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Bone Marrow-derived CD8+ T Cells From Pediatric Leukemia Patients Express PD1 and Expand Ex Vivo Following Induction Chemotherapy

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Summary: Adoptive cell therapy (ACT) of chimeric antigen receptor T cells has demonstrated remarkable success for the treatment of pediatric B-cell leukemia. For patients who are not candidates for chimeric antigen receptor T-cell therapy, ACT using tumor antigen-experienced polyclonal T cells may be a treatment option. Since leukemic blasts reside in the bone marrow and bone marrow is a preferred site for homeostatic proliferation of cytotoxic memory $CD8^+$ T cells, we hypothesized that bone marrow would be a source of activated T cells. The aim of this study was to determine the feasibility of using bone marrow-derived T cells following post-induction chemotherapy for use in adoptive cell transfer. Matched patient samples of bone marrow and peripheral blood-derived T cells expanded ex vivo and displayed similar apoptotic profiles. Before activation and expansion, there was a significant increase in the percentage of bone marrow-derived $CD8^+$ T cells expressing activation markers PD1, CD45RO, and CD69 as compared with peripheral blood $CD8^+$ T cells. Considering, melanoma-reactive $CD8^+$ T cells reside in the subset of $PD1^+CD8^+$ T cells, the bone marrow may be an enriched source leukemic-specific T cells that can be used for ACT.

Key Words: $PD1^+$ T cells, bone marrow, adoptive cell therapy, pediatric leukemia

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During the past decade, immunotherapy has been a scientific breakthrough for the treatment of cancer. Adoptive cell therapy (ACT) using genetically modified T cells expressing chimeric antigen receptors (CAR) has demonstrated remarkable success in the treatment of pediatric B-cell leukemia with response rates as high as 70% to 90%.[1–3] However, there are subtypes of pediatric leukemia with unknown tumor antigens, or the targeted tumor antigens are not expressed and CAR cell therapy is not an option. Therefore, there remains a need to identify leukemia-specific T cells that can be used for ACT. For patients who are not candidates for CAR T-cell therapy, ACT using leukemia antigen-experienced polyclonal T cells (in combination with chemotherapy or other targeted therapies) may be an option.

The T-cell immune inhibitory receptor, PD1, is upregulated following T-cell receptor activation and remains elevated with chronic antigen stimulation.[4] Recently, melanoma-reactive $CD8^+$ T cells were found to reside in the subset of $PD1^+CD8^+$ melanoma-infiltrating lymphocytes.[5,6] Therefore, enrichment of $PD1^+$ T cells is a reasonable strategy to optimize the anticancer efficacy of ACT. Since bone marrow is a reservoir for leukemic blasts, we hypothesized that bone marrow-derived T cells would express PD1 and may serve a source of leukemia-reactive cells. The objective of this study was to phenotype bone marrow-derived T cells from pediatric leukemia patients to determine whether these T cells would be amenable for ACT. Since many leukemia patients are heavily pretreated with chemotherapy before immunotherapy, T cells were analyzed following induction chemotherapy. Using matched pairs of T cells obtained from bone marrow and peripheral blood, our results show the percentage of $CD8^+$ T cells expressing activation markers PD1, CD69, and CD45RO is significantly elevated in bone marrow as compared with peripheral blood. Importantly, bone marrow-derived T cells expanded ex vivo when obtained following induction chemotherapy.

MATERIALS AND METHODS

Patient Samples

Bone marrow and peripheral blood samples were obtained in accordance with guidelines set forth in the approved Children's Hospital of Wisconsin Institutional Review Board protocol, IBC20150066. Informed consent was obtained.

T-Cell Purification and Ex Vivo Expansion

Mononuclear cells were purified from bone marrow and peripheral blood using SepMate tubes (StemCell Technologies, Vancouver, Canada) according to the manufacturer's guidelines. Cells were activated once with anti-CD3 and anti-CD28-conjugated magnetic Dynabeads (Thermo Fisher Scientific, Carlsbad, CA) at a 1:1 ratio of cells to beads. Cells were cultured in RPMI 1640 media (Gibco) with 20 U/mL interleukin 2 (IL2), 5 ng/mL IL7, and 5 ng/mL IL15 for 9 to 12 days. Media and cytokines were replaced every 2 to 3 days during culture.

