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Review article

Leukapheresis guidance and best practices for optimal chimeric antigen receptor T-cell manufacturing

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ABSTRACT

Chimeric antigen receptor (CAR) T-cell therapy is an individualized immunotherapy that genetically reprograms a patient's T cells to target and eliminate cancer cells. Tisagenlecleucel is a US Food and Drug Administration-approved CD19-directed CAR T-cell therapy for patients with relapsed/refractory (r/r) B-cell acute lymphoblastic leukemia and r/r diffuse large B-cell lymphoma. Manufacturing CAR T cells is an intricate process that begins with leukapheresis to obtain T cells from the patient's peripheral blood. An optimal leukapheresis product is essential to the success of CAR T-cell therapy; therefore, understanding factors that may affect the quality or T-cell content is imperative. CAR T-cell therapy requires detailed organization throughout the entire multistep process, including appropriate training of a multidisciplinary team in leukapheresis collection, cell processing, timing and coordination with manufacturing and administration to achieve suitable patient care. Consideration of logistical parameters, including leukapheresis timing, location and patient availability, when clinically evaluating the patient and the trajectory of their disease progression must be reflected in the overall collection strategy. Challenges of obtaining optimal leukapheresis product for CAR T-cell manufacturing include vascular access for smaller patients, achieving sufficient T-cell yield, eliminating contaminating cell types in the leukapheresis product, determining appropriate washout periods for medication and managing adverse events at collection. In this review, the authors provide recommendations on navigating CAR T-cell therapy and leukapheresis based on experience and data from tisagenlecleucel manufacturing in clinical trials and the real-world setting.

Introduction

Chimeric antigen receptor (CAR) T-cell therapy is an individualized immunocellular therapy that genetically reprograms a patient's T cells to target and eliminate cancer cells. Tisagenlecleucel is an approved CD19-directed CAR T-cell therapy for patients with relapsed/refractory (r/r) B-cell acute lymphoblastic leukemia (ALL) and r/r diffuse large B-cell lymphoma (DLBCL). Manufacturing CAR T cells is a complex, multistep process that begins with leukapheresis to obtain viable T cells from the patient's peripheral blood. An optimal leukapheresis product is critical for the success of manufacturing; therefore, understanding factors that may affect the leukapheresis product quality or T-cell content is imperative.

The first step in CAR T-cell therapy is leukapheresis for T-cell collection. Procedures that ensure successful collection are necessary for successful manufacturing and treatment, as higher T-cell numbers in the leukapheresis product may be associated with achievement of remission [1]. Previous studies have identified patient and disease characteristics associated with low lymphocyte collection efficiency (defined as the lymphocyte count per product volume divided by the average lymphocyte count per processed peripheral blood volume) that include advanced age, diagnosis of ALL and high platelet count prior to leukapheresis [2]. Heavily pre-treated patients may have high numbers of circulating malignant cells or lymphopenia, which may be suboptimal for leukapheresis collection. Advancing age

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(every 10 years) and disease factors (including disease type) are associated with low lymphocyte collection efficiency (<40%). Although further data are needed, high platelet count is thought to lead to occult platelet clumping in the apheresis centrifuge that may result in narrowing of the mononuclear cell interface. Poorly understood, but of great interest, is identifying optimal conditions for leukapheresis in very young patients, which will enhance leukapheresis yield and enable therapy delivery. Real-world data have demonstrated the efficacy and safety of CAR T-cell therapy in pediatric and young adult patients with r/r ALL and the ability to collect leukapheresis product from very young patients (<3 years old) [3–6].

