (090239 and 110164) or AML3-OCI. Percent inhibition of killing was determined as described in Materials and Methods section. Experiments were done in triplicates, and representative data from four separate experiments are shown. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, using unpaired, two-tailed Student t test.
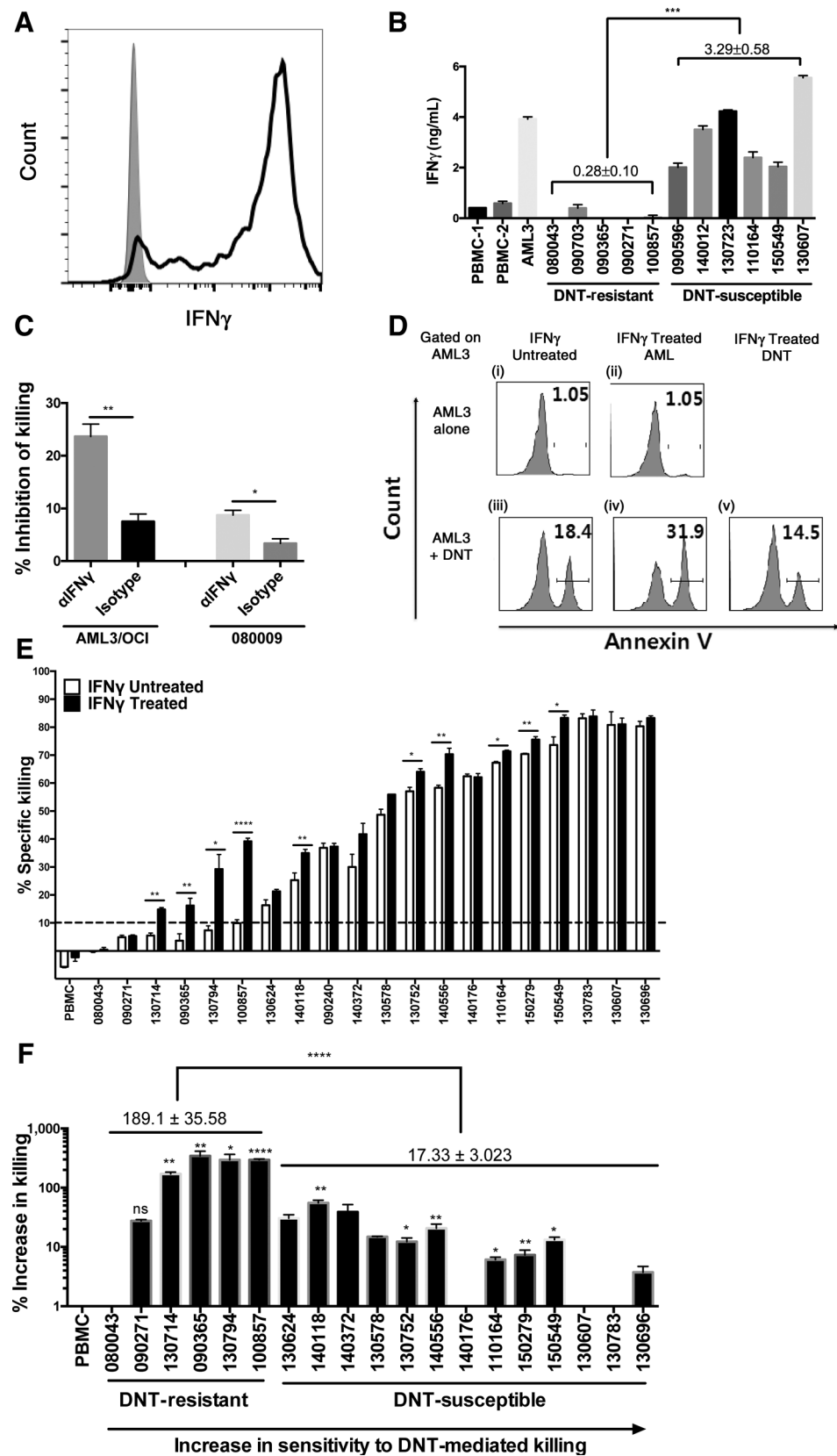
recognition and targeting of AMLs by DNTs, which are being currently explored.

