



# Exercise-induced $\beta$ 2-adrenergic Receptor Activation Enhances the Antileukemic Activity of Expanded $\gamma\delta$ T-Cells via DNAM-1 Upregulation and PVR/Nectin-2 Recognition

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## ABSTRACT

Exercise mobilizes cytotoxic lymphocytes to blood which may allow superior cell products to be harvested and manufactured for cancer therapy. Gamma-Delta ( $\gamma\delta$ ) T-cells have shown promise for treating solid tumors, but there is a need to increase their potency against hematologic malignancies. Here, we show that human  $\gamma\delta$  T-cells mobilized to blood in response to just 20 minutes of graded exercise have surface phenotypes and transcriptomic profiles associated with cytotoxicity, adhesion, migration, and cytokine signaling. Following 14 days *ex vivo* expansion with zole-dronic acid and IL2, exercise mobilized  $\gamma\delta$  T-cells had surface phenotypes and transcriptomic profiles associated with enhanced effector functions and demonstrated superior cytotoxic activity against multiple hematologic tumors *in vitro* and *in vivo* in leukemia-bearing xenogeneic mice. Infus-

ing humans with the  $\beta$ 1+ $\beta$ 2-agonist isoproterenol and administering  $\beta$ 1 or  $\beta$ 1+ $\beta$ 2 antagonists prior to exercise revealed these effects to be  $\beta$ 2-adrenergic receptor (AR) dependent. Antibody blocking of DNAM-1 on expanded  $\gamma\delta$  T-cells, as well as the DNAM-1 ligands PVR and Nectin-2 on leukemic targets, abolished the enhanced antileukemic effects of exercise. These findings provide a mechanistic link between exercise,  $\beta$ 2-AR activation, and the manufacture of superior  $\gamma\delta$  T-cell products for adoptive cell therapy against hematologic malignancies.

**Significance:** Exercise mobilizes effector  $\gamma\delta$  T-cells to blood via  $\beta$ 2-adrenergic signaling which allows for generation of a potent expanded  $\gamma\delta$  T-cell product that is highly cytotoxic against hematologic malignancies.

## Introduction

Physical activity and structured exercise are known to reduce cancer risk and progression (1, 2). This is linked to shifts in hormone levels, reduced adiposity, lowered systemic inflammation, and improvements in immune function

(3–5). Every bout of moderate to vigorous intensity exercise dramatically increases circulating catecholamine levels, which drive the mobilization of highly cytotoxic lymphocytes to the blood compartment and coupled with the release of myokines facilitates their ability to infiltrate tumors (6). Considering the significant role that exercise-mobilized lymphocytes can play in strengthening immune surveillance and restraining tumor development, we have sought to investigate whether they may be more suitable candidates for adoptive cell therapies (7).

Among the various subsets of effector lymphocytes that exhibit high responsiveness to exercise,  $\gamma\delta$  T-cells appear to be a promising option for the treatment of both solid and hematologic malignancies (8–12).  $\gamma\delta$  T-cells, which represent about 5% of circulating lymphocytes, recognize a broad range of tumors in a non-MHC-restricted manner via their  $\gamma\delta$ -TCR or natural killer (NK)-cell-like activating receptors such as natural killer group 2D (NKG2D) DNAX-activating molecule (DNAM-1), and TNF-related apoptosis-inducing ligand (TRAIL; ref. 13). They are also capable of direct anti-tumor immunity through the release of cytotoxic granules, or indirectly through the production of effector cytokines such as IFN $\gamma$  and TNF $\alpha$ . A subset of  $\gamma\delta$  T-cells, V $\gamma$ 9V $\delta$ 2 T-cells, can be stimulated and expanded with aminobisphosphonates, such as

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Zoledronate (ZOL), and the *ex vivo* expansion of these cells with ZOL+IL2 has been shown to induce cytotoxic effects against many hematologic malignancies and solid tumors in both preclinical and clinical trials (14–18). There is, however, a critical need to increase the potency of expanded  $\gamma\delta$  T-cells, particularly against hematologic malignancies. For instance, the adoptive transfer of *ex vivo* expanded  $\gamma\delta$  T-cell has been shown to improve disease progression but failed to improve overall survival (OS) in multiple cancers (19–25). The results were similar when ZOL was used to promote the *in vivo* expansion of  $\gamma\delta$  T-cells, with little to no effect in most patients (26–32). Critically, approximately 58% of patients receiving *in vivo* ZOL failed to reach  $\gamma\delta$  T-cell numbers associated with increased OS following allogeneic hematopoietic cell transplantation. Moreover, *ex vivo* expansion has sometimes been unsuccessful due to low numbers of  $\gamma\delta$  T-cells in circulation (33, 34). We have shown that a single bout of exercise in humans mobilizes  $\gamma\delta$  T-cells to the peripheral blood and augments their *ex vivo* expansion and anti-tumor activity against a range of hematologic tumors *in vitro* (10). We also found an upregulation of key activating receptors on  $\gamma\delta$  T-cells expanded after exercise, and antibody blocking of NKG2D abrogated the augmented cytotoxic effects of exercise against a multiple myeloma (U266) but not a chronic myeloid leukemia (K562) cell line (10).

In the current study, we built on these findings by showing that  $\gamma\delta$  T-cells mobilized with exercise have transcriptomic profiles associated with cytotoxicity, adhesion, migration, and cytokine signaling, and that their increased cytotoxicity against K562 leukemic cells is dependent on an upregulation of DNAM-1 at the cell surface, allowing them to kill K562 cells via poliovirus receptor (PVR) and Nectin-2. The increased anti-leukemic effects of exercise were replicated when  $\gamma\delta$  T-cells were collected from the blood of humans during isoproterenol [ISO; a synthetic  $\beta_1+\beta_2$ -AR (adrenergic receptor) agonist] infusion. In addition, the mobilization and enhanced expansion of  $\gamma\delta$  T-cells were abolished after administering a  $\beta_1+\beta_2$ -AR but not a  $\beta_1$ -AR antagonist prior to exercise. These findings provide a mechanistic link between exercise,  $\beta_2$ -AR activation, and the manufacture of enhanced  $\gamma\delta$  T-cell products for adoptive cell therapy against hematologic malignancies.

## Materials and Methods

### Participants

We recruited 26 (9 female) healthy, physically active adults to participate in this study. 10 participants (height:  $174 \pm 10.1$  cm, body mass:  $69.2 \pm 9.1$  kg; age:  $29.6 \pm 6.5$  years, female:  $n = 3$ ) completed the exercise only trials, 9 participants (height:  $175.7 \pm 7.9$  cm, body mass:  $75 \pm 6.9$  kg; age:  $28.3 \pm 5.4$  years, female:  $n = 3$ ) completed the randomized placebo-controlled cross-over trial involving exercise and beta blockers, and 7 participants (height:  $173 \pm 11.5$  cm, body mass:  $68.2 \pm 11.9$  kg; age:  $26.8 \pm 5.4$  years, female:  $n = 2$ ) completed the ISO infusion experiments. Participants were excluded if they were current or recent (within 6 months) users of tobacco products, physically inactive [physical activity readiness (PAR) score  $<4$ ], obese [body mass index  $\geq 30$  kg/m<sup>2</sup> or waist girth  $>102$  cm (men) and 88 cm (women)], pregnant, or had any illness, disease, or disorder that could affect the immune system. Enrolled participants were not taking medications (except for oral contraceptives for some female participants) and were classified as “low risk” for graded exercise testing in accordance with ACSM/American Heart Association criteria. Participants were required to abstain from alcohol, caffeine, and physical activity 24 hours prior to trials, as well as eliminate vitamin/mineral supplementation and medications that could modulate the immune system at least 4 weeks prior to involvement

in the study. Adherence to the pretest guidelines were confirmed verbally with the participants prior to their arrival at the laboratory.

### Experimental Design

All laboratory visits occurred between 7:00 am and 10:00 am and, when multiple visits were required, these were separated by 1–3 weeks. Participants were required to fast overnight (8–12 hours), abstain from alcohol, and refrain from vigorous activities for 24 hours prior to each visit. The study was conducted in accordance with the Declaration of Helsinki and approval from the Human Subjects Protection Program at the University of Arizona (Tucson, AZ; #1801161041 and #1711017841), with written consent from all participants.

### Standard Exercise Protocol

Participants completed two exercise trials performed at least a week apart, the first being a maximal exercise test to determine  $\dot{V}O_{2max}$  ( $44.9 \pm 8.7$  mL/kg/minute), and the second being a steady-state or graded bout of exercise up to intensities equivalent to 80%  $\dot{V}O_{2max}$  on an indoor cycling ergometer, as described previously (10, 35). Heart rate, electrocardiogram (ECG) activity, and respiratory gas exchange were recorded continuously throughout the test (Cosmed CPET). Prior to exercise (REST) and during the last 3–5 minutes of the exercise bout (EX), blood samples were collected from an indwelling catheter into vacuum-sealed tubes containing EDTA or citric acid, sodium citrate, and dextrose (ACD; Becton-Dickinson).

### Exercise and Beta Blocker Protocol

Participants completed the same maximal exercise test to determine  $\dot{V}O_{2max}$  ( $40.3 \pm 10.2$  mL/kg/minute), as stated above, but were required to visit the laboratory again on four separate occasions with a period of 7 days interspersed between visits (10). Participants performed the same 30-minute steady state or 20-minute graded bout of cycling exercise on three separate occasions after ingesting either (i) 10 mg bisoprolol ( $\beta_1$ -AR antagonist), (ii) 80 mg nadolol (nonselective  $\beta_1+\beta_2$ -AR antagonist), or (iii) a placebo, exactly 3 hours prior to exercise (6). Blood samples were collected prior to drug/placebo ingestion (REST) and during the last 3–5 minutes of the EX. Heart rate, ECG activity, and respiratory gas exchange were recorded continuously throughout all trials. The tablets were administered in a double-blind fashion and the trials were performed using a block randomization design.