In addition to volume and quality of the leukapheresis product, another important factor in the leukapheresis process is the timing with respect to the patient's disease trajectory and treatment. Performing leukapheresis prior to beginning salvage therapy to treat disease relapse depends on the clinical status and disease burden of the patient. Evidence suggests that the choice of salvage therapy may adversely affect subsequent leukapheresis attempts as it relates to T-cell count recovery and quality [7]. The timing of leukapheresis also depends on the history of the patient and disease refractoriness. In patients with highly refractory disease, trying to decrease disease burden prior to leukapheresis may be counterproductive given the prior lack of disease response. In pediatric patients with ALL, some clinicians may consider early leukapheresis in patients with a high risk of relapse (e.g., Philadelphia chromosome+ and infant ALL) and when bone marrow transplant outcomes have not been favorable. Recently, real-world data have demonstrated that leukapheresis and tisagenlecleucel manufacturing have been successful in patients <3 years old [8,9]. Patient leukapheresis product collected for tisagenlecleucel manufacturing can be cryopreserved for up to 30 months prior to use in manufacturing. Some centers have established procedures that facilitate leukapheresis collection earlier in the disease course, which allows flexibility in scheduling, shipping and patient management [10]. The possibility of long-term storage of cryopreserved leukapheresis product may be beneficial for pediatric patients with ALL and patients with high-risk non-Hodgkin lymphoma who have early r/r disease prior to additional salvage and bridging. Notably, if a patient is planning to proceed with allogeneic stem cell transplant (alloSCT) and requires CAR T-cell therapy for post-alloSCT relapse, it is unlikely that the leukapheresis product stored from pre-alloSCT would be utilized, and a fresh collection would be preferred [11,12]. The optimal timing for cryopreservation is as soon as possible after collection, but at most within 24 h of collection [10]. Although there are many challenges involved in coordinating leukapheresis, CAR T-cell manufacturing has evolved to be able to handle a wide range of starting material and continues to improve. Currently, each CAR T-cell therapy manufacturer provides specifications and requirements for collection and handling of leukapheresis product that is acceptable for manufacturing; however, further understanding of how these specifications and requirements relate to patient clinical outcomes is needed. As experience is gained in the real-world setting, it may be possible to increase the flexibility of leukapheresis specifications and requirements and may allow further harmonization across different CAR T-cell therapy manufacturers.

Although additional factors related to the cellular characteristics of the leukapheresis product, such as T-cell subsets and activation/senescence status of patient T cells, may affect manufacturing and CAR T-cell efficacy [10], this article focuses on the authors' experiences and recommendations pertaining to overcoming the challenges of obtaining optimal leukapheresis product for CAR T-cell manufacturing from patients with B-cell malignancies. An algorithm to improve leukapheresis collection efficiency is presented to better predict the amount of processed blood volume required to minimize the amount of time the patient spends in apheresis. Herein the authors provide our experience based on CD19-targeted CAR T-cell therapy and leukapheresis data from the larger tisagenlecleucel manufacturing experience.

