Highlights:

- CD14+ depletion allows T cell expansion from cryopreserved G-CSF-stimulated PBSCs
- Naïve and central memory T cell precursors are present in cryopreserved PBSC units
- CAR-T cells generated from cryopreserved PBSC units displayed anti-tumor activity
- Cryopreserved PBSC units can serve as an alternative source for CAR-T cell products
- Use of stored apheresis units can extend the options for heavily treated patients
Manufacture of CAR-T Cells from Mobilized Cyropreserved Peripheral Blood Stem Cell Units Depends on Monocyte Depletion

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Short title
Frozen PBSC units as CAR-T cell source

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Abstract

Cytotoxic chemotherapy and radiation can render lymphocyte repertoires qualitatively and quantitatively defective. Thus, heavily treated patients are often poor candidates for the manufacture of autologous CAR-T cell products. In the USA and Europe, children with high-risk neuroblastoma undergo apheresis early in the course of their treatment to collect peripheral blood stem cells (PBSCs) for cryopreservation in preparation for high-dose chemotherapy followed by autologous stem cell rescue. Here, we investigate whether these cryopreserved chemotherapy and G-CSF-mobilized PBSCs can be utilized as starting material for CAR-T cell manufacturing. We evaluated T cell precursor subsets in cryopreserved PBSC units from 8 patients with neuroblastoma using FACS-based analysis. Every cryopreserved unit collected early in treatment contained both CD4 and CD8 precursors with significant numbers of naïve and central memory precursors. Significant numbers of Ki67+/PD1+ T cells were detected, presumably the result of chemotherapy-induced lymphopenia and subsequent homeostatic proliferation. Cryopreserved PBSC units containing from 56-112x10^6 T cells were amenable to immunomagnetic selection, CD3x28 bead activation, lentiviral transduction, and cytokine-driven expansion, provided that CD14 monocytes were depleted prior to the initiation of cultures. Second and third generation CD171-CAR+ CD4 and CD8 effector cells derived from cryopreserved units displayed anti-neuroblastoma lytic potency and cytokine secretion comparable to those derived from a healthy donor, and mediated in vivo anti-tumor regression in NSG mice. We conclude that cryopreserved PBSCs standardly procured during early treatment can serve as an alternative
starting source for CAR-T cell manufacturing, extending the options for heavily treated patients.

Keywords: Autologous cryopreserved G-CSF-mobilized PBSCs, CAR-T cell manufacturing, CD14 depletion

Introduction

The robust clinical activity of CD19-specific CAR-T cell immunotherapy in the treatment of B-cell malignancies has generated interest in developing similar strategies for solid tumor immunotherapy. Neuroblastoma is the most common extracranial malignant solid tumor in children, arising from the sympathoadrenal lineage of the neural crest. Treatment protocols for high-risk neuroblastoma in the USA and Europe currently include the harvest of granulocyte colony stimulating factor (G-CSF)-mobilized apheresis products, which are intended for autologous rescue following myeloablative therapy. Although polychemotherapy, autologous stem cell transplant, anti-GD2 monoclonal antibody and retinoic acid regimens induce initial remissions in a majority of patients, high-risk neuroblastoma frequently relapses as highly resistant disease demonstrating the urgent need for additional consolidative modalities, such as CAR-T cell immunotherapy.

We previously defined CD171 (also known as L1CAM) as a target antigen for CAR-T cell therapy in pediatric neuroblastoma and have defined second and third generation CAR constructs that selectively target a subset of CD171 molecules enriched on tumor cells based on aberrant glycosylation. CD171 is a homotypic adhesion molecule that contributes to the malignant biology of solid tumors, and, is ubiquitously expressed in a homogeneous pattern by
neuroblastoma at the time of diagnosis and after relapse.\textsuperscript{6-9} A Phase I clinical trial in children with refractory or relapsed neuroblastoma has been initiated and the tolerability of defined CD4/CD8 T cell composition CD171-CAR-T cell products is being studied (Clinical Trial.Gov identifier NCT02311621; IND FDA#16139).\textsuperscript{6}

Pediatric neuroblastoma patients are intensively treated with upfront cytotoxic regimens, and upon relapse frequently receive I-131 MIBG radiotherapy in addition to additional rounds of chemotherapy.\textsuperscript{10} The T lymphocyte pool in refractory patients is damaged and depleted making these patients a challenge in CAR-T cell trials that perform apheresis upon trial enrollment.\textsuperscript{11-13} PBSC harvesting after initial chemotherapy cycles is standard of care in many centers, and because patients typically have more PBSC units cryopreserved than is required for the consolidative autologous transplant, we sought to assess the feasibility of manufacturing CAR-T cell products from this starting material. While the successful generation of virus-specific T cells from fresh, G-CSF-mobilized apheresis products obtained from healthy donors has been previously described\textsuperscript{14-16}, the generation of tumor-specific CAR-T cells from cryopreserved G-CSF-mobilized apheresis products obtained from cancer patients is novel. Furthermore, the high myeloid cell content in these G-CSF-mobilized units has created the general belief in the field that T cells derived from G-CSF-mobilized products are incapable of proliferation.\textsuperscript{17} Here, we demonstrate that PBSC products are replete with naïve and central memory precursors that are, after myeloid cell depletion, amenable to isolation, activation, transduction and expansion into functional CAR effector cells.
Material and Methods