### ISO Infusion Protocol

Participants were required to visit the Clinical and Translational Sciences Research Center (CATS) at The University of Arizona to complete the nonselective  $\beta$ -AR agonist infusion trial. Prior to infusion, two indwelling catheters (Becton-Dickinson) were placed inside bilateral antecubital veins to allow for simultaneous infusion of ISO and blood collection. Participants were infused with ISO (50 ng/kg/minute) for 20 minutes and blood samples were collected before (REST) and during the last 10 minutes of ISO infusion. Heart rate, ECG activity, and blood pressure were monitored during the trial.

### Blood Sample Analysis and Processing

Complete blood counts were immediately performed on whole blood samples treated with EDTA using an automated hematology analyzer (AcT 5Diff CP, Beckman Coulter). The enumeration of leukocytes and leukocyte subtypes in whole blood was performed using direct immunofluorescence assays and up to 8-color flow cytometry (MACSQuant 10, Miltenyi Biotec). All

antibodies were purchased from Miltenyi Biotec unless otherwise stated. Briefly, peripheral blood mononuclear cells (PBMC) were stained with the following antibodies; CD8-VioBlue, CD14-VioGreen, CD3-FITC, CD4-PE, CD20-PerCP-Vio700, CD45-APC, CD56-APC-Vio770. Blood samples treated with ACD were used to isolate PBMCs (Ficoll-Paque PLUS, Cytiva), which were cryopreserved in liquid nitrogen at a concentration of  $10 \times 10^6$  cells/mL in freezing media (90% FBS, 10% DMSO) until they were used to phenotype and expand  $\gamma\delta$  T-cell *ex vivo*. Briefly, PBMCs were stained with the following antibodies; CD8-VioBlue, CD16-VioBlue, CD56-VioBlue, TRAIL-BV421 (BD Biosciences), CCR5-BV421 (BioLegend), CD3-VioGreen, V $\delta$ 2-FITC, CD4-PE, CD62L-PE, NKG2D-PE, CXCR3-PE, CD45-PerCP-Vio700, PD1-PerCP-eFlour (BD Biosciences), 2B4-PerCP-Cy5.5 (BioLegend), BTLA-PerCP-Cy5.5 (BioLegend), TCR $\alpha\beta$ -PE-Vio770, CD45RA-PE-Vio770, FasL-PE-Vio770, NKG2C-PE-Vio770, CCR7-PE-Vio770, V $\delta$ 1-APC, DNAM1-APC, NKG2A-APC, CD161-APC, TCR $\gamma\delta$ -APC-Vio770, V $\delta$ 1-APC-Vio770. Extra aliquots were frozen from 3 participants for subsequent RNA isolation and single-cell RNA sequencing (scRNA-seq) using the 10x genomics platform.

### Expansion of $\gamma\delta$ T-cells

$\gamma\delta$  T-cells were expanded using methods described previously (10, 34). Briefly, PBMCs were thawed, enumerated, and seeded at a concentration of  $1 \times 10^6$  cells/mL in a 24 well-plate with culture media (RPMI1640 media +10% FBS and 10% penicillin-streptomycin) containing 300 IU/mL of IL2 (Miltenyi Biotec) and 5  $\mu$ mol/L of ZOL (Sigma-Aldrich). Culture media was changed on days 3, 7, and 10, with fresh culture media culture containing IL2 (300 IU/mL) only. After 14 days, expanded V $\gamma$ 9V $\delta$ 2+ T-cells were harvested to determine number, phenotype, and function by flow cytometry (MACSQuant 10; Miltenyi Biotec). Expanded V $\gamma$ 9V $\delta$ 2+ T-cell phenotypes were assessed in a similar manner as previously described in the aforementioned section but with a slightly altered phenotype panel by replacing, V $\delta$ 1-APC-Vio770 with TCR $\gamma\delta$ -APC-Vio770 and NKG2C-PE-Vio770 with FasL-PE-Vio770. The expanded V $\gamma$ 9V $\delta$ 2+ T-cells were cryopreserved in liquid nitrogen at a concentration of  $10 \times 10^6$  cells/mL in freezing media (90% FBS, 10% DMSO) until use in the *in vitro* cytotoxicity assays, adoptive transfer experiments in xenogeneic mice, and bulk RNA-seq analysis.

### V $\gamma$ 9V $\delta$ 2+ T-cell Cytotoxicity Assays

The *in vitro* cytotoxic function of the EX and ISO expanded V $\gamma$ 9V $\delta$ 2+ T-cells were tested against the chronic myeloid leukemia cell line K562 (ATCC: CCL-243), multiple myeloma cell line U266 (ATCC: TIB-196), and Burkitt lymphoma cell line Daudi (ATCC: CCL-213). All target cells were maintained in a glutamine-enriched RPMI1640 media (+10% FBS and 10% penicillin-streptomycin). All experiments were performed with target cells between passages 3 and 9, to ensure all target cells used in this study were equivalent. *Mycoplasma* testing was routinely performed but we did not authenticate the cell lines. In some experiments, the target cells were incubated with 5  $\mu$ mol/L of ZOL for 20 hours. On the day of the V $\gamma$ 9V $\delta$ 2+ T-cell cytotoxicity assay,  $2 \times 10^6$  target cells were removed and labeled with an anti-CD71-FITC antibody. The target cells were washed and resuspended in 5 mL of RPMI1640 media (+10% FBS and 10% penicillin-streptomycin). The expanded V $\gamma$ 9V $\delta$ 2+ T-cells were then cocultured with the CD71-labeled target cells in a 96-well plate at 0:1, 1:1, 5:1, 10:1, and 20:1  $\gamma\delta$  T-cell:target cells (E:T) ratios in a final volume of 200  $\mu$ L. The 0:1 E:T ratio was used to determine the spontaneous death of the target cells. After 4 hours of incubation at 37°C, the cytotoxicity of  $\gamma\delta$  T-cell was assessed on the MACSQuant 10 (Miltenyi Biotec). Propidium iodide was

added to each well on the 96-well plate immediately before analysis to quantify cell death. V $\gamma$ 9V $\delta$ 2+ T-cell cytotoxic activity was quantified as specific lysis (% total lysis – % spontaneous death).

The receptor-ligand mechanism by which expanded V $\gamma$ 9V $\delta$ 2+ T-cell recognize and lyse K562 cells was determined using antibody blocking experiments. To block activation of V $\gamma$ 9V $\delta$ 2+ T-cells through the NKG2D, TRAIL, DNAM-1, and TCR $\gamma\delta$  receptors, expanded V $\gamma$ 9V $\delta$ 2+ T-cells were incubated with either media alone, an irrelevant antibody, or anti-mAb against the activating receptors for 30 minutes prior to performing the V $\gamma$ 9V $\delta$ 2+ T-cell cytotoxicity assay. Concanamycin A (CMA) was used to inhibit the perforin-mediated killing of target cells by V $\gamma$ 9V $\delta$ 2+ T-cells. CMA was incubated with V $\gamma$ 9V $\delta$ 2+ T-cells at a concentration of 15 nM for 30 minutes at 37°C prior to coculture with target cells, without further washing. To block recognition of tumor cells through the PVR, Nectin-2, TRAIL-R1, and TRAIL-R2, K562 cells were incubated with either media alone, an irrelevant antibody, or anti-mAb against the respective tumor ligands for 30 minutes at 37°C prior to performing the V $\gamma$ 9V $\delta$ 2+ T-cell cytotoxicity assay.

### Xenogeneic Mouse Experiments

NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup> Tg(IL15)1Sz/SzJ [NSG-Tg(Hu-IL15)] mice (Jackson Labs, stock no: 030890; RRID:MGI:6201748) between the ages of 8–12 weeks were used for xenotransplantation of expanded V $\gamma$ 9V $\delta$ 2+ T-cells and K562-luc2 tumor cells (ATCC: CCL-243-LUC2). All experiments were performed with K562-luc2 between passages 3 and 4, to ensure all target cells used in this study were equivalent. *Mycoplasma* testing was routinely performed but we did not authenticate the cell line. K562-luc2 cells were maintained at 37°C, 5% CO<sub>2</sub> in Iscove's DMEM supplemented with 10% FBS and 8  $\mu$ g/mL blasticidin and prepared for tail vein injection as described previously (35). Mice were irradiated with 100 cGy, using a Cesium 137 irradiator, one day (day –2) before intravenous injection of expanded V $\gamma$ 9V $\delta$ 2+ T-cells on day –1, mice not receiving expanded  $\gamma\delta$  T-cells (tumor-only) were injected with an equal volume of saline on the same day. The following day (day 0),  $1 \times 10^6$  K562-luc2 cells were injected intravenously through the lateral tail vein. One day after tumor injection (day 1), mice will be exposed to bioluminescent imaging (BLI), to monitor tumor progression using a LagoX (Spectral Instruments Imaging). Mice were monitored daily until sacrifice. In some experiments, ZOL was injected intraperitoneal on day 1 and repeated weekly to sensitize the tumors to  $\gamma\delta$  T cell-mediated lysis. BLI was repeated every 3–4 days to track tumor progression. Briefly, D-luciferin, potassium salt (15 mg/mL; Gold Biotechnologies) was injected intraperitoneally at a concentration of 10  $\mu$ L/g of body weight (BW). Data were expressed as photons/second to allow for comparisons between exposure times. Although expanded  $\gamma\delta$  T-cells do not elicit GvHD, symptoms were monitored to ensure the small proportion of contaminating  $\alpha\beta$  T-cells (~5%–10%) did not propagate GvHD. Mice were weighed and scored every 3–4 days to monitor GvHD. The following symptoms were assessed to determine GvHD status: Skin Integrity (0–2): 0 = normal, healthy skin; 1 = Scaling of paws/tail; 2 = dehydrated, obvious areas of denuded skin; Fur Integrity (0–2): 0 = normal, fluffy, and elastic fur; 1 = mild to moderate ruffling; 2 = soiled, stiff, and rough fur; Posture (0–2): 0 = normal posture; 1 = hunching only at rest; 2 = severe hunching, sunken or distended abdomen; Activity (0–2): 0 = normal, responsive and vocal; 1 = mild to moderately decreased; 2 = unresponsive, separates from group, circling, head pressing; Weight Loss (0–2): 0  $\leq$  10%; 1 = 10% to 20%; 2  $\geq$  20%; Diarrhea (0–1): 0 = no; 1 = yes. All animal procedures were performed in accordance

with protocols approved by the University of Arizona Institutional Animal Care and Use Committee (#17-338).