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Flow Cytometry

Cells were stained with antibodies to T-cell markers: CD3 (UCHT1), CD4 (OKT4), CD8a (SK1); inhibitory receptors: CD279 (PD1, clone J105) and CD223 (LAG3, clone 17B4); activation markers: CD69 (FN50), CD45RO (UCHL1), and memory markers: CD197 (CCR7, clone 3D12), CD122 (mikβ3), and CD127 (ebioRDR5). Cells were stained with 7-AAD viability dye and gated on live cells. Flow cytometric analysis was performed on a BD Biosciences LSRII (Franklin Lakes, NJ) flow cytometer, and resulting data analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Apoptosis

The Caspase-Glo 3/7 assay (Promega, Madison, WI) was used according to the manufacturer's protocol. Cells were assayed between 9 and 12 days of ex vivo expansion. Thereafter, 4 μM camptothecin was added to cells for 2.5 hours as a positive control.

RESULTS

Following Induction Chemotherapy, Bone Marrow-derived CD8+ T Cells Have an Activated Phenotype

Bone marrow and peripheral blood samples were obtained from pediatric leukemia patients following one month of induction chemotherapy. Induction chemotherapy included vincristine, pegaspargase, dexamethasone, and daunorubicin with a subset of patients also receiving bortezomib. Table 1 shows patient clinical descriptors. Since bone marrow is a preferred site for homeostatic proliferation of cytotoxic memory $CD8^+$ T cells,[7,8] it was of interest to determine if activated bone marrow-derived T cells would be present and able to expand ex vivo following induction chemotherapy. This was particularly relevant since there is an anticipated need to obtain T cells for ACT in patients following chemotherapy. Patient matched bone marrow and peripheral blood buffy coats were stained with antibodies and analyzed for T-cell subsets by flow cytometry. For patients 006, 008, and 010, the bone marrow sample was not adequate to be used for flow cytometric analysis. There was a significant ($P < 0.05$) difference in the percentage of bone marrow-derived $CD8^+$ T cells expressing activation markers PD1, CD45RO, and CD69 as compared with peripheral blood $CD8^+$ T cells (Fig. 1A). This difference was not observed for $CD4^+$ T cells (Fig. 1B). Figure 1C shows expression of the activation markers relative to each other on bone marrow-derived $CD8^+$ T cells. There was no difference in the expression of memory markers, CD197 (CCR7), CD122, or CD127 on $CD4^+$ and $CD8^+$ T cells obtained from the bone marrow and peripheral blood when analyzed before activation and expansion (data not shown).

Following Ex Vivo Expansion, PD1 and LAG3 are Upregulated on CD4+ and CD8+ T Cells Obtained From Bone Marrow and Peripheral Blood

To demonstrate the ability to expand T cells ex vivo, cells were activated once with anti-CD3/CD28-conjugated beads and cultured in the presence of low dose IL2 (20 U/mL), IL7 (5 ng/mL), and IL15 (5 ng/mL) for 9 to 12 days. Although there were individual differences in the ability of bone marrow-derived T cells to expand, there was >10-fold expansion in 8 of 10 bone marrow samples tested (Fig. 2A). There was significantly ($P < 0.05$) greater fold expansion of peripheral blood-derived T cells as compared with bone marrow-derived T cells. When tested for apoptosis postexpansion, there was no difference between bone marrow and peripheral blood-derived T cells (Fig. 2B). Importantly, Figure 2C shows that despite a low postinduction chemotherapy white blood count in patients 003, 004, and 010, their bone marrow-derived T cells expanded >25-fold. These data show that in the majority of patients tested, bone marrow-derived T cells expanded ex vivo and had an apoptotic profile similar to peripheral blood-derived T cells.

Prior to ex vivo expansion, the CD4:CD8 ratio was ~1:1 for both peripheral blood and bone marrow-derived T cells (Fig. 2D). Following ex vivo expansion, the CD4:CD8 T-cell ratio was 1:4 regardless of the T-cell source. These data suggest $CD8^+$ T cells have a greater ex vivo expansion potential than $CD4^+$ T cells. Following ex vivo expansion, T cells were analyzed for expression of checkpoint receptors PD1 and LAG3. Regardless of the T-cell source, there was a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells expressing PD1 and LAG3 (Fig. 2E). An increase in the percentage of T cells expressing PD1 and LAG3 was not surprising as activated or chronically stimulated T cells upregulate coinhibitory molecules.[9]

TABLE 1. Clinical Characteristics and Disease Burden of Pediatric Leukemia Patients