Retrospective patient samples

Research followed the tenets of the Declaration of Helsinki, and was approved by the institutional review board of Seattle Children’s Hospital (IRB#13740). The study utilized apheresis products from 8 patients diagnosed with neuroblastoma and treated between 2000 and 2013 in the Department of Pediatric Hematology-Oncology of Seattle Children’s Hospital. All human participants gave written informed consent. Blood samples drawn from healthy donors (HD) served as controls.

CAR construction and lentiviral production

CD171-specific CARs used herein were previously described. Briefly, the CE7 mAb scFv was codon optimized and linked to a 12 AA (“hinge-only”) spacer derived from human IgG4-Fc followed by the transmembrane domain of human CD28 and by signaling modules comprising either (i) the cytoplasmic domain of 4-1BB alone (2nd generation CAR) or (ii) of CD28 (mutant) and 4-1BB (3rd generation CAR). The cDNA clones encoding CAR variants were linked to a downstream T2A ribosomal skip element and truncated EGFR receptor (EGFRt) and cloned into the epHIV7 lentiviral vector. The lentiviral vectors were produced at the Center for Biomedicine and Genetics at City of Hope (COH) in Duarte, CA under current good manufacturing practices (cGMP) (BB-MF 13830 – Lentiviral Vector Manufacturing and Testing – City of Hope).

T cell culture

Frozen apheresis products from neuroblastoma patients were thawed in Normosol R + 10% human albumin. Peripheral blood mononuclear cells
(PBMCs) were isolated by standard protocol using Ficoll-Paque (GE Healthcare) either from thawed patients' apheresis products or from a fresh collected healthy donor apheresis product. Cells were then used for flow cytometry staining or, after CD14 depletion using CD14 magnetic beads (Miltenyi), stimulated with anti-CD3 and anti-CD28 beads (TransAct®, Miltenyi) and cultured in X-Vivo media (Lonza) supplemented with 10% fetal bovine serum (FBS; Atlas), recombinant human Interleukin (IL)2 (50 U/ml) and IL15 (0.5 ng/μl). Transduction was performed one day following activation by centrifugation at 800g for 30 minutes at 32°C with lentiviral supernatant (multiplicity of infection [MOI] = 0.25 - 1) supplemented with 1 mg/mL protamine sulfate (APP Pharmaceuticals). Approximately 10 days later, EGFRt+ T cells were enriched by immunomagnetic selection with biotin-conjugated Erbitux (Bristol-Myers-Squibb) and streptavidin-microbeads (Miltenyi) as previously described. At the end of the stimulation cycle, cells were cryopreserved in aliquots as cell bank and thawed subsequently for in vitro and in vivo experiments.

Further expansion of CAR-T cells was carried out by co-culture with irradiated PBMCs and EBV-transformed lymphoblastoid cell lines (TM-LCLs) with OKT3 (30ng/ml) in RPMI media (Gibco) supplemented with 10% FBS (HyClone) and IL15 (0.5 ng/ml) plus IL2 (50 U/ml) for CD8+ cells, and IL15 (0.5 ng/ml) plus IL7 (5 ng/ml) for CD4+ cells. In vitro experiments were performed on day 11 of expansion cultures. For in vivo experiments cells were expanded until day 13, cryopreserved in aliquots as cell banks and thawed prior to injection.

Cell lines
The neuroblastoma cell lines SK-N-BE(2) and SK-N-DZ were obtained from the American Type Culture Collection (ATCC). The IL2 secreting, firefly luciferase (ffLuc) expressing SK-N-DZ was generated as previously described. All neuroblastoma cell lines were cultured in DMEM (Cellgro) supplemented with 10% FBS and 2mmol/L L-glutamine.

**Protein expression**

Western Blot. T cells were harvested, washed twice in PBS and lysed in RIPA lysis buffer containing protease inhibitor (Millipore). Proteins were analyzed using SDS/PAGE followed by Western blotting using anti-CD247 (CD3-ζ, BD Biosciences), according to manufacturer’s instructions. Protein bands were detected using an Odyssey Infrared Imager (LI-COR).