### RNA-seq Analysis

We used scRNA-seq ( $n = 3$ ) to identify transcriptomic changes within exercise-mobilized  $V\gamma 9V\delta 2+$  T-cells, and bulk RNA-seq to identify transcriptomic shifts among  $V\gamma 9V\delta 2+$  T-cells expanded, from the same participants, after both exercise ( $n = 2$ ) and ISO infusion ( $n = 2$ ). PBMCs or expanded  $V\gamma 9V\delta 2+$  T-cells (>95% purity) were thawed and resuspended in PBS and RNAlater solution and processed by the University of Arizona Genetics Core for scRNA-seq analysis using the 10x Genomics platform and bulk RNA-seq using the Illumina NextSeq500 platform. The scRNA-seq library generation, sequencing, and analysis were performed as described previously (35, 36). For bulk RNA-seq, RNA samples' quality was assessed with a high sensitivity RNA Fragment Analyzer Kit (Advanced Analytics) and quantity with an RNA HS assay kit (Qubit). Quality samples were used for library builds with the Rapid RNA Library Kit (Swift) and Dual Combinatorial Indexing Kit (Swift). Samples had quality and average fragment size assessed with the High Sensitivity next-generation sequencing (NGS) Analysis Kit (Advanced Analytics). Quantity was assessed with the Kapa Library Quantification kit (Illumina), and then samples were equimolar-pooled and clustered for sequencing using Illumina NextSeq500 run chemistry (NextSeq 500/550 High Output v2 kit 150 cycles). Data were analyzed by the Bioinformatics core. For each differential expression analysis comparison, gene set enrichment analysis (GSEA), with a FDR of (0.25), was performed and annotated to both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms.

### Statistical Analysis

Statistical analyses were completed using GraphPad Prism 9 (GraphPad Software) or SPSS (v24.0 IBM). All data are represented as mean  $\pm$  SD unless otherwise stated. Paired  $t$  tests or Wilcoxon matched-pair signed rank test were used to detect differences in the proportion, total number, and phenotypes of isolated  $\gamma\delta$  T-cells or expanded  $V\gamma 9V\delta 2+$  T-cells before and during exercise or ISO infusion. Multiple linear mixed models (LMM) with Bonferroni correction for multiple comparisons were built to detect main effects of group (REST vs. EX/ISO), dose (E:T ratios), condition (antibody blockade), treatment (ZOL sensitization), and multiple interaction effects for *in vitro* cytotoxic function of the EX and ISO expanded  $V\gamma 9V\delta 2+$  T-cells. Multiple LMMs with Bonferroni correction for multiple comparisons were used to detect main effects of group, time, treatment, and multiple interaction effects for leukemic burden (BLI) and BW. Simple OS (Kaplan–Meier) was used to detect differences in OS and tumor-free survival (TFS). Significance was accepted at  $P < 0.05$ . For GSEA, the FDR was used separately for each database (GO and KEGG) to correct for multiple hypothesis testing and given the exploratory nature of our analysis, we selected an FDR threshold of  $<0.25$ , which denotes the confidence of “possible” or “hypothesis,” while an FDR  $<0.05$  denotes “high confidence” or “statistical significance” (13). We used the less stringent FDR for our GSEA to avoid overlooking potentially meaningful changes in enriched gene sets in response to exercise.

### Data Availability

The scRNA-seq and bulk RNA-seq data generated in this study are publicly available in NCBI's Gene Expression Omnibus at accession numbers GSE212740 and GSE263181, respectively. All other data generated during this study are available from the corresponding author upon request.

## Results

### Acute Exercise Mobilizes $\gamma\delta$ T-cells with a Surface Phenotype and Transcriptomic Profile Associated with Increased Effector Function

A single bout of cycling exercise mobilized lymphocytes to the blood compartment, increasing total numbers of all major lymphocyte subtypes ( $CD3^+$  T-cells,  $CD4^+$  T-cells,  $CD8^+$  T-cells, NK-cells,  $\gamma\delta$  T-cells; refs. 10, 37–39). Both  $V\delta 2+$  and  $V\delta 1+$  T-cells made up a larger proportion of  $CD3^+$  T-cells during the EX (Table 1). Our flow cytometric panel was designed to detect exercise-induced changes among circulating  $V\delta 2+$  and  $V\delta 1+$  T-cells for markers associated with activation, inhibition, homing, and exhaustion. Exercise increased the percentage of  $V\delta 2+$  and  $V\delta 1+$  T-cells expressing CD56 and 2B4 (Fig. 1A). While the percentage of NKG2D+ and CD16+ and mean fluorescence intensity (MFI) of DNAM-1, CXCR3, and CCR5 were also elevated among the exercise-mobilized  $V\delta 2+$  T-cells (Fig. 1A; Supplementary Fig. S1). Exercise reduced the percentage of PD-1+, CXCR3+, and CCR7+ cells among the  $V\delta 1+$ , while the MFI of NKG2A was decreased among the  $V\delta 2+$  T-cells (Fig. 1A; Supplementary Fig. S1).  $V\delta 1+$  and  $V\delta 2+$  T-cell expression of NKG2C, TRAIL, Fas Ligand (FasL), CD272, and CD161 was unaffected by exercise (Fig. 1A; Supplementary Fig. S1). To understand if exercise altered gene transcription among  $\gamma\delta$  T-cells in blood, we identified pan  $\gamma\delta$  T-cells by scRNA-seq using the Azimuth map for human PBMCs before quantifying differentially expressed genes (DEG) and performing GSEA on the  $\gamma\delta$  T-cell cluster, which we annotated to GO and KEGG terms ( $n = 3$ ; Fig. 1B and C). The top 15 pathways upregulated and downregulated in response to exercise are shown. Notable enriched gene sets among  $\gamma\delta$  T-cells mobilized by exercise include “G protein-coupled receptor signaling,” “citrate cycle (tricarboxylic cycle),” “cell adhesion,” “neuroactive ligand-receptor interaction,” “chemotaxis,” and “NK cell-mediated cytotoxicity” (Fig. 1D). Collectively, these findings indicate that exercise mobilizes  $\gamma\delta$  T-cells with a surface protein phenotype and transcriptomic profile associated with increased effector functions, including those related to cytotoxicity, migration, activation, and signaling.

### Acute Exercise Augments the *Ex Vivo* Expansion and Antileukemic Activity of $V\gamma 9V\delta 2+$ T-cells *In Vitro* and *In Vivo*

We showed previously that exercise augmented the *ex vivo* expansion of  $V\gamma 9V\delta 2+$  T-cells and increased their ability to kill K562, U266, and 221AEH target cells *in vitro* (10). Here, we added an additional lymphoma cell line (Daudi) and determined whether ZOL sensitization would enhance the antileukemic effects of the  $V\gamma 9V\delta 2+$  T-cells expanded after exercise (Fig. 2A). Exercise increased the total number of  $V\gamma 9V\delta 2+$  T-cells expanded over 14 days, even after adjusting for  $V\gamma 9V\delta 2+$  T-cell numbers among the stimulated PBMC fractions at day 0 (Fig. 2B). The purity ( $\sim 90\%$   $\gamma\delta$  T-cells) and subset [naïve, central memory (CM), effector memory (EM), effector memory RA (EMRA)] composition of the expanded products was not altered by exercise (Fig. 2B). However, the  $V\gamma 9V\delta 2+$  T-cells expanded after exercise have significantly enhanced cytotoxicity against K562, Daudi and U266 target cells *in vitro* both with and without ZOL sensitization, indicating that  $V\gamma 9V\delta 2+$  T-cells expanded from exercise-mobilized cells have enhanced anti-tumor activity against multiple hematologic cancer cell lines (Fig. 2C). To determine whether  $V\gamma 9V\delta 2+$  T-cells expanded after exercise would persist and control leukemic growth *in vivo*, we engrafted irradiated NSG(Tg)-IL15 mice with a luciferase tagged K562 cells ( $1 \times 10^6$ ), and, the following day, injected a vehicle control

**TABLE 1** The effect of acute exercise on lymphocyte subsets

	REST	EX	t or z score	P-value
Lymphocytes				
cells/ $\mu$ L	1,586 $\pm$ 464	3,233 $\pm$ 1,112****	$t = 7.327$	<0.0001
CD3 <sup>+</sup> T-cells				
cells/ $\mu$ L	1,204 $\pm$ 385	1,877 $\pm$ 642*	$t = 6.319$	0.004
of Lymphocytes (%)	72.9 $\pm$ 5.2	57 $\pm$ 5.7****	$t = 11.11$	<0.0001
CD4 <sup>+</sup> T-cells				
cells/ $\mu$ L	745 $\pm$ 220	955 $\pm$ 296*	$t = 5.162$	0.0013
of Lymphocytes (%)	45.4 $\pm$ 5	30.8 $\pm$ 5.4*	$t = 14.72$	<0.0001
of CD3 <sup>+</sup> T-cells (%)	62.3 $\pm$ 4.9	53.9 $\pm$ 7.2***	$t = 7.275$	0.0010
CD8 <sup>+</sup> T-cells				
cells/ $\mu$ L	369 $\pm$ 126	673 $\pm$ 275*	$t = 5.312$	0.0011
of Lymphocytes (%)	22.2 $\pm$ 2.8	20 $\pm$ 3*	$t = 2.476$	0.0425
of CD3 <sup>+</sup> T-cells (%)	30.5 $\pm$ 3.4	35.2 $\pm$ 5**	$t = 4.658$	0.0020
$\gamma\delta$ T-cells				
cells/ $\mu$ L	86 $\pm$ 77	225 $\pm$ 183**	$t = 3.534$	0.0095
of Lymphocytes (%)	4.9 $\pm$ 3	6.7 $\pm$ 3.7**	$t = 3.654$	0.0081
of CD3 <sup>+</sup> T-cells (%)	6.8 $\pm$ 4	11 $\pm$ 6.3**	$t = 3.955$	0.0055
V $\delta$ 2 <sup>+</sup> T-cells				
cells/ $\mu$ L	76 $\pm$ 74	181 $\pm$ 177*	$t = 2.851$	0.0247
of Lymphocytes (%)	4.3 $\pm$ 3.1	5.4 $\pm$ 3.9*	$t = 2.796$	0.0267
of CD3 <sup>+</sup> T-cells (%)	6 $\pm$ 4.2	9.1 $\pm$ 6.7*	$t = 3.125$	0.0167
V $\delta$ 1 <sup>+</sup> T-cells				
cells/ $\mu$ L	5 $\pm$ 8	29 $\pm$ 62**	$z = 2.521$	0.0120
of Lymphocytes (%)	0.3 $\pm$ 0.6	0.8 $\pm$ 1.7*	$z = 2.100$	0.0360
of CD3 <sup>+</sup> T-cells (%)	0.6 $\pm$ 0.7	1.9 $\pm$ 2.5*	$z = 2.521$	0.0120
NK-Cells				
cells/ $\mu$ L	188 $\pm$ 79	928 $\pm$ 426***	$t = 5.797$	0.0007
of Lymphocytes (%)	11.1 $\pm$ 3.2	26.6 $\pm$ 6****	$t = 11.14$	<0.0001