Challenges Associated with Leukapheresis

Patient evaluation and vascular access for leukapheresis

Prior to leukapheresis, patients are evaluated for their general suitability to receive CAR T-cell therapy. The factors evaluated include age, Eastern Cooperative Oncology Group performance status (ECOG PS) or Karnofsky/Lansky performance status, history of prior malignancies, timing of prior alloSCT as it relates to immunosuppression status and graft-versus-host disease (GVHD), prior CD19-directed therapy or CAR T-cell therapy clinical trials or commercial product, active infections and history of central nervous system disease [7]. Beyond these initial patient and disease characteristics, clinical studies have also evaluated how patient characteristics may influence leukapheresis outcomes. In a retrospective study of three clinical trials in pediatric patients with r/r B-cell malignancies, 15% (n = 11, N = 71) of patients experienced leukapheresis complications, including paresthesia and nausea, but these did not correlate with patient age or weight [13]. Similarly, in the PLAT-02 and ENCIT-01 trials, age and weight (<40 kg versus >40 kg) in pediatric patients did not affect leukapheresis collection, but the majority of patients (17 of 19, 89%) required a blood prime, likely due to their total blood volume and low body weight [14]. Adult patients who have undergone CAR T-cell therapy in clinical trials have also been evaluated for factors that may influence leukapheresis outcomes. In a single-center study among adult patients with advanced B-cell malignancies (N = 92), having ALL, advanced age and higher platelet counts were associated with poor leukapheresis collection efficiency [2]. The potential influence of other laboratory parameters that may indicate hepatic involvement or organ failure, such as absolute neutrophil count, bilirubin, aspartate aminotransferase/alanine aminotransferase and serum creatinine [7,15,16], and the subsequent effect on CAR T-cell product attributes are not well understood. Patient characteristics and collection parameters that may affect leukapheresis collection efficiency were evaluated at the Moffitt Cancer Center in a retrospective study among patients with r/r DLBCL treated with tisagenlecleucel [17]. In adult patients (N = 23), absolute lymphocyte count (ALC) and peripheral blood CD3+ cell count significantly correlated with leukapheresis product CD3+ cell counts ($P < 0.0001$ and $P < 0.03$, respectively). The authors reported that high T-cell collection efficiency was possible among heavily pre-treated patients who had received ≥3 prior lines of therapy and had low peripheral blood lymphocyte counts (ALC <100/μL) provided that an adequate volume of blood was processed [17]. Patients with high circulating blasts in peripheral blood may have difficulty meeting the leukapheresis product requirements for CD3+ cell count and CD3+%. Overall, the authors found that peripheral blood ALC and CD3+ cell counts were the best indicators of obtaining a sufficient CD3+ cell count in the leukapheresis product. An analysis of real-world leukapheresis and tisagenlecleucel manufacturing in young pediatric patients (<3 years of age) with r/r ALL demonstrated improved outcomes from the first US Food and Drug Administration approval of a CAR T-cell product in 2017 through 2021 [8]. Based on these data, some of the key author recommendations for optimizing leukapheresis in patients with low body weight included verification of adequate ALC or CD3+ counts the day before leukapheresis, maintenance of hematocrit levels at 40% prior to collection and allowing more than 1 day for collection based on individual patient needs [8].

Patient comorbidities and disease history may also affect leukapheresis outcomes. Current best practice recommendations for managing patients undergoing CAR T-cell therapy include pre-

leukapheresis screening to assess eligibility and fitness for CAR T-cell therapy [18]. In clinical trials of CAR T-cell therapy, eligibility criteria typically exclude patients with comorbidities that could have a negative impact on clinical outcomes, some of which could also affect leukapheresis and CAR T-cell manufacturing outcomes. However, in real-world clinical practice, sicker patients with more severe comorbidities are being treated with CAR T-cell therapy and achieving clinical outcomes that are similar to those reported in CAR T-cell therapy clinical trials. For example, an analysis of real-world data from 17 treatment centers in the United States found that patients with r/r DLBCL underwent successful leukapheresis collections for the manufacture of axicabtagene ciloleucel (N = 298), even the 43% of patients with comorbidities and baseline characteristics that would have excluded them from participating in the registrational ZUMA-1 trial of axicabtagene ciloleucel in adult patients with r/r large B-cell lymphoma. Criteria that would have resulted in exclusion from the ZUMA-1 trial included ECOG PS >1, platelet count <75 000/μL, central nervous system disease, renal insufficiency, bilirubin >1.5 g/dL and prior CD19-directed therapy [19]. Of these, ECOG PS, platelet count and renal insufficiency should be evaluated prior to leukapheresis and may lead to adverse events (AEs) from leukapheresis, including electrolyte imbalance and the need for transfusion. However, these AEs can typically be resolved with proper treatment [7] and do not typically affect leukapheresis outcomes. Overall, CAR T-cell manufacturing was successful in 92% of patients who underwent leukapheresis; however, the researchers noted lower progression-free survival and overall survival among patients with comorbidities that would have made them ineligible for ZUMA-1 [19].