Flow Cytometry. Immunophenotyping was conducted with fluorophore-conjugated mAbs: CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD27, CD28, CD45RA, CD45RO, CD56, CD62L, CD69, CD123, CD127, Ki67, TIM-3, PD1 (BD Biosciences and Biolegend) and LAG3 (R&D Systems). Dead cells were excluded from analysis using a fixable viability stain (BD Biosciences). Cell surface expression of CD171 was analyzed using a fluorophore-conjugated mAb (Clone 014, Sino Biological) or the biotinylated CE7 mAb with a fluorophore-conjugated streptavidin secondary reagent. EGFRt expression was analyzed using a fluorophore-conjugated Cetuximab (Bristol-Myers-Squibb and BD biosciences). Flow analyses were performed on an LSRFortessa (BD Biosciences) and data were analyzed using FlowJo software (Treestar).

*In vitro T cell assays*
Cytotoxicity measured by Chromium Release Assay (CRA). Target cells were labeled with $^{51}$Cr (Perkin Elmer), washed and incubated in triplicate at 5x10$^3$ cells per well with T cells at various effector to target (E:T) ratios. Supernatants were harvested after a 4-hour incubation for γ-counting using Top Count NTX (Perkin Elmer) and specific lysis was calculated as previously described.$^{19}$

Cytokine release. A total of 5x10$^5$ T cells were plated with stimulator cells at an E:T ratio of 2:1 for 24 hours. Interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and IL2 in the supernatant were measured using Bio-Plex cytokine assay and Bioplex-200 system (Bio-rad Laboratories).

*In vivo* experiments

NOD/SCID/γc-/- mice. NSG mouse tumor model was conducted under the SCRI IACUC approved protocol #13853. Adult male NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ [NOD scid gamma(NSG)] mice were obtained from the Jackson Laboratory or bred in-house. Mice were injected intracranially (i.c.) on day 0 with 2x10$^5$ IL2 secreting, ffLuc expressing SK-N-DZ tumor cells 2mm lateral, 0.5mm anterior to the bregma and 2.5mm deep to the dura. Mice received a subsequent intra-tumoral injection of 2x10$^6$ mock transduced or CD171-CAR-modified T cells seven days later. For bioluminescent imaging of tumor growth, mice received intra-peritoneal (i.p.) injections of D-luciferin (Perkin Elmer; 4.29mg/mouse). Mice were anesthetized with isoflurane and imaged using an IVIS Spectrum Imaging System (Perkin Elmer) 15 minutes after D-luciferin injection. Photon flux was analyzed within regions of interest using Living Image Software Version 4.3 (Perkin Elmer).

Statistical analyses
Statistical analyses were conducted using Prism Software (GraphPad). Data are presented as means ± standard deviation (SD). Means of all groups were compared for statistical differences by Student’s t test or for statistical analyses of survival by log-rank testing. Results with a P value less than 0.05 were considered significant.

Results

T cell composition of patient PBSC units.

The T cell populations in patient derived cryopreserved G-CSF-mobilized PBSCs obtained early during the treatment protocol for high-risk neuroblastoma (Figure 1) were examined by multiparameter FACS analysis. We investigated cryopreserved samples from 8 patient PBSC units with high-risk neuroblastoma enrolled in COG neuroblastoma trials and treated at our Seattle Children’s Hospital.20,21 We analyzed mononuclear cells (MNCs) including T cell subsets and markers for T cell exhaustion and T cell activation status (gating strategies for FACS analysis are shown in Supplemental Figures 1&2). The T, B, NK and dendritic cell populations were heterogeneous between different patients. CD3+ T cells comprised 23.3% (mean, range=12.9-44.3%) of MNCs (Table 1), with the majority expressing the CD8 marker for T cells (mean=63.9%, range=34.6-88.7%; Figure 2A). The majority of CD3+ T cells from the 5 healthy donors, in contrast, expressed CD4 (mean=64.8%, range=60.3-71.4%). Frequencies of T cells displaying a central memory phenotype (T_{CM}, defined as CD45RO+CD62L+) or naïve phenotype (T_{N}, defined as CD45RO−CD62L+) were lower in pediatric patients than in adult healthy donors (patients: CD4+T_{CM}+ mean=15.4%, range=3.4-46.4%; CD8+T_{CM}+ mean=7.9%, range=0.2-16.0%; CD4+T_{N}+ mean=15.6%,
range=0.5-39.8%; CD8⁺T₉⁺ mean=17.4%, range=1.4-44.0%). Most patient T cells were effector memory (Tₑₑₑₑ, defined as CD45RO⁺CD62L⁻; CD4⁺Tₑₑₑₑ⁺ mean=36.3%, range=17.7-67.3%; CD8⁺Tₑₑₑₑ⁺ mean=30.6%, range=5.7-43.1%) or effector T cells (Tₑₑₑₑ, defined as CD45RO⁺CD62L⁻; CD4⁺Tₑₑₑₑ⁺ mean=32.7%, range=7.3-63.8%; CD8⁺Tₑₑₑₑ⁺ mean=44.1%, range=26.0-77.8%; Figure 2B). Patient products contained only few activated T cells (<15% CD25⁺ or CD69⁺), however both CD4 and CD8 T cells in the products were proliferating (CD4⁺Ki67⁺ mean=39.3%, range=9.4-63.3%; CD8⁺Ki67⁺ mean=53.3%, range=5.5-88.2%; Figure 2C). In contrast to healthy donors, high frequencies of CD4⁺ and CD8⁺ patient T cells expressed the inhibitory receptor programmed cell death protein 1 (PDCD1, also known as PD-1; Patients: CD4⁺PD1⁺ mean=53.3%, range=22.7-97.4%; CD8⁺PD1⁺ mean=49.6%, range=18.5-74.7%; Healthy donors: CD4⁺PD1⁺ mean=3.0%, range=0.9-5.7%; CD8⁺PD1⁺ mean=3.6%, range=1.3-6.2%; Figure 2D). Some patient CD8⁺ cells additionally expressed Hepatitis A virus cellular receptor 2 (HAVCR2, also known as TIM-3; CD8⁺TIM-3⁺ mean=15.1%, range=1.9-36.9%), consistent with previously described markers of T cells undergoing lymphopenic proliferation rather than functional exhaustion (Figure 2D).²² Consistent with this, T cells co-expressing LAG3, a marker of exhaustion, (CD4⁺LAG3⁺ mean=2.3%, range=0.8-4.7%; CD8⁺LAG3⁺ mean=0.8%, range=0.3-1.5%) were rare.