NOTE: The absolute number and percentage of lymphocytes and lymphocyte subsets before (REST) and during exercise (EX). Depending on the statistical test used a t-score or z-score is reported, respectively. Data are mean  $\pm$  SD ( $n = 8$ ). Statistical differences from REST are indicated by \*,  $P > 0.05$ ; \*\*,  $P > 0.01$ ; \*\*\*,  $P > 0.001$ ; \*\*\*\*,  $P > 0.0001$ .

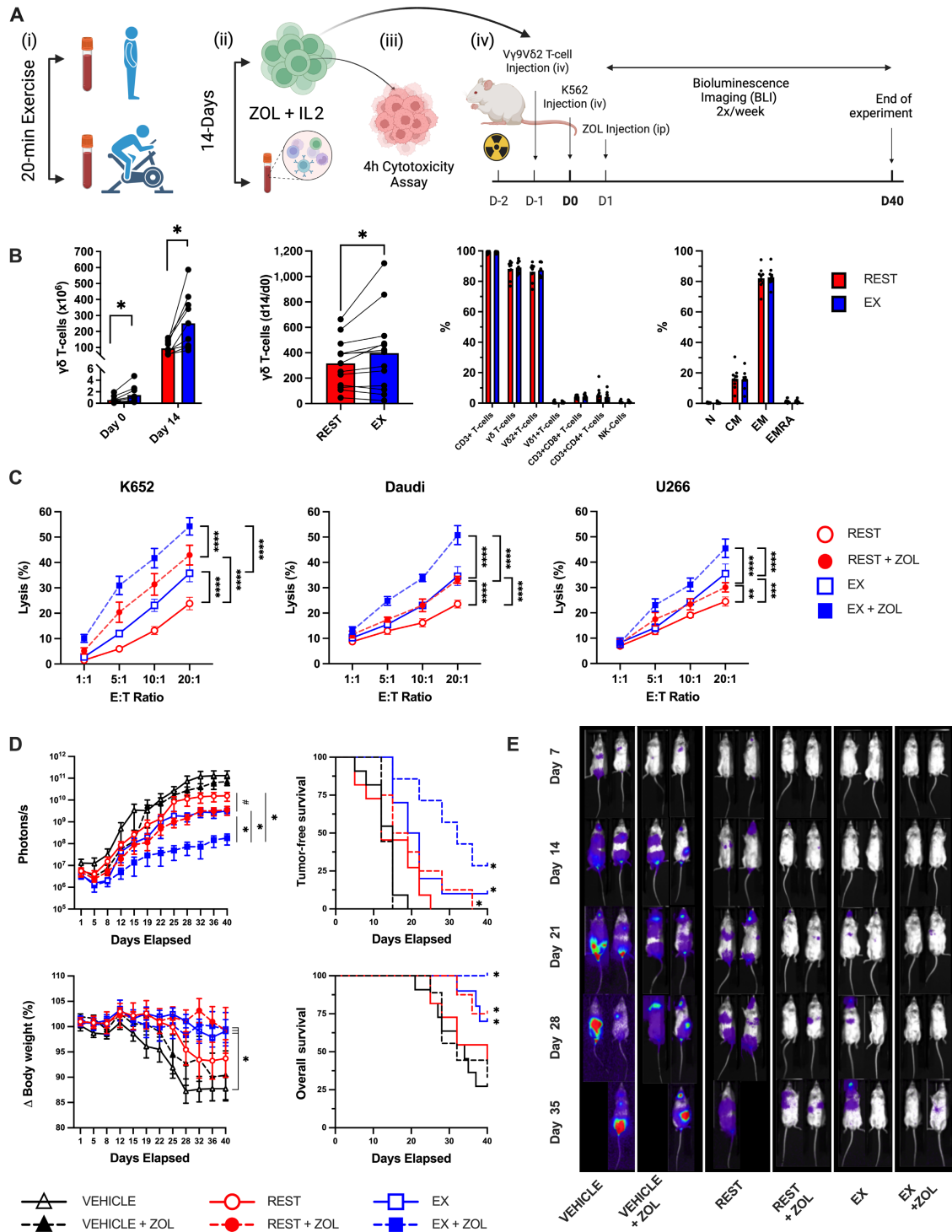
(saline) or V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells ( $10 \times 10^6$ ) expanded from resting or exercise-mobilized PBMCs (Fig. 2A). Half of the mice in each group received weekly injections of ZOL starting on day+ 2 and all mice were monitored for up to 40 days. Mice were removed if their BW dropped by 20% or more, or if they presented with a morbidity score  $\geq 8$ . BLI performed 2x/week revealed significantly lower tumor burden (photons/second) in mice receiving V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells expanded after exercise when combined with ZOL (Fig. 2D and E). TFS (BLI score > baseline) and OS was significantly greater in mice receiving V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells expanded after exercise with ZOL compared with all other groups (Fig. 2D). Collectively, these findings indicate that exercise mobilizes V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells to blood which when expanded *ex vivo* have enhanced anti-tumor activity both *in vitro* and *in vivo*.

### $\beta$ 2 but not $\beta$ 1-AR Activation is Responsible for Mobilizing V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells to Blood and Augmenting Their Cytotoxic Activity Following *Ex Vivo* Expansion

To determine the role of  $\beta$ 1- and  $\beta$ 2-AR signaling on the exercise effects, we first administered a placebo, a selective  $\beta$ 1-AR antagonist (10 mg bisoprolol), or a nonselective  $\beta$ 1+ $\beta$ 2-AR antagonist (80 mg, nadolol) to healthy participants

3 hours prior to exercise. The three exercise trials were performed in a double-blind randomized cross-over design with each subject acting as his/her own control. A 7-day wash out period was interspersed between each 20-minute exercise trial and blood was collected before and during the last 5 minutes of exercise. Absolute cycling power (watts) was controlled across all three exercise trials. Bisoprolol and nadolol did not affect exercising oxygen uptake or plasma catecholamines (epinephrine and norepinephrine) but did lower exercising heart rate and systolic blood pressure by a similar extent compared with placebo (Fig. 3A). This indicates that hemodynamic forces (e.g., shear stress), which can also mobilize lymphocytes to the circulation, was similar between the bisoprolol and nadolol trials (6). Nadolol but not bisoprolol significantly blunted both the mobilization and *ex vivo* expansion of V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells in response to exercise, revealing that the exercise effects are dependent on  $\beta$ 2-AR but not  $\beta$ 1-AR activation (Fig. 3B and C). We next aimed to determine whether systemic  $\beta$ -AR activation in the absence of exercise could also mobilize and increase the function of expanded V $\gamma$ 9V $\delta$ 2<sup>+</sup> T-cells. To do this, we infused healthy participants ( $n = 7$ ) with the synthetic  $\beta$ 1+ $\beta$ 2-AR agonist, ISO (50 ng/kg/minute) continuously for 20 minutes under resting conditions (participants remained supine during the infusion; Fig. 4A). Blood was collected