During patient preparation for leukapheresis, vascular access must be evaluated regardless of patient age, and assessment by experienced apheresis nurses can help determine the appropriate type of access for each patient (i.e., peripheral versus central line). Peripheral access considerations include vein location, integrity, elasticity, size and whether the vein can support the blood flow rates of an average collection (85 mL/min) [20]. Central access is used if the patient's veins do not meet the standards for peripheral venous access. Among pediatric patients who undergo collection of peripheral blood cells, the majority require a central venous catheter to initiate and maintain blood flow [21], including temporary or permanent tunneled double-lumen apheresis catheters. The size of the catheter depends on the patient's weight; patients >30 kg can have either an 8-French or a 10-French double-lumen catheter placed [14]. In the authors' experience, a 7-French catheter has been used as the lower limit for smaller patients. Although not routinely performed, the team performing leukapheresis collection determines if sedation is needed, specifically for smaller pediatric patients or patients with clinical concerns. Potential complications that are monitored for during and after collection include pneumothorax, bleeding, infection, electrolyte issues (magnesium, calcium, potassium) and catheter site pain. Among adult patients, leukapheresis collection using peripheral venous access is common, but in cases where central catheter placement is needed, procedures are similar to those used for pediatric patients.

Blood volume exchange and special considerations for specific clinical management strategies depend on patient age/weight and leukapheresis center guidelines and may include the need for transfusion, calcium supplementation and heparin use (additional details regarding the use of anticoagulants are discussed later in this article). Blood priming of the apheresis system using donated packed red blood cells (PRBCs) should be considered for patients weighing <25 kg to ensure that the patient is isovolemic during the collection process. Blood prime in low-weight patients can compensate for potentially high percentages (>15%) of extracorporeal blood volume in the collection equipment, which can allow patients to remain stable and ensure that an adequate volume of blood is able to be processed during collection. To minimize potential contamination of the leukapheresis product with T cells from the blood donor, leukoreduction filters and irradiation of PRBCs can be used to avoid transfer of intact viable allogeneic T cells.

Table 1
Recommendations for optimizing leukapheresis in low-weight pediatric patients.

Timing	Actions
Before leukapheresis	Verify adequate ALC and/or CD3+ cell count day prior to leukapheresis. Transfuse PRBCs prior to leukapheresis to raise hematocrit to 40% in patients <10 kg (preferably <15 kg) to maintain hemodynamic stability during procedure. Blood prime leukapheresis instrument tubing with irradiated and leukoreduced PRBCs for patients <25 kg. Anticoagulant with mixture of ACD-A and heparin to minimize risk of ACD-A–induced hypocalcemia. Allow for >1 day of leukapheresis if needed to meet acceptance criteria in small patients. Benefits and risks of additional days of leukapheresis (>1) should be assessed on a case-by-case basis based on leukapheresis product cell count (TNC and CD3+). Risk to the patient must be collaboratively evaluated across the stem cell laboratory and clinical teams.
During leukapheresis	Prevent hypothermia using warming blanket or in-line blood warmer. Observe patients for hypocalcemia, hypomagnesemia and alkalosis (clinical signs and symptoms of irritability, inconsolable crying and heart rate and blood pressure instability) by monitoring ionized calcium, magnesium and blood gases and replacing as needed and consider prophylactic IV calcium and magnesium supplementation during the procedure. Monitor rate and color of in-line collection for non-mobilized MNC collection (maintain in-line salmon color consistent with a leukapheresis product, hematocrit at 3–4% and rate of approximately 0.8–1.2 mL/min or less in small children). Monitor collection volume (not to exceed 10 mL/kg per AABB standards) and perform partial rinse-back at the end of each leukapheresis day. In low-weight patients, four to six blood volume exchanges may be necessary for adequate collection during a single day of leukapheresis.

AABB, Association for the Advancement of Blood & Biotherapies; ACD-A, anticoagulant citrate dextrose solution A; ALC, absolute lymphocyte count; CD, cluster of differentiation; IV, intravenous; MNC, mononuclear cell; TNC, total nucleated cell. Provided courtesy of L. Cough [8,22].