		Differential				
		Baseline		Day 29 (Post-induction Chemotherapy)		
Patient Number	Diagnosis	BM blasts (%)	PB blasts (%)	BM blasts (%)	PB blasts (%)	White Blood Count (WBC) (10^3/μL)
001	B-ALL/T-ALL	ND	96	4 myeloblasts	0	3.7
002	B-ALL	80	1	0	0	10.7
003	B-ALL	97	2	0	0	0.5
004	B-ALL	96	73	1 myeloblasts	ND	0.8
005	B-ALL	80	59	0	0	8.7
006	T-ALL	86	3	0	0	1.4
007	B-ALL	94	5	1 lymphoblasts1 myeloblasts	0	18.1
008	B-ALL	57	0	0	0	7.2
009	B-ALL	91	53	0	0	4.4
010	T-ALL	81	83	0	0	0.3

ALL indicates acute lymphocytic leukemia; BM, bone marrow; ND, not determined; PB, peripheral blood.

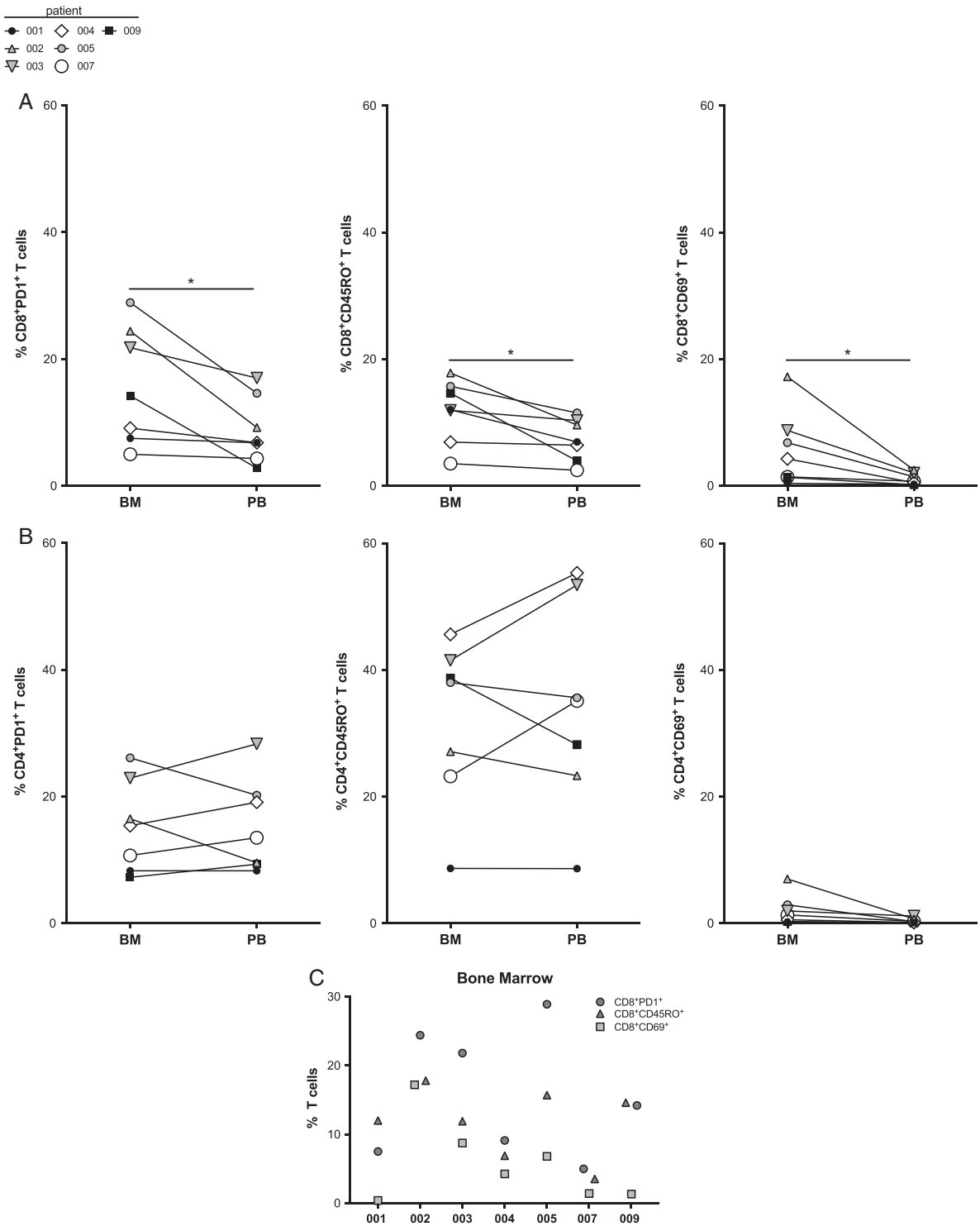

FIGURE 1. T-cell subsets in bone marrow and peripheral blood of pediatric patients following postinduction chemotherapy. Percentage of PD1+, CD69+, and CD45RO+ CD8+ T cells (A) and CD4+ T cells (B) in BM and PB. C, The expression of PD1, CD69, and CD45RO in individual patients. *$P < 0.05$ as calculated by Wilcoxon matched pairs signed rank test. BM indicates bone marrow; PB, peripheral blood.

DISCUSSION

Following induction chemotherapy for pediatric acute lymphocytic leukemia, the bone marrow was shown to have a greater percentage of PD1$^+$CD8$^+$ T cells than peripheral blood. There is evidence that CD8$^+$ T cells expressing PD1 contain the subset of T cells that are functional against cancer.[5,6] In murine models of multiple myeloma and acute myeloid leukemia, our own data has demonstrated PD1 as a