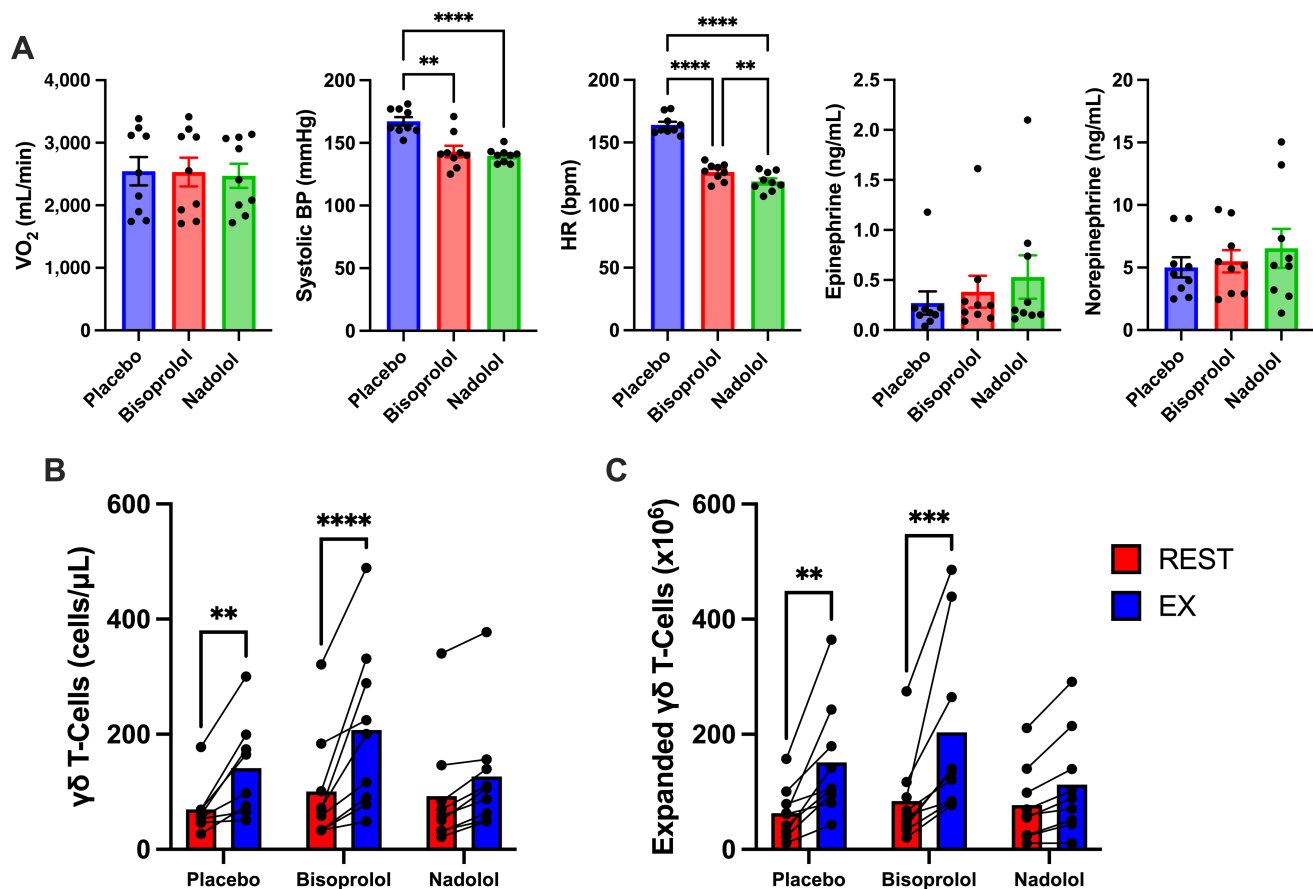


**FIGURE 2** Acute exercise enhances the antileukemic activity of *ex vivo* expanded  $V\gamma 9V\delta 2+$  T-cells *in vitro* and *in vivo*. **A**, Schematic of the experimental design for *in vitro* and *in vivo* experiments. **B**, The total number ( $1 \times 10^6$ ) of  $V\gamma 9V\delta 2+$  cells isolated before (REST) and during the final 5 minutes of exercise (EX) at day 0, and the total number of  $V\gamma 9V\delta 2+$  T-cells generated in the expanded cell products after stimulation with ZOL+IL2 for 14 days. The number of  $V\gamma 9V\delta 2+$  cells generated at day 14 relative to the number of  $\gamma\delta$  cells in the PBMC fractions at day 0. The cellular composition of the expanded  $V\gamma 9V\delta 2+$  T-cell products at day 14. The proportions of naïve (N), CM, EM, and CD45RA+ EMRA cells among  $V\gamma 9V\delta 2+$  T-cells after 14 days of expansion ( $n = 10$ ). **C**, The *in vitro* anti-tumor activity of *ex vivo* expanded  $V\gamma 9V\delta 2+$  T-cells against K562, Daudi, and U266 with and without ZOL sensitization. Target cells were exposed to  $5 \mu\text{mol/L}$  of ZOL for 20 hours and cytotoxicity of  $V\gamma 9V\delta 2+$  cells was assessed via flow cytometry-based assays. **D**, To determine the *in vivo* GvL effect of exercise expanded  $V\gamma 9V\delta 2+$ , NSG-IL15 (Continued on the following page.)

(Continued) mice were injected with REST or EX expanded  $V\gamma 9V\delta 2+$  ( $10 \times 10^6$ ) and challenged with  $1 \times 10^6$  luciferase tagged human chronic myeloid leukemia cells (K562-luc). A subset of mice in each group were also provided weekly injections of ZOL (REST+ZOL; EX+ZOL; VEHICLE +ZOL). The BLI (photons/second) scores, TFS, change in BW, and overall probability of survival (OS) after injection of expanded  $V\gamma 9V\delta 2+$  T-cells with or without ZOL sensitization ( $n = 8-11/\text{group}$ ). BLI significant difference from EX+ZOL and EX conditions indicated by \* and #, respectively (all conditions were significantly different from vehicle controls). TFS, BW, and OS significant difference from vehicle controls were indicated by \*. **E**, Representative bioluminescence images of leukemia-bearing mice that received (from left to right) vehicle, vehicle + ZOL, REST expanded  $V\gamma 9V\delta 2+$  T-cells, REST expanded  $V\gamma 9V\delta 2+$  T-cells + ZOL, EX expanded  $V\gamma 9V\delta 2+$  T-cells, and EX expanded  $V\gamma 9V\delta 2+$  T-cells + ZOL. BLI intensity on a scale from low (purple) to high (red). Data are represented as mean  $\pm$  SEM; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Student two-tailed paired  $t$  test (B); repeated measures two-way ANOVA or LMM with Bonferroni *post hoc* test (C-D); log-rank (Mantel-Cox) test (D).

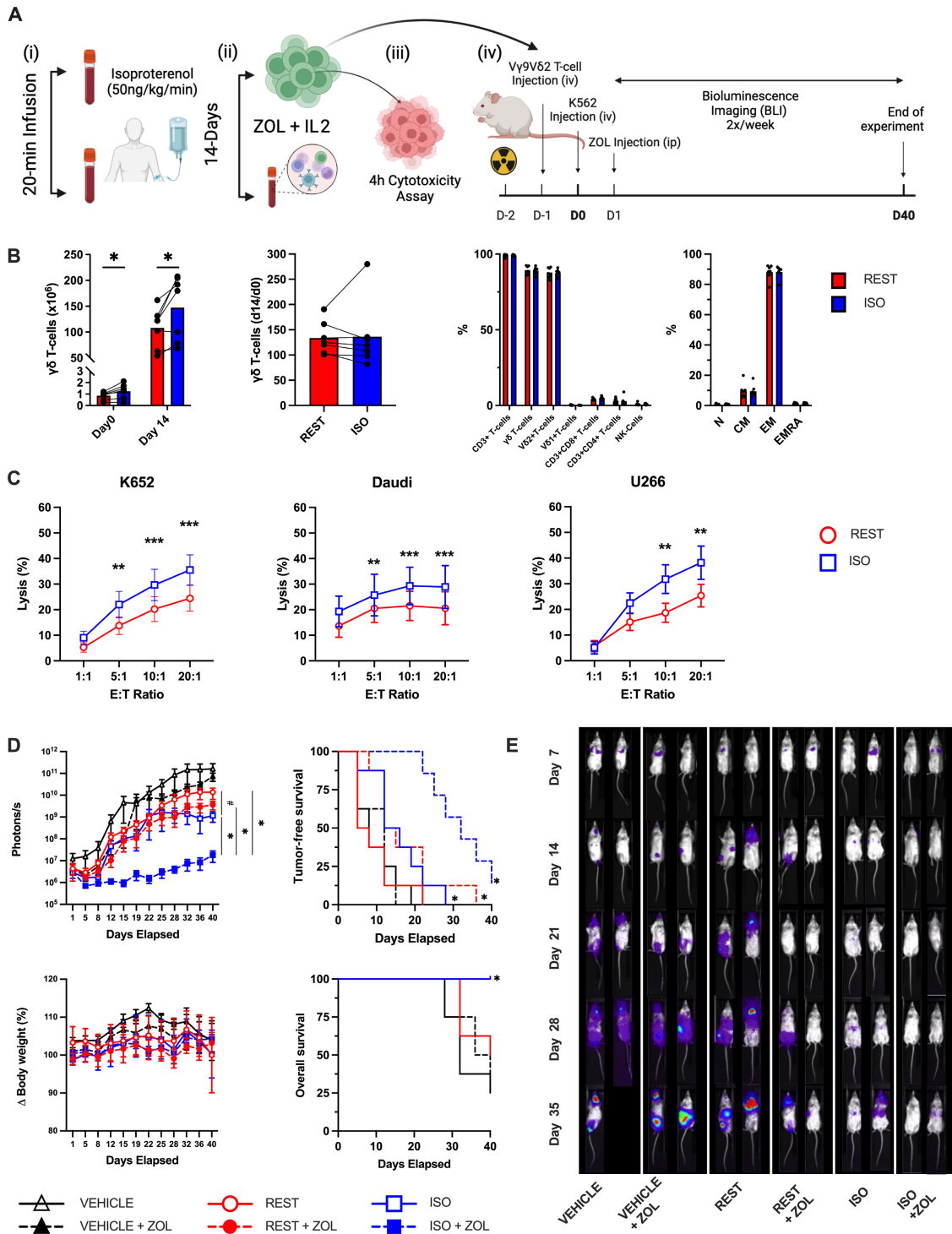
before and during the last 5 minutes of infusion. Like exercise, ISO mobilized  $V\gamma 9V\delta 2+$  T-cells to blood and increased the total number of  $V\gamma 9V\delta 2+$  T-cell expanded with ZOL+IL2 over 14 days (Fig. 4B). Contrary to exercise, the numbers of  $V\gamma 9V\delta 2+$  T-cells expanded at day 14 was proportional to the numbers of  $V\gamma 9V\delta 2+$  T-cells in the PBMC fractions stimulated at day 0 (Fig. 4B). However, like exercise,  $V\gamma 9V\delta 2+$  T-cells expanded from cells collected during ISO infusion were still superior at killing K562, Daudi and U266 target cells *in vitro* (Fig. 4C). We then tested whether  $V\gamma 9V\delta 2+$  T-cells expanded

after ISO infusion would also exert better control of leukemic growth *in vivo*. Using the same NSG(Tg)IL15 mouse model for the previously described exercise trials, we observed significantly lower tumor burden (photons/second) in mice receiving  $V\gamma 9V\delta 2+$  T-cells expanded after ISO infusion when combined with ZOL (Fig. 4D and E). Similar to exercise, TFS (BLI score > baseline) and OS was significantly greater in mice receiving  $V\gamma 9V\delta 2+$  T-cells expanded after ISO infusion with ZOL compared with all other groups (Fig. 4D). Collectively, these findings indicate that  $\beta 2$  but not  $\beta 1$ -AR activation is required to



**FIGURE 3** Blockade of  $\beta 2$ -AR blunts the mobilization and expansion of  $V\gamma 9V\delta 2+$  T-cells. **A**, The physiologic effects of a placebo,  $\beta 1$ -AR antagonist (10 mg bisoprolol), or a nonselective  $\beta 1+\beta 2$  antagonist (80 mg, nadolol) on  $VO_2$  (mL/minute), systolic blood pressure (mmHg), HR (bpm), epinephrine (ng/mL), and norepinephrine (ng/mL) during the last minutes of acute exercise ( $n = 9$ ). The effects of a placebo,  $\beta 1$ -AR antagonist, or a nonselective  $\beta 1+\beta 2$  antagonist on the total number of  $V\gamma 9V\delta 2+$  T-cells mobilized with exercise (**B**) and the *ex vivo* expansion of  $V\gamma 9V\delta 2+$  T-cells after 14 days with ZOL+IL2 ( $n = 9$ ; **C**). Data are represented as mean  $\pm$  SEM; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by repeated measures one-way ANOVA with Bonferroni *post hoc* test.