IFNγ is a well-known inflammatory cytokine with a pleiotropic function that can elicit both pro- and antitumorigenic effects (33, 40–43). A previous clinical trial using IFNγ as a monotherapy

failed to achieve a clinical response in AML patients (44), which is in agreement of our finding that IFNγ alone does not induce AML cell death directly. However, we found that IFNγ renders AML blasts more susceptible to DNTs. Six primary AML blast samples obtained from 10 patients (60%) that were initially resistant to

Figure 5.
DNTs release IFNγ upon recognizing susceptible AML cells, and IFNγ sensitizes the AML targets to DNT-mediated cytotoxicity. **A,** Intracellular staining of IFNγ in activated DNTs. The filled graph is the FMO control. The result shown is a representative of two separate experiments done with 4 HD DNTs. **B,** *Ex vivo* expanded DNTs were coincubated with allogeneic PBMCs, AML3/OCI, DNT-resistant ($n = 5$), or DNT-susceptible ($n = 6$) primary AML samples at 4:1 effector-to-target ratio, and the level of IFNγ in the culture supernatants was determined by ELISA. The number represents the average amount $\pm$ SEM of IFNγ produced from each coculture. The data are representative of three independent experiments each with triplicates. **C,** *Ex vivo* expanded DNTs were pretreated with 10 μg/mL anti-IFNγ antibody or isotype control antibody for 30 minutes before coincubation with AML3/OCI or primary blasts. The results represent three independent experiments each with triplicates. **D–F,** AML3/OCI, DNTs (**D**), primary AML samples, or allogeneic HD PBMCs (**E** and **F**) were pretreated or untreated with recombinant IFNγ (rIFNγ, 50 ng/mL) for 1 hour and cocultured with DNTs, and % specific killing was determined. The graphs shown are representative of 3, 4, 3, and 6 separate experiments for PBMC, AML3/OCI, DNT, and primary AML samples, respectively, and each experiment was done in triplicates. **F,** Percent increase in DNT-mediated killing was determined as described in Materials and Methods section. Number above represents the average % increase in killing $\pm$ SEM for DNT-resistant ($n = 6$) or -susceptible ($n = 14$) primary AML samples upon rIFNγ pretreatment. The result is the summary of 6 separate experiments, each with triplicates. n.s., not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, using unpaired, two-tailed Student t test or linear regression test.
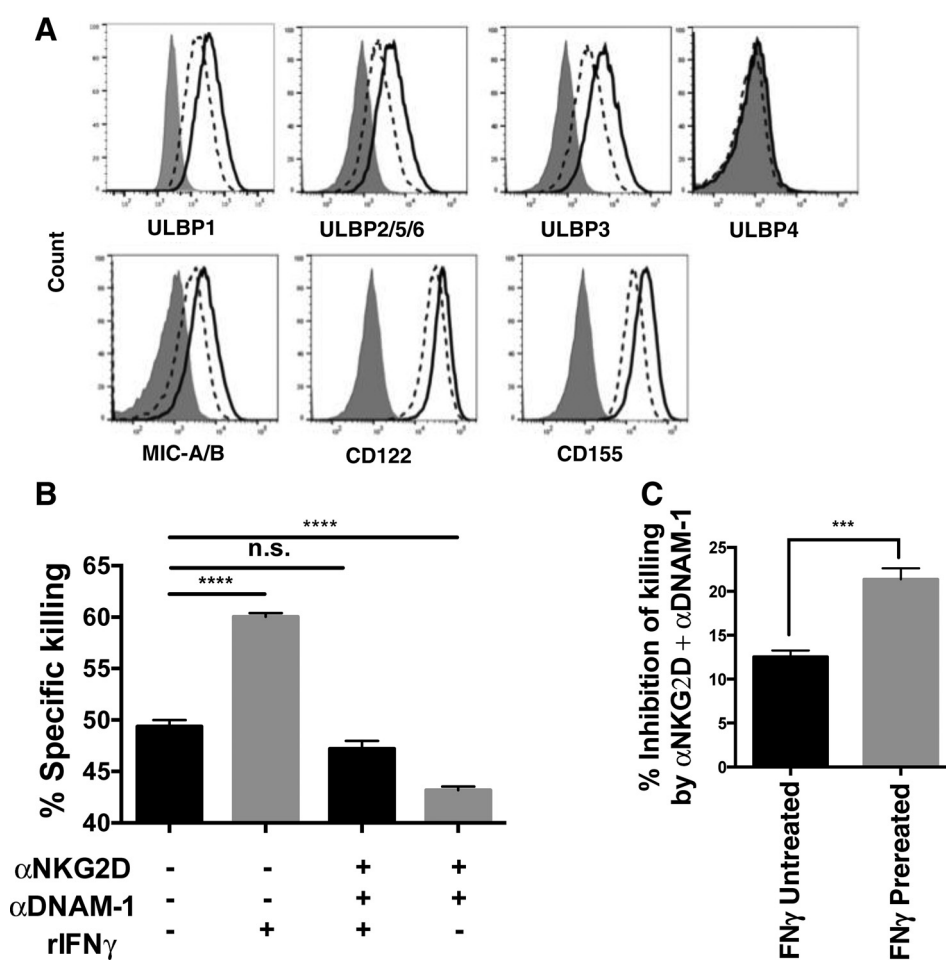
A,