Monocyte depletion of thawed PBSC units is required for subsequent T cell activation and proliferation.

Initial attempts to thaw and expand T cells present in cryopreserved G-CSF-stimulated PBSCs failed because of the outgrowth of monocytes.
Monocytes outnumbered lymphocytes by >4-fold (range=4.2 – 20.2) in 6 of the 8 cryopreserved samples, while there were more lymphocytes than monocytes in healthy donor derived non G-CSF-stimulated PBSCs (Figure 3A).23,24 Following CD14 immunomagnetic depletion leading to a near 100% CD14 negative population, CD3x28 bead activated CD4 and CD8 T cells could be expanded more than 2-fold within 9 days of culture while numbers of CD3x28 bead activated CD4 and CD8 T cells in non depleted preparations decreased (Figure 3B). Therefore, we established a manufacturing protocol containing magnetic CD14 depletion in order to eliminate the growth-inhibiting monocytes before T cell activation (Figure 3C). Mimicking the GMP manufacturing process used in the ongoing ENCIT-01 clinical trial, cells were transduced on day 1 with clinical grade lentiviral vector containing either the 2nd or 3rd generation CD171-CAR transgene. CAR-T cell products manufactured from 3 unique G-CSF-stimulated apheresis products (collected from 3 neuroblastoma patients) exhibited 7.9- to 26.3-fold expansion (Figure 3D) achieving T cell numbers necessary for clinical trials administering up to 1x10^7 CAR-T cells/kg body weight. The transduction efficiency for the CD4^+ T cells ranged from 59.6-74.7% for the 2nd generation CAR-T cell products with a mean of 64.8% and from 52.8-78.8% for the 3rd generation CAR-T cell products with a mean of 66.4%. The transduction efficiency for the CD8^+ T cells ranged from 61.0-72.8% for the 2nd generation CAR-T cell products with a mean of 65.4% and from 71.1-73.0% for the 3rd generation CAR-T cell products with a mean of 72.1% (Figure 4A). On day 14 of ex vivo culture, CAR-T cell products displayed a viability >80% and were enriched for homogeneous levels of EGFRt expression by cetuximab immunomagnetic positive selection.18 Following EGFRt selection and thus enrichment of CAR-
expressing T cells, the viability dropped for both 2nd and 3rd generation CAR-T cell products from patient #1 requiring a dead cell removal using Ficoll gradient centrifugation (Figure 4B). The 2nd and 3rd generation vectors performed equally well in CD4+ or CD8+ T cell subsets, using T cell expansion, transduction efficiency and viability as endpoints. Protein expression of the CAR variants was confirmed by Western blotting for CD3ζ (Figure 4C). Using the same transduction and expansion strategy, we also generated control CD171-CAR-T cells obtained from a fresh healthy donor apheresis product.

CAR-T cells generated from cryopreserved patient-derived PBSC products display anti-tumor activity in vitro and in vivo.