**FIGURE 4** ISO infusion enhances the antileukemic activity of *ex vivo* expanded V $\gamma$ 9V $\delta$ 2+ T-cells *in vitro* and *in vivo*. **A**, Schematic of the experimental design for *in vitro* and *in vivo* experiments. **B**, The total number ( $1 \times 10^6$ ) of V $\gamma$ 9V $\delta$ 2+ cells isolated before (REST) and during the final 5 minutes of ISO infusion at day 0, and the total number of V $\gamma$ 9V $\delta$ 2+ T-cells generated in the expanded cell products after stimulation with ZOL+IL2 for 14 days. The number of V $\gamma$ 9V $\delta$ 2+ cells generated at day 14 relative to the number of  $\gamma\delta$  cells in the PBMC fractions at day 0. The cellular composition of the expanded V $\gamma$ 9V $\delta$ 2+ T-cell products at day 14. The proportions of naive (N), CM, EM, and CD45RA+ EMRA cells among V $\gamma$ 9V $\delta$ 2+ T-cells after 14 days of expansion ( $n = 7$ ). **C**, The *in vitro* anti-tumor activity of *ex vivo* expanded V $\gamma$ 9V $\delta$ 2+ T-cells against K562, Daudi, and U266 cells via flow cytometry-based assays. Significance indicated by \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ). **D**, To determine the *in vivo* GvL effect of ISO expanded V $\gamma$ 9V $\delta$ 2+, NSG-IL15 mice were injected with REST or ISO expanded V $\gamma$ 9V $\delta$ 2+ ( $10 \times 10^6$ ) and challenged with  $1 \times 10^6$  luciferase tagged human chronic myeloid leukemia cells (K562-luc). A subset of mice in each group were also provided weekly injections of ZOL (REST+ZOL; ISO+ZOL; VEHICLE +ZOL). The BLI (photons/second) scores, TFS, change in BW, and probability of survival after injection of expanded (Continued on the following page.)

(Continued)  $V\gamma 9V\delta 2+$  T-cells with or without ZOL sensitization ( $n = 7-8/\text{group}$ ). BLI significant difference from EX+ZOL and EX conditions indicated by \* and #, respectively (all conditions were significantly different from vehicle controls). TFS, BW, and OS significant difference from vehicle controls were indicated by \* (TFS; EX, EX+ZOL, and REST+ZOL significance indicated by a single marker). **E**, Representative bioluminescence images of leukemia-bearing mice that received (from left to right) vehicle, vehicle + ZOL, REST expanded  $V\gamma 9V\delta 2+$  T-cells, REST expanded  $V\gamma 9V\delta 2+$  T-cells + ZOL, ISO expanded  $V\gamma 9V\delta 2+$  T-cells, and ISO expanded  $V\gamma 9V\delta 2+$  T-cells + ZOL. BLI intensity on a scale from low (purple) to high (red). Data are represented as mean  $\pm$  SEM; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by Students two-tailed paired *t* test (B); Repeated measures two-way ANOVA or LMM with Bonferroni *post hoc* test (C-D); log-rank (Mantel-Cox) test (D).

mobilize  $V\gamma 9V\delta 2+$  T-cells to blood that can be expanded into a superior product with enhanced cytotoxic activity against human hematologic malignancies.

### Exercise and ISO Infusion Evoke Similar Surface Phenotype and Transcriptomic Shifts Among *Ex Vivo* Expanded $V\gamma 9V\delta 2+$ T-cells

To identify potential phenotypic determinants of the exercise/ISO effects, we performed extended flow cytometry and bulk RNA-seq of the  $V\gamma 9V\delta 2+$  T-cell products expanded after exercise and ISO infusion. Pairwise comparisons were made with  $V\gamma 9V\delta 2+$  T-cells expanded from resting blood samples. Shifts in the surface phenotypes of  $V\gamma 9V\delta 2+$  T-cell expanded after exercise were strikingly similar to those expanded after ISO infusion (Fig. 5A). Surface expression of markers associated with cytotoxicity and migration such as CD56, TRAIL, DNAM-1, NKG2D, CXCR3, CCR5, and CD161 were significantly elevated among  $V\gamma 9V\delta 2+$  T-cells expanded after both exercise and ISO infusion (Fig. 5A). Conversely, NKG2A, a potent inhibitory receptor expressed by NK-cells and  $V\gamma 9V\delta 2+$  T-cells, was significantly lower after both exercise and ISO infusion (Fig. 5A). Bulk RNA-seq revealed several enriched gene sets among  $V\gamma 9V\delta 2+$  T-cells expanded after exercise including those related to “NK-cell mediated cytotoxicity,” “cell adhesion,” “T-cell activation,” “cAMP response,” and “antigen processing and presentation” (Fig. 5B). Among  $V\gamma 9V\delta 2+$  T-cells expanded after ISO infusion was an enrichment of gene sets related to “membrane protein targeting,” “oxidative metabolism,” “NK-cell mediated immunity,” “immune responses to tumor cell,” and “cytokine signaling” (Fig. 5C). Collectively, these findings indicate that exercise promotes the mobilization of  $V\gamma 9V\delta 2+$  T-cells that can be expanded into a cell product with a surface phenotype and transcriptomic profile associated with enhanced effector functions, and that the exercise effects can be replicated with systemic  $\beta 2$ -AR activation *in vivo*.

### Exercise Arms Expanded $V\gamma 9V\delta 2+$ T-cells with DNAM-1 to Increase Cytolysis of K562 via PVR and Nectin-2

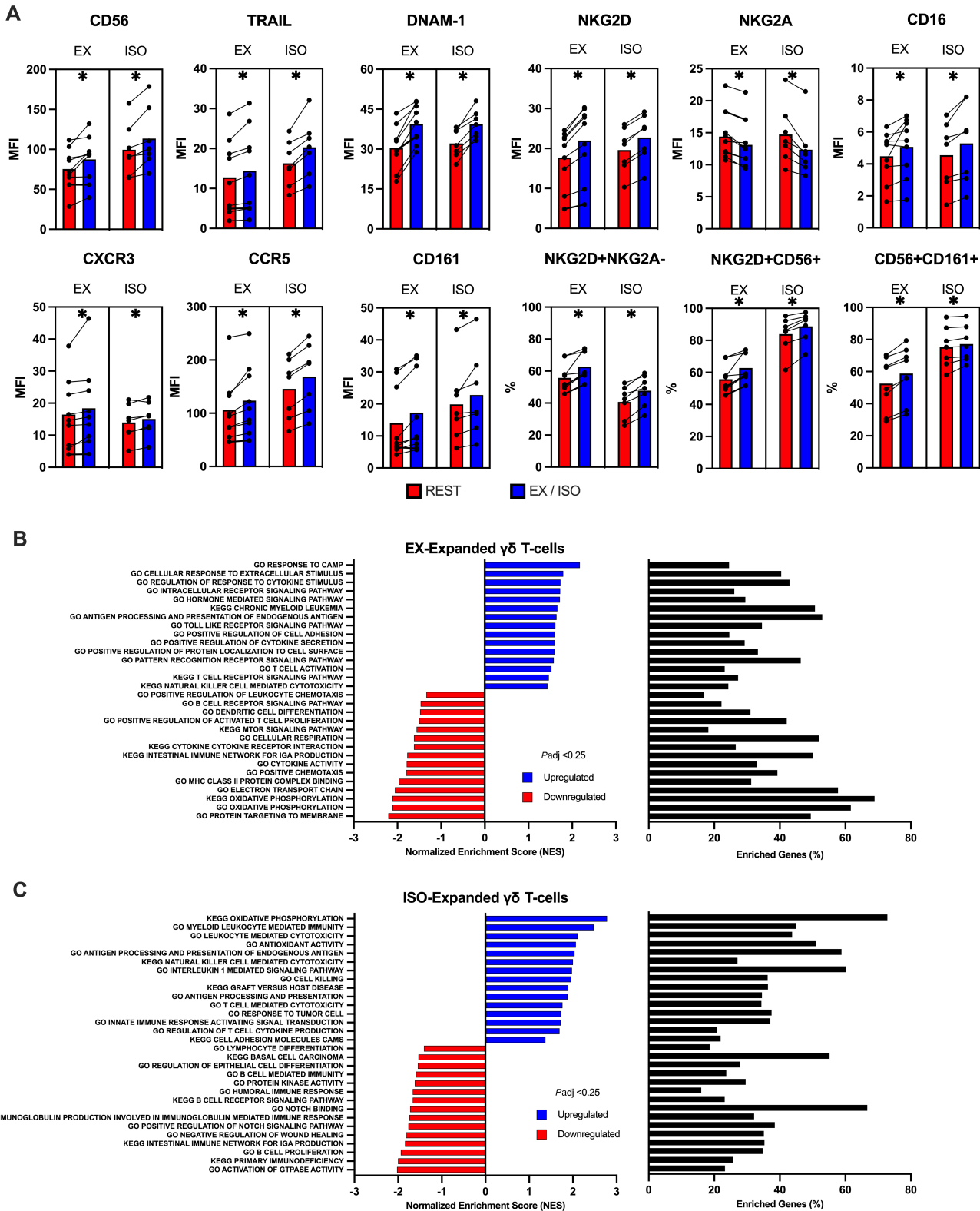
Our next step was to determine whether phenotypic shifts among the  $V\gamma 9V\delta 2+$  T-cells expanded after exercise were responsible for their enhanced cytotoxic activity (Fig. 6A). We showed previously that an upregulation of NKG2D on  $V\gamma 9V\delta 2+$  T-cells expanded after exercise played a mechanistic role in improved killing of the U266 multiple myeloma cell line, but not against the K562 chronic myeloid leukemia cell line (10). This was most likely due to K562-expressing lower levels of the NKG2D ligands ULBP-1 and ULBP-3 (Fig. 6B). We screened K562 cells for ligands of known activating and inhibitory receptors expressed by  $V\gamma 9V\delta 2+$  T-cells (Fig. 6B). We found that they expressed ligands for DNAM-1 in PVR and Nectin-2, and for TRAIL in TRAIL-R1 and TRAIL-R2 (Fig. 6B). We, therefore, identified DNAM-1 and/or TRAIL upregulation as a potential mechanism for the enhanced antileukemic effects of  $V\gamma 9V\delta 2+$  T-cells expanded after exercise.  $V\gamma 9V\delta 2+$  T-cells expanded after rest and exer-