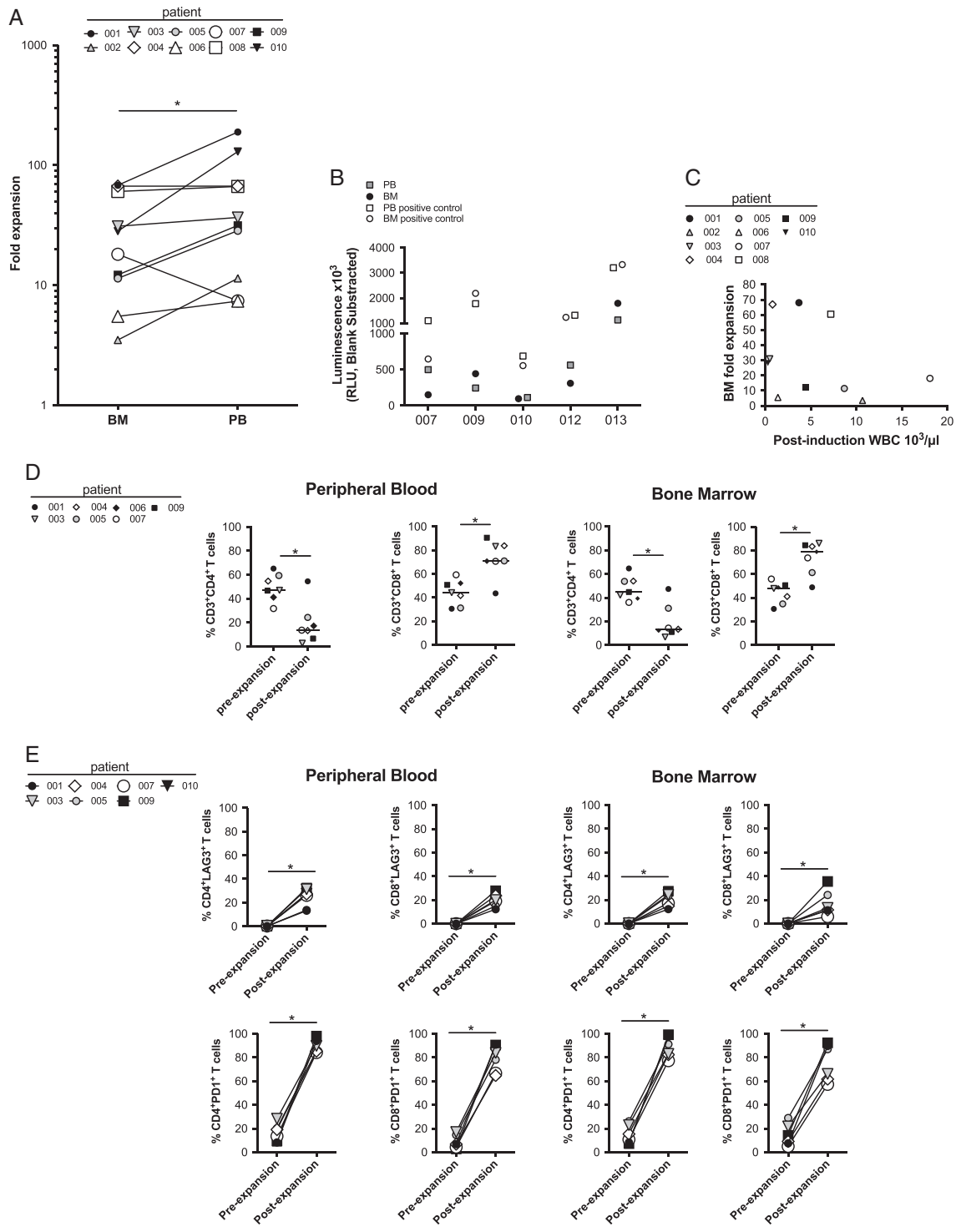

FIGURE 2. Fold expansion, apoptosis, and checkpoint receptor expression on ex vivo expanded T cells. A, Comparison of fold expansion between BM-derived and PB-derived T cells. B, Caspase 3/7 apoptosis of BM-derived and PB-derived T cells. C, Correlation of post-induction chemotherapy complete white blood cell count and fold expansion of BM-derived T cells. D, Percentage of $CD4^+$ and $CD8^+$ T cells present in the PB and BM pre-activation and expansion (pre-expansion) and post-activation and expansion (post-expansion). E, PD1 and LAG3 checkpoint receptor expression pre-expansion and post-expansion. *$P < 0.05$ as calculated by Wilcoxon matched pairs signed rank test. BM indicates bone marrow; PB, peripheral blood.

biomarker for functional leukemia-reactive T cells that are effective against leukemia in vivo when administered as ACT.[10] Therefore, PD1 expression on T cells obtained from pediatric leukemia patients may be a marker of leukemia reactivity. Despite a greater percentage of $PD1^+$ T cells in the bone marrow, it is important to note that $PD1^+$ T cells were also present in peripheral blood. It is possible that both bone marrow and peripheral blood-derived $PD1^+CD8^+$ T cells have anti-leukemia reactivity. Peripheral blood-derived T cells are more easily obtained and have greater expansion potential than bone marrow-derived T cells, thus peripheral blood-derived T cells enriched for PD1 could also be a source of anti-leukemic cells.

Following ex vivo expansion there was a significant increase in the percentage of T cells expressing PD1 and LAG3. In a murine model of ovarian cancer, PD1 and LAG3 were shown to physically associate, traffic to the immunologic synapse and synergize to dampen T-cell signaling.[11] Blockade of LAG3 and PD1 pathways in preclinical cancer models has had synergistic effects increasing the antitumor $CD8^+$ T-cell response.[12] These data suggest that administration of anti-PD1 and anti-LAG3 blocking antibodies in combination with ACT of T cells expressing PD1 and/or LAG3 is a reasonable approach to facilitate the antitumor efficacy of adoptively transferred T cells.