Cryopreserved CD171-CAR-T cells were thawed and analyzed for viability using trypan blue staining. All CD171-CAR-T cell products displayed a viability >60% after thaw meeting GMP release criteria (Supplemental Figure 3A). The phenotypic classification of the final CD171-CAR-T cell products generated from cryopreserved G-CSF-stimulated PBSC apheresis products was analyzed using FACS-based analysis. The 3rd generation CAR-T cell products from all 3 patients contained numerically higher amounts of CD8+ T cells than the 2nd generation CAR-T cell product (2nd generation CD8+ T cells mean=32.2%, range=29.0-36.7%, 3rd generation CD8+ T cells mean=48.6%, range=39.2-58.6%, p=0.06; Supplemental Figure 3B) but the difference did not reach statistical significance. We detected wide ranges of expression levels of CD45RO and CD62L between the manufactured products compared to the starting apheresis specimens. CD45RO and CD62L are two commonly used surface markers to define T cell subsets displaying a naïve-, effector-,
effector memory- or central memory-like phenotype. CAR-expressing CD4+ T cells displayed a 1.8-fold increase (2nd generation) and a 2-fold increase (3rd generation) in CD45RO+CD62L+ cells compared to the starting material (CD45RO+CD62L+ mean in starting material=21.5%, range=3.4-46.4%; CD45RO+CD62L+ mean in manufactured 2nd generation product=38.4%, range=32.9-43.2%; CD45RO+CD62L+ mean in manufactured 3rd generation product=42.5%, range=27.8-58.3%) while the CAR-expressing CD8+ T cells displayed a 1.3-fold increase (2nd generation) and a 0.1-fold decrease (3rd generation) in CD45RO+CD62L+ expression (CD45RO+CD62L+ mean in starting material=10.0%, range=0.8-16.0%; CD45RO+CD62L+ mean in manufactured 2nd generation product=13.1%, range=4.6-18.2%; CD45RO+CD62L+ mean in manufactured 3rd generation product=8.7%, range=4.8-13.4%). In general, the CAR-expressing CD8+ T cells remained CD45RO+CD62L- (2nd generation: 1.1-fold increase, 3rd generation: 0.2-fold decrease with CD45RO+CD62L- mean in starting material=25.6%, range=8.8-34.6%; CD45RO+CD62L- mean in manufactured 2nd generation product=26.8%, range=12.4-38.0%; CD45RO+CD62L- mean in manufactured 3rd generation product=20.0%, range=11.6-28.3%) and CD45RO’CD62L- (2nd generation: 1-fold increase, 3rd generation: 1.1-fold increase with CD45RO’CD62L- mean in starting material=45.7%, range=26.0-77.8%; CD45RO’CD62L- mean in manufactured 2nd generation product=44.2%, range=28.7-73.9%; CD45RO’CD62L- mean in manufactured 3rd generation product=48.8%, range=30.2-64.9%) following ex vivo culture (Figure 5A). Cytolytic activity was determined in vitro using 4-hour chromium and 24-hour cytokine release assays and in vivo using a xenograft mouse model. Both 2nd and 3rd generation CAR-T cells resulted in lysis of CD171+ neuroblastoma
target cell lines; although higher lysis was observed following exposure to 3rd
generation CAR-T cells (50:1 E:T ratio: lysis of 2nd generation CAR-T cells=21.8%, range=10.3-32.8%; lysis of 3rd generation CAR-T cells=36.7%, range=21.2-44.8%; p<0.0001; Figure 5B). Both patient derived 2nd and 3rd
generation CAR-T cells released IFNγ (2nd generation: mean=367.8 pg/ml, range=268.8-466.3 pg/ml; 3rd generation: mean=918.8 pg/ml, range=755.7-1192.6 pg/ml), IL2 (2nd generation: mean=49.7 pg/ml, range=37.3-68.7 pg/ml; 3rd generation: mean=66.9 pg/ml, range=33.3-115.8 pg/ml) and TNFα (2nd generation: mean=658.8 pg/ml, range=433.3-1024.7 pg/ml; 3rd generation: mean=509.0 pg/ml, range=173.5-962.5 pg/ml) during co-culture with CD171+ tumor cells with a significant difference between 2nd and 3rd generation CAR-T cells for IFNγ (p<0.0001; Figure 5C).

To assess anti-tumor activity in vivo, we performed adoptive transfer
experiments in NSG mice with established human neuroblastoma xenografts
stereotactically implanted in the cerebral hemisphere (Figure 6A). All SK-N-DZ tumor–engrafted mice treated with intratumoral injection of 2x10^6 2nd or 3rd
generation CD171-CAR-T cells generated from patient derived G-CSF-
stimulated apheresis products demonstrated tumor regression and prolonged
survival compared to non-CAR expressing negative mock control T cells (2nd
generation: p<0.0001 and 3nd generation: p=0.0001; Figures 6B&C).