cise were cocultured with K562 cells with an anti-DNAM-1 antibody or isotype control for 4 hours ( $n = 3$ ). DNAM-1 blockade not only lowered K562 killing, but also abrogated the enhanced cytotoxic effects seen with exercise-expanded  $V\gamma 9V\delta 2+$  T-cells (Fig. 6C). Furthermore, blocking the DNAM-1 ligands PVR and Nectin-2 expressed on the K562 cells similarly reduced K562 killing and abrogated the exercise effects (Fig. 6C). In a similar approach, blocking TRAIL was found to reduce K562 killing but the cytotoxic effects of exercise-expanded  $V\gamma 9V\delta 2+$  T-cells was still enhanced, indicating that the upregulation of TRAIL on  $V\gamma 9V\delta 2+$  T-cells expanded after exercise is not mechanistically involved in the enhanced cytotoxic effects against K562 (Fig. 6D). As expected, therefore, blocking TRAIL ligands (R1 and R2) on K562 cells did not alter the exercise effects (Fig. 6D). Antibody blocking the  $\gamma\delta$ -TCR and depleting perforin with CMA confirmed that the expanded  $V\gamma 9V\delta 2+$  T-cells exert their cytotoxic effects via activation of the  $\gamma\delta$ -TCR and release of cytolytic granules (Supplementary Fig. S2). These findings indicate that exercise upregulates DNAM-1 expression on *ex vivo* expanded  $V\gamma 9V\delta 2+$  T-cells, allowing them to exert greater cytotoxic effects against K562 via PVR and Nectin-2.

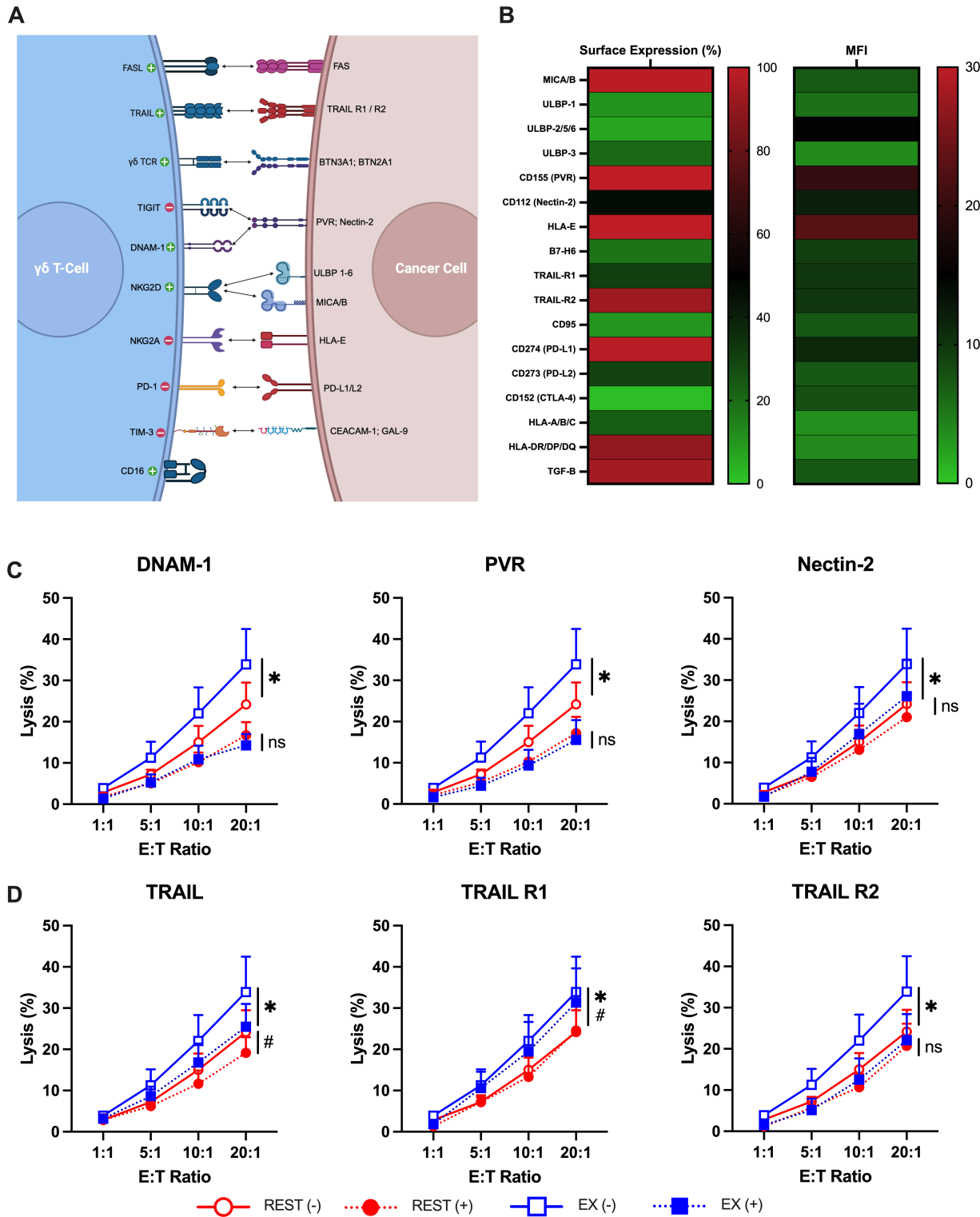
## Discussion

$\gamma\delta$  T-cells are promising candidates for allogeneic cell therapies due to their anti-tumor activity and ability to function across MHC barriers without causing GvHD (40). While  $\gamma\delta$  T-cells, expanded *in vivo* or *in vitro* prior to adoptive transfer, have demonstrated anti-tumor activity in preclinical and early phase clinical trials, their efficacy against hematologic malignancies has thus far been modest (12). In this article, we demonstrate that a single session of exercise activates the catecholamine,  $\beta 2$ -AR signaling pathway, leading to mobilization of  $\gamma\delta$  T-cells with enhanced anti-tumor activity into the bloodstream (Supplementary Fig. S3). We further found that  $\gamma\delta$  T-cells manufactured using exercise or ISO-mobilized cells displayed a cytotoxic phenotype and transcriptomic profile associated with augmented anti-tumor activity *in vitro* and *in vivo*. Inhibiting  $\beta 2$ -AR but not  $\beta 1$ -AR signaling abrogated the mobilization and *ex vivo* expansion of  $\gamma\delta$  T-cells with exercise. We also found that an upregulation of the NK-cell cytotoxicity trigger molecule DNAM-1 on  $\gamma\delta$  T-cells expanded after exercise and  $\beta 2$ -AR agonist infusion, and its ability to ligate with the PVR and Nectin-2 expressed by leukemic targets, were directly involved in the anti-tumor response. These findings provide a mechanistic link between exercise,  $\beta 2$ -AR activation, and the manufacture of superior  $\gamma\delta$  T-cell products for adoptive cell therapy against hematologic malignancies (Supplementary Fig. S3).

Exercise has long been known to preferentially mobilize cytotoxic lymphocytes (e.g., NK-cells, CD8<sup>+</sup> T-cells,  $\gamma\delta$  T-cells) to the peripheral blood compartment which promptly exit the bloodstream upon cessation of exercise to survey peripheral tissues (37, 41). Recent work has shown that these mobilized cells subsequently infiltrate tumors and are essential in constraining tumor



**FIGURE 5** Systemic  $\beta$ -AR activation through either acute exercise or ISO infusion evoke similar transcriptomic and surface phenotype shifts among *ex vivo* expanded  $V\gamma 9V\delta 2+$  T-cells. **A**, The percentage of activating, inhibitory, and chemokine receptors among expanded  $V\gamma 9V\delta 2+$  T-cells before (REST) and during (EX) exercise or ISO infusion. ( $n = 8$ ). Statistical significance set as \*,  $P < 0.05$ . GSEA performed using KEGG and GO terms showing enriched pathways on expanded  $V\gamma 9V\delta 2+$  T-cells that were expanded after **(B)** exercise or **(C)** ISO infusion. Graphs show enriched upregulated (blue) and downregulated (red) pathways as well as the percentage of DEGs contributing to each mechanism. Statistical significance set as  $FDR \leq 0.25$ . Data are represented as mean.