Pediatric patients with low body weight can present specific leukapheresis collection challenges and require specific optimization strategies (Table 1) [8,22]. Recent experience with commercial tisagenlecleucel in 31 pediatric patients <3 years of age with r/r ALL and low weight (<10 kg) identified factors for optimizing leukapheresis [22,23]. For instance, raising the hematocrit to 40% with PRBCs may be necessary to stabilize the patient during apheresis. Specifically, the volume to be processed is based on linear regression of the target number of CD3+ cells versus the number of peripheral CD3+ lymphocytes collected per volume of blood processed. The peripheral CD3+ lymphocyte count can be obtained through the patient pre-screening process. Additional factors that can be optimized for leukapheresis in patients weighing <10 kg include central venous catheter size, blood prime of the leukapheresis instrument, hypothermia prevention during collection and an allowance of >1 day for collection (if needed) [22,23].

During leukapheresis collection, the use of anticoagulants is necessary to maintain blood fluidity in the apheresis system [24]. In this setting, anticoagulant citrate dextrose solution A (ACD-A) is preferred, and although the permitted ratio of whole blood to ACD-A for anticoagulants varies among institutions, a 12:1 ratio has been used

as a default starting point. Smaller patients are at a higher risk of hypocalcemia following leukapheresis when ACD-A is used as a result of the typically higher number of blood volume exchanges during leukapheresis resulting in an unacceptable level of ACD-A being introduced to the patient. To reduce the risk of hypocalcemia, in conjunction with adjusting the flow rate, heparin can be added to ACD-A to decrease the citrate concentration to avoid clumping and maintain fluidity. In combination with heparin, using less ACD-A allows for a higher inlet flow and less time on the apheresis instrument [24].

Leukapheresis logistics

Timely coordination across the multidisciplinary team is necessary to achieve an efficient leukapheresis collection [20]. Coordination can be improved by holding weekly team meetings that include physicians, apheresis teams, nurses, pharmacists and social workers [25]. In some instances, smaller patients and those with lower ALC and CD3+ cells may require an additional leukapheresis collection day. Pediatric and young adult patients, however, do not always require additional collection. Teamwork and partnering between the CAR T-cell therapy provider and manufacturer are essential in medically determining when and if additional collection days are necessary on an individual patient basis.

Successful manufacture of CAR T-cell products is dependent on smooth coordination of supply chain logistics after collection. Leukapheresis sites must label material efficiently for processing and communicate with couriers to ensure proper handling procedures with optimal cryogenic shippers with temperature control mechanisms [25,26]. Specific areas of improvement for the manufacture of CAR T-cells include optimizing the T-cell enrichment process to reduce variability [27] and reducing the time taken to perform sterility testing by using polymerase chain reaction-based mycoplasma sterility assay [26]. The leukapheresis process needs to be as efficient and timely as possible to avoid disease progression and decline in patient performance status.

Leukapheresis product can be cryopreserved for long-term storage. For tisagenlecleucel, leukapheresis product is approved for cryopreserved storage for up to 30 months before manufacturing without negatively affecting the post-thaw cell viability and recovery of mononuclear cells (Novartis Pharmaceuticals Corporation data on file). For hematopoietic progenitor cell transplant, some stem cells have been reported to have been frozen for 15 years before use, with post-thaw cell viability assessed by *in vitro* colony formation assays [28]. It is possible that storage time for tisagenlecleucel leukapheresis product could be extended in the future with the appropriate stability studies. For example, cord blood units can be stored for more than 20 years with minimal hematopoietic stem cell loss as assessed by post-thaw *in vitro* colony formation and T-cell activity [29]. Because of the ability to store leukapheresis product for longer-term periods, there are opportunities for leukapheresis collections earlier in the course of disease, which may correlate with higher cell counts and quality, potential higher manufacturing success and a robust CAR T-cell product [10]. T-cell function (tumor necrosis factor alpha secretion in response to CD19 antigen) and expansion decrease with cycles of chemotherapy [30,31]; therefore, earlier collection should be considered to ensure the highest possible activity for future cellular therapies.