Following cell expansion, both, CD4+ and CD8+ CD171-CAR-T cells
developed increased expression of the inhibitory receptors LAG3 and TIM-3
compared to the starting material (CD4+LAG3+ T cells: 2nd generation: 13.6-
fold increase, mean=18.0%, range=15.8-20.0%, 3rd generation: 17.3-fold
increase, mean=22.9, range=20.5-26.3; CD8+LAG3+ T cells: 2nd generation:
6.7-fold increase, mean=5.3%, range=3.0-7.3, 3rd generation: 7.7-fold
increase, mean=6.2%, range=5.2-7.3%; CD4^+TIM-3^+ T cells: 2\textsuperscript{nd} generation: 94.4-fold increase, mean=72.1%, range=64.4-79%, 3\textsuperscript{rd} generation: 93.0-fold increase, mean=71.0%, range=60.3-86.5%; CD8^+TIM-3^+ T cells: 2\textsuperscript{nd} generation: 10.7-fold increase, mean=89.0%, range=84.2-92.2%, 3\textsuperscript{rd} generation: 10.6-fold increase, mean=87.8%, range=84.0-90.1%), while only the CD4^+ cells had an increased expression of PD1 (CD4^+PD1^+ T cells: 2\textsuperscript{nd} generation: 1.1-fold increase, mean=61.8%, range=58.4-67.5%, 3\textsuperscript{rd} generation: 1.3-fold increase, mean=73.7%, range=71.2-77.0%; CD8^+PD1^+ T cells: 2\textsuperscript{nd} generation: 4.6-fold decrease, mean=9.1%, range=5.3-15.4%, 3\textsuperscript{rd} generation: 2.7-fold decrease, mean=15.3%, range=8.0-24.4%; Figure 6D). Since the up-regulation of multiple inhibitory receptors did not impact the \textit{in vivo} efficacy of the CD171-CAR-T cells, the up-regulation was most likely due to T cell activation and not to exhaustion.\textsuperscript{26} This was in line with our previous finding that PD1 expression goes up upon initial T cell stimulation using CD3/CD28 beads but retains to basal levels after 6 days of expansion underlining its role as an early activation marker (unpublished data). Further, there was a significant difference between the 2\textsuperscript{nd} and 3\textsuperscript{rd} generation final products for CD4^+PD1^+ cells only (p=0.0094), but not for the other markers assessed.
Discussion

CAR-T cell therapy is the subject of a considerable number of early-phase clinical trials. Two CAR-T cell products received FDA approval in 2017 (Kymriah™ from Novartis Pharmaceuticals Corporation and Yescarta™ from Kite Pharma/Gilead Sciences). Nevertheless, manufacturing of CAR-T cell products from heavily pretreated patients is still challenging since those patients are often severely lymphopenic and have higher $T_{EM}$ frequencies in their CD4 and CD8 T cell subsets, which are known to have shorter persistency and inferior antitumor immunity compared to $T_{CM}$ subsets.27-29

Here we provide proof-of-concept that bioactive CAR-T cells can be manufactured from patient derived cryopreserved G-CSF-mobilized PBSC apheresis products.

Cryopreserved apheresis products isolated early after diagnosis have enormous potential as an alternative T cell source to blood draws or apheresis at tumor relapse or recurrence. The use of cryopreserved PBSC products as an alternative source for generating CAR-T cells has an economic impact, given this strategy would avoid not only the additional cost but also the patient discomfort associated with an additional apheresis procedure or high-volume blood draw. Furthermore, as CAR-T cell therapy becomes a more commonly accepted first-line therapy, the current treatment protocol using intensive chemotherapy regimens administered within only a few weeks might not allow sufficient bone marrow recovery necessary for CAR-T cell product manufacturing. The need may arise for PBSCs collected and cryopreserved early during treatment. The same applies for the administration of neuroblastoma-specific CAR-T cells in the minimal residual disease setting shortly following high-dose chemotherapy, as is currently done.
with NK-cells. Finally, utilizing a central specialized center or facility manufacturing CAR-T cells for different cancer entities would also become more feasible, since frozen starting products could be easily shipped to the manufacturing site for CAR-T cell production.

In order to use G-CSF-stimulated cryopreserved PBSCs, we demonstrated that monocyte depletion was essential before CD3x28 bead-based activation of isolated T cells, presumably because monocytes can phagocytose beads and potentially secrete soluble factors such as PGE\textsubscript{2} that inhibit T cell activation.\textsuperscript{30,31} Monocyte-depleted PBSC product-derived T cells were amenable to lentiviral vector transduction and were competent for subsequent \textit{ex vivo} proliferation to reach cell doses necessary for CAR-T cell therapy.