**FIGURE 6** Interactions between DNAM-1 and PVR/Nectin-2 enhances the antileukemic activity of exercise expanded  $V\gamma 9V\delta 2+$  T-cells. **A**, Illustration of the receptors expressed on  $V\gamma 9V\delta 2+$  T-cells and their corresponding ligands expressed on cancer cells. **B**, Heat map of the surface expression and MFI of ligands and receptors on K562 cells. **C** and **D**, The specific lysis of K562 cells by expanded  $V\gamma 9V\delta 2+$  T-cells under two conditions: unstained control (-) and respective blocking antibody (+), DNAM-1, PVR, Nectin-2, TRAIL, TRAIL-R1, and TRAIL-R2 ( $n = 3$ ). Significant differences between the unstained (-) and blocking conditions (+) were indicated by \* and #, respectively. Data are represented as mean  $\pm$  SEM; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by repeated measures two-way ANOVA with Bonferroni *post hoc* test (C-D).

growth in several murine cancer models (42). We recently introduced the innovative concept that lymphocytes mobilized by exercise enhance the graft-versus-leukemia (GvL) effects of donor lymphocyte infusions (35). In addition, an exercise session can improve the *ex vivo* production and effectiveness of virus-specific T-cells,  $\gamma\delta$  T-cells, and tumor antigen-specific T-cells (43–45). Previously, we reported that exercise augmented the *ex vivo* expansion of  $\gamma\delta$  T-cells and increased their ability to kill leukemia, lymphoma, and multiple myeloma target cells *in vitro* (10). While an upregulation of the activating receptor NKG2D was found to play a mechanistic role in the increased cytotoxic effects against U266, blocking NKG2D had no effect against K562 (10). Here we found that the activating receptors NKG2D, DNAM-1, TRAIL, CD16, CD56, and CD161, as well as the chemokine receptors CXCR3 and CCR5, were elevated among  $\gamma\delta$  T-cells expanded after exercise. Furthermore, exercise-expanded  $\gamma\delta$  T-cells had a lowered expression of the inhibitory receptor NKG2A and a greater proportion of cells with an NKG2D+/NKG2A– phenotype, which is highly predictive of cytotoxic potential (46). Indeed, NKG2A has been touted as a potential candidate for immune checkpoint inhibition (46, 47). The upregulation of DNAM-1 on  $\gamma\delta$  T-cells expanded after exercise was found to play a mechanistic role in the increased killing of K562 target cells. We found that blocking known DNAM-1 ligands, PVR and Nectin-2, on leukemic targets also abrogated the enhanced effects of exercise on  $\gamma\delta$  T-cell cytotoxicity. We also observed enhanced cytotoxic activity of exercise-expanded  $\gamma\delta$  T-cells against ZOL sensitized K562, U266 and Daudi cells. Moreover, our RNA-seq studies revealed an enrichment of gene sets in exercise-expanded  $\gamma\delta$  T-cells associated with cytokine stimulus and secretion, intracellular and hormone-mediated signaling pathways, antigen presentation and processing, T-cell activation and NK cell-mediated cytotoxicity, among others. It was not surprising, therefore, to find that  $\gamma\delta$  T-cells expanded after exercise were more capable of restricting K562 leukemic growth in NSG-IL15 mice, particularly when mice received a single dose of ZOL. The enrichment of genes annotated to GO terms such as “cellular response to extracellular stimulus,” may explain why exercise-expanded  $\gamma\delta$  T-cells were highly responsive to ZOL stimulation both *in vitro* and *in vivo*. Collectively, these findings indicate that exercise-mobilized  $\gamma\delta$  T-cells develop different phenotypic and transcriptomic characteristics after *ex vivo* expansion, increasing their responsiveness to exogenous antigens and making them more potent killers of multiple hematologic tumors.

The mobilization of  $\gamma\delta$  T-cells and other effector lymphocytes to blood is largely driven by catecholamines acting on the  $\beta$ 2-AR, which is a G protein-coupled receptor that is highly expressed in circulating  $\gamma\delta$  T-cells (6, 8, 10). At the transcriptional level, we found an enrichment of gene sets involved in G protein-coupled receptor and TOR signaling within exercise-mobilized  $\gamma\delta$  T-cells, and, within  $\gamma\delta$  T-cells expanded after exercise, an upregulation of genes involved in cAMP signaling—the major downstream messenger molecule following  $\beta$ 2-AR activation. This indicates that exercise-mobilized  $\gamma\delta$  T-cells are highly sensitive to catecholamines and  $\beta$ 2-AR signaling both before and after *ex vivo* expansion. Our interpretations of the single-cell and bulk RNA-seq data were bolstered by our randomized placebo-controlled exercise trial and synthetic  $\beta$ -AR agonist infusion experiments in humans, which clearly demonstrated involvement of the catecholamine-  $\beta$ 2-AR signaling axis in the mobilization and expansion of  $\gamma\delta$  T-cells. First, we showed that a nonselective  $\beta$ 1+ $\beta$ 2-AR antagonist (nadolol), but not a selective  $\beta$ 1-AR antagonist (bisoprolol), administered prior to exercise blocked  $\gamma\delta$  T-cell mobilization to blood and the subsequent enhanced *ex vivo* expansion. Second, when we infused “resting” participants with ISO—a synthetic  $\beta$ 1+ $\beta$ 2-AR agonist with approximately

4-fold preference for the  $\beta$ 2-AR—for 20 minutes, we were able to expand  $\gamma\delta$  T-cells from the ISO-mobilized cells that displayed increases in potency and phenotypic and transcriptomic alterations akin to exercise (48). It is noteworthy that the  $\gamma\delta$  T-cells expanded after ISO infusion controlled leukemic growth in the NSG-IL15 mice receiving ZOL more consistently than those expanded after exercise, possibly due to ISO being infused at a precise dose and rate compared with the inherent variability in endogenous catecholamine responses to fixed intensity acute exercise.

There is increasing evidence that adrenergic signaling is playing a pivotal role in the anti-tumor immune effects provided by exercise. Intermittent “spikes” in catecholamines and other acute exercise factors, such as the muscle-derived cytokines IL6 and IL15, can recruit effector lymphocytes to the blood and facilitate their trafficking to tumors leading to enhanced tumor infiltration and suppression of tumor growth over time (49). In a murine model of melanoma, voluntary wheel running was shown to increase NK-cell tumor infiltration in a manner that was dependent on IL6 and inhibited with nonselective beta blockade (propranolol; ref. 42). More recently, exercise was found to mobilize and accumulate tumor infiltration of IL15-responsive CD8<sup>+</sup> T-cells in a murine model of pancreatic ductal adenocarcinoma, an effect that was also blocked with propranolol (50). A limitation of these studies is that only a nonselective  $\beta$ 1+ $\beta$ 2-AR antagonist (propranolol) was used, whereas we have excluded involvement of  $\beta$ 1-AR signaling in the mobilization and redistribution of  $\gamma\delta$  T-cells with exercise by assessing the effects of both selective and nonselective  $\beta$ -AR antagonists. We have also shown previously that virus-specific T-cells, which can exert cytotoxic functions against viral infected tumors (e.g., Epstein-Barr Virus EBV+ lymphoma), are also preferentially redeployed with exercise via the catecholamine-  $\beta$ 2-AR signaling axis (44). Collectively, these findings have identified the  $\beta$ 2-AR as a therapeutic target that can potentially be exploited to improve anti-tumor immunity, and to mobilize more effective lymphocytes to blood where they can be readily accessed for the *ex vivo* manufacture of more potent cell products for cancer therapy.

In future studies, it will be important to determine whether expanding  $\gamma\delta$  T-cells with IL2 and IL15 can further augment potency, as it was recently shown that ZOL with IL2 and IL15 enhanced the cytotoxic function of the final  $\gamma\delta$  T-cell product compared with IL2 alone (51). Moreover, it remains to be seen how repeated  $\gamma\delta$  T-cell transfers perform in leukemia-bearing mice and to test their potency against a range of hematologic malignancies *in vivo*, both with and without combination therapeutics (e.g., immune checkpoint inhibitors, mAbs) or genetic manipulation (e.g., addition of a chimeric antigen receptor) designed to increase anti-tumor immunity. A minor limitation is our study participants were young (22–41 years), physically active (5–7 PAR score), and aerobically fit ( $\dot{V}O_{2max}$ : 44.9  $\pm$  8.7 mL/kg/minute). It was shown recently that physical fitness is a major determinant of  $\gamma\delta$  T-cell responsiveness, with successful *ex vivo*  $\gamma\delta$  T-cell expansions occurring in 100% of physically active donors (physically active >4 days/week) compared with just 25% of sedentary donors (52). It is possible therefore that the augmenting effects of acute exercise on  $\gamma\delta$  T-cell expansion and potency will be more prominent in sedentary individuals, but this remains to be investigated.

We conclude that exercise-induced  $\beta$ 2-AR activation preferentially mobilizes  $\gamma\delta$  T-cells with an effector phenotype and transcriptomic profile to the peripheral circulation. This allows for the collection and generation of a more potent *ex vivo* expanded  $\gamma\delta$  T-cell product that is highly effective in killing a broad range of hematologic tumor cells *in vitro* and exert better control of K562

leukemia growth *in vivo*. These findings have significant translational potential for using exercise as a nontoxic and economical therapeutic strategy—or in repurposing existing drugs that target  $\beta$ 2-AR signaling—to augment the manufacture of therapeutic cell products for the treatment of refractory and relapsed hematologic malignancies.

## Authors' Disclosures

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## Authors' Contributions

**F.L. Baker:** Conceptualization, resources, data curation, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. **K.A. Smith:** Resources, data curation, investigation, writing-review and editing. **P.L. Mylabathula:** Resources, data curation, investigation, writing-review and editing. **T.M. Zúñiga:** Resources, data curation, investigation, writing-review and editing. **D.M. Diak:** Resources, data curation, investigation, writing-review and editing. **H. Batatinha:** Resources, data curation, investigation, writing-review and editing. **G.M. Niemi:** Resources, data curation, investigation, writing-review and editing. **M.D.**

**Seckeler:** Writing-review and editing, medical Monitoring. **C.R. Pedlar:** Methodology, writing-review and editing. **D.P. O'Connor:** Formal analysis, writing-review and editing. **J. Colombo:** Writing-review and editing, medical Monitoring. **E. Katsanis:** Conceptualization, supervision, funding acquisition, methodology, writing-review and editing. **R.J. Simpson:** Conceptualization, data curation, supervision, funding acquisition, visualization, methodology, writing-original draft, project administration, writing-review and editing.

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## Note

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