Overall, cryopreservation ensures flexibility for the timing of leukapheresis because the hold time for cryopreserved leukapheresis cells used for tisagenlecleucel manufacturing has no effect on cell viability in the final product [10]. In addition, cryopreserving leukapheresis product brings potential supply chain benefits, including risk mitigation against transportation interruptions or delays due to unforeseen circumstances (e.g., current/future pandemics and severe weather events), as cryopreserved cells are stable for long periods compared with urgent fresh cell transport, which demonstrates shorter viability timelines. Analysis of data from patients enrolled in the ELIANA and JULIET clinical trials of tisagenlecleucel demonstrated that leukapheresis cells collected and cryopreserved on the same day had better post-thaw performance compared with cells that were cryopreserved the day after leukapheresis collection [10]. Hold times longer than 24 h before freezing had a negative effect on post-thaw cell viability and significantly reduced post-thaw cell recovery by up to 20% in patients with DLBCL. In this study, the viability of T cells in the post-thaw leukapheresis material was determined by microscopy using trypan blue exclusion and calculated based on hemocytometer counts [10]. In addition, the total number of CAR T cells during the manufacturing process was consistently higher when leukapheresis product was frozen on the same day as collection. In general, leukapheresis product performance was more negatively affected by a longer time between collection and cryopreservation, as characterized by low cell viability following recovery of the cellular leukapheresis product.

Characteristics of the leukapheresis product

Leukapheresis material must meet minimum specifications to be accepted for tisagenlecleucel manufacturing, including CD3+ lymphocyte count, total nucleated cell (TNC) count and TNC CD3+% [32]. The authors evaluated the relationship between characteristics of the incoming leukapheresis product and CAR T-cell manufacturing success (defined as sufficient cell growth to achieve final product specifications). Incoming leukapheresis material was characterized using the following variables (when available): leukapheresis parameters, sentinel vial measurements (% T cells, B cells, natural killer cells, monocytes) and incoming apheresis cell counts (TNC, CD3+ count,

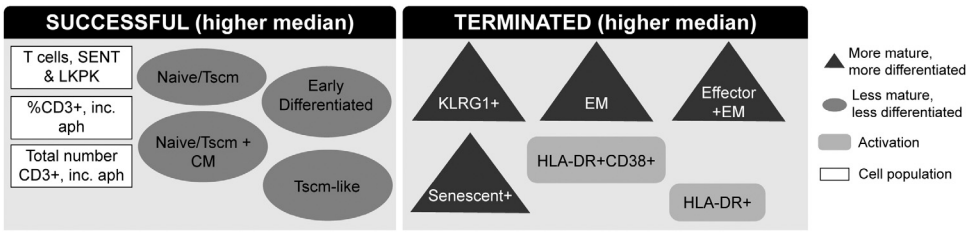

Fig. 1. Characteristics of leukapheresis products associated with successful CAR T-cell manufacturing. Apheresis products that result in successful CAR T-cell manufacturing have more T cells and more less mature and less differentiated T cells than manufacturing batches that are terminated. Sentinel vial measurements included the percentage of T cells, B cells, natural killer cells and monocytes. Incoming apheresis material measurements included total nucleated cells, total CD3 count and percent CD3+ cells. Effector T cells are short-lived and actively secrete cytokines. Effector memory T cells are responsible for cytotoxic action against pathogens and are typically found in the peripheral circulation and tissues. Central memory T cells augment immune response after reactivation and are typically found in the peripheral circulation and lymph nodes (Novartis Pharmaceuticals Corporation data on file). CM, central memory T cells; Effector, effector T cells; EM, effector memory T cells; HLA-DR, human leukocyte antigen - DR isotype; inc. aph, incoming apheresis material; KLRG1, killer cell lectin-like receptor G1; LKPK, leukapheresis material (tisagenlecleucel starting material); SENT, sentinel vial. Provided courtesy of Novartis Pharmaceuticals Corporation.