We evaluated the use of cryopreserved G-CSF-stimulated PBSCs collected by apheresis very early in the treatment protocol as a source of T cells for CAR-T manufacturing. Patients with high-risk neuroblastoma were selected as an example patient cohort who could benefit from this technological advancement. For instance, patients diagnosed with primary neuroblastoma classified as high-risk disease undergo dose-intensive therapy including myeloablative consolidation chemotherapy requiring PBSC rescue. Dose-intensive chemotherapy is also often a part of treatment for relapsed or refractory neuroblastoma. We experienced difficulties expanding T cells from 10.5% of the first neuroblastoma patients enrolled in the ENCIT trial.\textsuperscript{6,13} It is likely that the dose-intensive chemo- and radiotherapy to which the patients have been exposed during prior treatment has negatively impacted isolation and \textit{ex vivo} expansion of isolated T cells. This is in line with results from the University of Pennsylvania where 24% of leukemia patients were excluded.
from CD19-CAR manufacturing due to failure in the “test expansion” performed before patient enrollment into their clinical trials. The authors proposed that previous chemotherapy, especially therapy administered to high-risk leukemia patients, might have a negative impact on T cell expansion. To circumvent this problem, we propose using the cryopreserved G-CSF-stimulated PBSCs collected by apheresis early after diagnosis as a source of T cells for neuroblastoma patients.

The differentiation status of T cells present in the starting product emerges as an important parameter for generating CAR-T cell products capable of proliferation and persistence following adoptive T cell therapy. T cells used for adoptive therapy must retain their intrinsic capacity for self-renewal and proliferation in order to eradicate large, established tumors. Preclinical xenograft models have shown that CAR-T cells derived from CD8+ and CD4+ T\textsubscript{N} and T\textsubscript{CM} subsets are more potent than those manufactured from T\textsubscript{EM} subsets, and that a combination of the optimal CD8+ and CD4+ T cell subsets leads to synergistic anti-tumor activity. We demonstrated that cryopreserved G-CSF-stimulated apheresis products contain sufficient numbers of CD4 and CD8 T cell precursors having T naïve and central memory differentiation-subsets with replicating potential and the capacity to generate large numbers of effector T cells after tumor stimulation. A comparison of the T cell phenotype from the cryopreserved G-CSF-stimulated PBSCs to the T cell phenotype from the PBSCs of the first 5 patients enrolled in ENCIT-01, where the CAR-T cells were generated from freshly harvested apheresis products, revealed that the CD8 repertoire of the G-CSF-stimulated apheresis products contained higher frequencies of naïve and central memory T cells indicating a) a higher ex vivo expansion capability, b)
an ability to give rise to memory and effector T cell subsets, c) enhanced \textit{in vivo} persistence and d) improved anti-tumor activity.\textsuperscript{34,35}

We conclude that biobanked PBSCs collected by apheresis offer an excellent alternative source for CAR-T cell manufacture. Future detailed phenotypic and \textit{ex vivo} expansion analyses of starting materials, as well as pre-selection of defined functional T cell subsets and functional analysis of the final CAR-T cell products administered to patients will be essential to develop the most effective CAR-T cell products.

\textbf{Acknowledgement}

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References


Tables

Table 1. Lineage panel of thawed apheresis products from 8 neuroblastoma patients.

Table 1. Lineage panel

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<tr>
<th>Patient</th>
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<th>Monocytes</th>
<th>Lymphocytes</th>
<th>T cells</th>
<th>B cells</th>
<th>NK cells</th>
<th>pDC</th>
<th>mDC</th>
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</table>

All cells were gated on live cells. Monocytes were gated on CD45+CD14+CD56 CD3 CD19 CD16. Lymphocytes were gated on CD45+CD14+. T-, B-, NK- and dendritic cells were gated within the lymphocytes. T cells were gated on CD3+ cells, B cells on CD19+ cells. NK cells were gated on CD3 CD19 CD56+ cells. pDC (plasmacytoid dendritic cells) were gated on CD3 CD19 CD56 CD16 CD123+ cells. mDC (myeloid dendritic cells) were gated on CD3 CD19 CD56 CD16 CD11c+ cells.

Figure 1. Treatment schema for patients with high-risk neuroblastoma.

Current treatment schema for high-risk neuroblastoma patients responding to therapy. AHSCT, autologous hematopoietic stem cell transplantation; PBSC, peripheral blood stem cells.

Figure 2. Phenotypic analysis of previously collected and freshly thawed apheresis products from 8 neuroblastoma patients. (A) Flow cytometric quantification of CD4:CD8 ratio in thawed apheresis products.
derived from patients and in fresh blood derived from healthy donors (HD). (B) Flow cytometric quantification of CD45RO and CD62L surface expression ($T_{CM}$=central memory T cells=CD45RO$^+$CD62L$^+$, $T_{EM}$=effector memory T cells=CD45RO$^+$CD62L$^-$, $T_{EFF}$=effector T cells=CD45RO$^-$CD62L$^-$, $T_N$=naïve T cells= CD45RO$^-$CD62L$^+$). (C) Flow cytometric quantification of CD25, CD69 and Ki67 surface expression. (D) Flow cytometric quantification of PD1, LAG3 and TIM-3 surface expression. All cells were first gated on single cells, followed by live, CD3$^+$ lymphocytes. Bar graphs represent the mean ± SD. Pat, patient; HD, healthy donor.