ULBP1 ULBP2/5/6 ULBP3 ULBP4

MIC-A/B CD122 CD155

Count

B

% Specific killing

****
n.s.
****

65
60
55
50
45
40

αNKG2D	-	-	+	+
αDNAM-1	-	-	+	+
rIFNγ	-	+	+	-

C

% Inhibition of killing by αNKG2D + αDNAM-1

***

25
20
15
10
5
0

IFNγ Untreated   IFNγ Prereated

Figure 6.
IFNγ increases NKG2D and DNAM-1 ligand expression on AML cells and enhances their susceptibility to DNT-induced cytolysis. **A,** AML3/OCI cells were incubated with (solid lines) or without (dotted lines) 50 ng/mL rIFNγ overnight, and their expression of NKG2D and DNAM-1 ligands is shown. Filled histograms represent FMO controls. Graphs are representative of four separate experiments done with 3 AML cell lines AML3/OCI, KG1a, and MV4-11, each experiment with triplicates. **B** and **C,** AML3/OCI were pretreated or untreated with rIFNγ (50 ng/mL) then cocultured with DNTs in the presence of 10 µg/mL anti-NKG2D and DNAM-1 blocking antibodies or isotype control antibody. Percent specific killing of targets from each treatment is shown (**B**). Percent inhibition of DNT-mediated cytotoxicity by anti-NKG2D and DNAM-1 antibodies in a killing assay conducted against IFNγ-pretreated and untreated targets was calculated as described in Materials and Methods section (**C**). Results represent four separate experiments each with triplicates. ***, $P < 0.001$; ****, $P < 0.0001$, using unpaired, two-tailed Student t test.

DNT-mediated cytotoxicity became susceptible after pretreatment with exogenous IFNγ. In combination with IFNγ, >85% (40/46) of tested AML samples were effectively killed by DNTs. Unlike solid tumors (33, 34), IFNγ induced higher expression of NKG2D and DNAM-1 ligands on AML cells, which rendered them more sensitive to DNTs and theoretically to other cytotoxic cells such as NK cells. Notably, normal PBMCs express low levels of NKG2D and DNAM-1 ligands, which were not upregulated by IFNγ, and rIFNγ treatment did not render normal cells sensitive to DNTs. These findings help to explain why DNTs can selectively target AML but not normal cells. Furthermore, our data suggest that rIFNγ and DNT combination therapy may result in a synergistic effect, leading to a greater efficacy against AML via sensitizing leukemic cells to DNT-mediated cytotoxicity and other modes of antileukemic activity of IFNγ (41–43, 45, 46).

Resistance to chemotherapy results in refractory AML and disease relapse, both of which significantly hamper clinical outcomes in patients. Alternative forms of treatment to target these cells are in urgent need. Here, we demonstrate that a majority of primary AML cells obtained from chemotherapy-resistant and relapsing patients are susceptible to DNT-mediated cytotoxicity both *in vitro* and *in vivo*. Expression of NKG2D and DNAM-1 ligands is regulated by the DNA damage repair pathway (47, 48), explaining higher levels of ligand expression

on transformed cells (31, 37, 38, 49). The majority of chemotherapy drugs cause DNA damage and interrupt the cell cycle; hence, treatment of myeloma with doxorubicin and bortezomib has been shown to increase expression of NKG2D and DNAM-1 ligands (48, 50). These findings support the potential application of DNT therapy after conventional chemotherapy, which may yield synergistic effects via NKG2D and DNAM-1 pathways to target chemotherapy-resistant residual disease to prevent disease relapse.

We show in PDX models that 1 or 3 DNT infusions significantly reduced leukemia load, but complete eradication of AML cells may be needed to prevent relapse. A higher dose or more DNT treatments or DNT in combination with other forms of therapies may be needed to eradicate the disease. In this study, DNTs were infused 3 to 14 days after AML infusion when the leukemia load is relatively low. Whether DNTs given at later time points where recipients have a higher leukemia load are effective needs to be determined.

In summary, we demonstrate that allogeneic human DNTs have potent antileukemic activity against primary AML cells, including chemotherapy-resistant cells, both *in vitro* and *in vivo* in PDX models, without observed toxicity to normal cells and tissues and elucidate the underlying mechanism of the selective toxicity of DNTs toward AML. Therapeutically, our findings

support the use of DNTs expanded from HDs as a new "off-the-shelf" nontoxic cellular immunotherapy to target chemotherapy-resistant AML populations following conventional chemotherapy to improve patient survival.

Disclosure of Potential Conflicts of Interest

J.E. Dick is a consultant/advisory board member for Trillium Therapeutics. L. Zhang is a consultant for WYZE Biotech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: J. Lee, E. Streck, L. Zhang
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Lee, E. Streck, S. Kang, R.W. Childs, L. Zhang
Writing, review, and/or revision of the manuscript: J. Lee, M.D. Minden, B. Chen, D. Ly, C. D'Souza, R.W. Childs, J.E. Dick, L. Zhang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Lee, M.D. Minden, B. Chen, H. Kang, Y. Li, R.W. Childs
Study supervision: J. Lee, J.E. Dick, L. Zhang

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