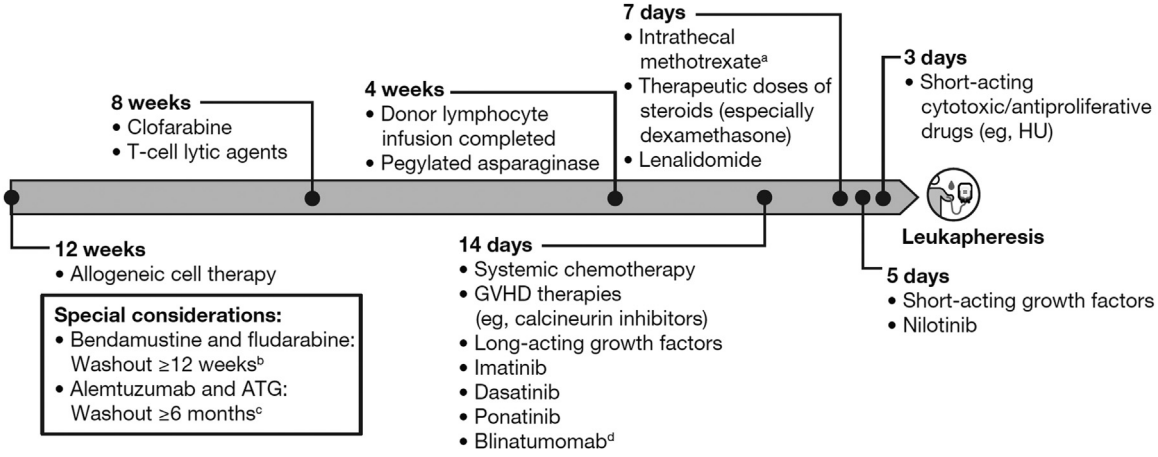

Fig. 2. Recommended washout periods prior to leukapheresis for CAR T-cell manufacture and timing for discontinuation of prior therapy if the patient's condition and disease status allow. Recommendations are based on previously published guidance [7,39] and the authors' clinical experience. [a]If indicated, intrathecal cytarabine can be given up to a day prior to leukapheresis. For an intravenous cytarabine dose <100 mg/m^2, a washout of 7 days is recommended; for a dose ≥100 mg/m^2, a washout of 14 days is recommended. [b]For bendamustine and fludarabine, allow adequate washout and avoid use for ≥12 weeks prior to leukapheresis because of the potential long-term effects on T cells; however, limited data are available for these agents in the context of CAR T-cell therapy for these agents. [c]For alemtuzumab and ATG (T-cell lytic agents), allow adequate washout, avoid use for ≥6 months prior to leukapheresis and consider the potential prolonged effects on T cells. [d]Although the half-life of blinatumomab is short (approximately 2 h), a washout of 1–2 weeks is recommended prior to leukapheresis. The inotuzumab elimination half-life (12.3 days) is too long to wait for a washout period of five half-lives in most patients. ATG, anti-thymocyte globulin; GVHD, graft-vs-host disease; HU, hydroxyurea; pALL, pediatric acute lymphoblastic leukemia. Provided courtesy of Novartis Pharmaceuticals Corporation. Copyright Novartis Pharmaceuticals Corporation 2021.

CD3+%). The relative importance of the leukapheresis variables for manufacturing success was assessed using univariate (box plots, Wilcoxon significance test, adjusted *P* values) and multivariate (decision tree/random forest, logistic regression, elastic net) analyses. In general, patients with T cells that are less mature and less differentiated at collection tend to achieve more successful manufacturing outcomes than patients with more mature and more differentiated/activated T cells (Figure 1). Overall, successful tisagenlecleucel manufacturing for patients with DLBCL has been associated with the collection of more T cells and tends to include T cells that are less mature and less differentiated and exhibit higher expression of CD25 (e.g., naive/stem memory T cells [TSCMs], naive/TSCMs + memory T cells, early differentiated T cells and TSCM-like cells; Figure 1). Because terminated batches often have higher expression of activation markers (HLA-DR or HLA-DR and CD38), which are also upregulated upon T-cell activation, the lower expression of CD25 observed in terminated batches may be due to decreased IL-2 uptake, which can negatively impact T-cell growth.