**Figure 3. Manufacturing CD171-CAR-T cell products from frozen apheresis products.** (A) Flow cytometric quantification of lymphocyte:monocyte ratio in thawed apheresis products derived from patients and in fresh blood derived from healthy donors (HD). (B) Expansion curves of PBSCs isolated from a frozen apheresis product derived from a neuroblastoma patient treated with and without immunomagnetic CD14 bead depletion. (C) CD171-CAR-T cell manufacturing schema. (D) Growth curves of 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR-T cell products manufactured from frozen apheresis products from 3 patients with high-risk neuroblastoma. HD, healthy donor; 2G, 2$^{nd}$ generation; 3G, 3$^{rd}$ generation.

**Figure 4. Enrichment for CD171-CAR expression.** (A) EGFRt surface expression detected with Cetuximab in CD4$^+$ and CD8$^+$ 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR-T cell products before and after sorting for EGFRt. (B) Viability curves of 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR-T cell products from 3 neuroblastoma patients. (C) Expression levels of 2$^{nd}$ and 3$^{rd}$ generation CAR detected by Western blotting with an antibody against CD3ζ. 2G, 2$^{nd}$ generation; 3G, 3$^{rd}$ generation.
**Figure 5. Phenotypic analysis and evaluation of final CAR-T cell products.** (A) Flow cytometric quantification of CD45RO and CD62L expression in CD4$^+$ and CD8$^+$ 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR-T cell products after thawing. Bar graphs represent the mean ± SD. (B) Anti-tumor lytic activity of 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR CTLs determined by CRA. (C) Stimulation of cytokine secretion in mixed 2$^{nd}$/3$^{rd}$ generation-CAR-CTL tumor cultures. Error bars represent the standard deviation of the mean of triplicates. HD, healthy donor control; 2G, 2$^{nd}$ generation; 3G, 3$^{rd}$ generation; *P < 0.05.

**Figure 6. In vivo evaluation of CD171-CAR-T cell products.** (A) Schema of the intracranial (IC) NSG mouse neuroblastoma xenograft therapy model and biophotonic signal of ffLuc$^+$ SK-N-DZ tumors on day 6 following stereotactic implantation. (B) Biophotonic tumor signal response to intratumorally injected 2$^{nd}$ and 3$^{rd}$ generation CAR CTLs (n=5 mice per group). Mock-treated control cohort was euthanized on day 20 due to tumor-related animal distress. (C) Kaplan–Meier survival plots of treated cohorts described in B. (D) Flow cytometric quantification of PD1, LAG3, TIM-3, CD27 and CD127 expression in CD4$^+$ and CD8$^+$ 2$^{nd}$ and 3$^{rd}$ generation CD171-CAR-T cell products after thawing. All cells were first gated on single cells, followed by live CD3+ lymphocytes. Bar graphs represent the mean ± SD. 2G, 2$^{nd}$ generation; 3G, 3$^{rd}$ generation; *P < 0.05.

**Supplemental Figure 1. Gating strategy for lineage panel.** (A) Gating strategy for lymphocytes and viability staining flow cytometric analysis. (B) Gating strategy for monocyte and dendritic cell staining flow cytometric analysis. (C) Gating strategy for NK cell staining flow cytometric analysis. (D) Gating strategy for T cell staining flow cytometric analysis.
Supplemental Figure 2. Gating strategy for T cell phenotype. \( T_N = \) CD45RO\(^{-}\)/CD62L\(^{+}\), \( T_{CM} = \) CD45RO\(^{+}\)/CD62L\(^{-}\), \( T_{EM} = \) CD645RO\(^{+}\)/CD62L\(^{-}\), \( T_{Eff} = \) CD45RO\(^{-}\)/CD62L\(^{-}\). N, naïve; CM, central memory; EM, effector memory; Eff, effector.

Supplemental Figure 3. Characterization of final CAR-T cell products after thawing. (A) Viability of final CAR-T cell products assessed by trypan blue staining. (B) Flow cytometric quantification of CD4:CD8 ratio.
Figure 1

Induction

Cycle 1  Cycle 2  Cycle 3  Cycle 4  Cycle 5  Cycle 6

PBSC harvest

Consolidation

AHSC  Radio-therapy  Isotre-tinoin

PBSC infusion
Figure 4

A

EGFR+ [%]

CD4+ before sort after sort

P#1 2 3 P#1 2 3

CD8+ before sort after sort

P#1 2 3 P#1 2 3

B

Viability [%]

CD171 2G CD171 3G

Pat #1 Pat #2 Pat #3 Pat #1 Pat #2 Pat #3

C

CAR ζ chain
internal ζ chain
mock P#1 2 3 HD
mock P#1 2 3 HD
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