There are multiple variables to consider when producing effective antileukemia T cells for ACT. During induction chemotherapy, patients are treated with multiple drugs and the direct effects (eg, induction of apoptosis) or indirect effects (eg, elimination of leukemia blast cells or lymphopenia[13]) of these therapies on T-cell activation, ex vivo proliferation and effector responses is largely unknown. It has been reported that bortezomib (a proteasome inhibitor) augments $CD8^+$ T-cell effector function.[14] However, it has also been reported that bortezomib induces apoptosis of activated T cells.[15] It would be of interest to investigate the effects of bortezomib and other chemotherapeutics on ex vivo T-cell expansion and anti-leukemia effector function.

How best to ex vivo activate and expand T cells to produce durable and effective anti-leukemia T cells is also under investigation. In this study, low dose IL2 (20 U/mL) with 5 ng/mL IL7 and 5 ng/mL of IL15 was used to support lymphocyte proliferation. The influence of cytokines and culture conditions on differentiation or proliferation of T-cell subsets requires further study.

In summary, we have shown that peripheral blood and bone marrow-derived T cells can be expanded ex vivo when obtained from pediatric leukemia patients following induction chemotherapy. There is a greater percentage of $PD1^+$ T cells in the bone marrow as compared with peripheral blood. The bone marrow is a reservoir for leukemic blasts, thus PD1 expression may be a marker of activated leukemia-experienced T cells. However, a comparison of the anti-leukemia specificity (against patient leukemia blast cells) of bone marrow and peripheral blood-derived $CD8^+$or $CD8^+PD1^+$ T cells is required to determine the best personalized source of anti-leukemic T cells to be used for ACT.

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