Terminated batches (in which manufacturing failed) often have more mature and differentiated T cells (including killer cell lectin-like receptor G1+ cells, terminally senescent effector memory cells and activated HLA-DR+/CD38+ and HLA-DR+ T cells), more B-lineage cells (including lymphoblasts) and more TNCs. Killer cell lectin-like receptor G1+ expression increases with patient age, reaching 90% on CD8+ T cells in individuals >65 years [33], and increases with T-cell maturation, with the highest expression seen in differentiated end-stage cells [34]. Similarly, among pediatric and young adult patients with ALL, successful tisagenlecleucel manufacturing has been associated with the collection of more total T cells, with a higher proportion of T cells that are less mature and less differentiated, and CD4+ T cells, with a higher ratio of CD4+:CD8+ cells. The results observed in patients with pediatric ALL are consistent with recent data showing that the maturation and differentiation states of T-cell subpopulations have an impact on the manufacture of CAR T-cell products [30,35]. In general, a less mature and less differentiated T-cell subtype is beneficial for generation of proliferating CAR T cells. Leukapheresis material enriched for naive and stem central memory T cells expanded well *in vitro,* but studies have shown chemotherapy-related depletion and a corresponding decline in T-cell expansion [30,35].

Leukapheresis is used to obtain T cells from the patient's peripheral blood for CAR T-cell manufacturing; however, as the leukapheresis collection separates blood components by density gradient, the process yields a variety of cells and not a pure CD3+ T-cell product. Monocytes in the leukapheresis collection material have been considered contaminants because they eventually become macrophages and may engulf and digest cellular constituents, including the antibody-conjugated beads used for CAR T-cell manufacturing [36]. Therefore, monocyte contaminants may result in a reduction in the viable CAR T-cell counts required to meet dosing requirements in the final product. Granulocyte colony-stimulating factors prescribed to patients post-chemotherapy and radiation to mitigate neutropenia can affect cell populations (e.g., increased levels of monocytes) [37], which may alter final CAR T-cell counts and expansion potential. For these reasons, protocols have been developed to deplete myeloid cells by plastic adherence [13]. Circulating tumor cells in patients with ALL, chronic lymphocytic leukemia and non-Hodgkin lymphoma contribute to higher B-cell and toxic malignant cell counts that can interfere with healthy T-cell numbers and expansion. Therefore, treating physicians should provide therapy to reduce the number of circulating blasts prior to leukapheresis, especially in pediatric ALL patients with very high circulating blast counts, underlying chronic lymphocytic leukemia and/or circulating DLBCL blasts.

Washout of medications prior to leukapheresis

Patients with r/r B-cell ALL and DLBCL have likely received prior aggressive chemotherapies and/or high-dose steroids that can have a detrimental effect on patients' T cells. Therefore, appropriately timed washout of chemotherapy, medications and other agents prior to leukapheresis collection is important to optimize T-cell fitness and achieve manufacturing success. For the majority of anti-neoplastic agents, a washout period equal to five half-lives for the particular agent/drug has been suggested to allow for sufficient clearance to obtain optimal leukapheresis collection material (Figure 2) [7,22,38,39]. However, agents that directly affect T cells or CD19 expression may require a washout period longer than five half-lives [7,22,39].

Based on individual agent/drug half-lives and the known or potential effects on T-cell fitness, short-acting cytotoxic, anti-

proliferative drugs can be administered up to 3 days prior to leukapheresis [7]. Short-acting growth factors and nilotinib should have a washout period of 5 days prior to leukapheresis, with the washout period being longer than five half-lives of the therapeutic agents. Intrathecal methotrexate, therapeutic doses of steroids (especially dexamethasone) [7] and lenalidomide are recommended to be discontinued at least 7 days prior to leukapheresis. However, steroids used for physiological replacement require no washout [7]. This includes <12 mg/m^2/day and <40 mg/day of hydrocortisone or equivalent in pediatric patients and adult patients, respectively [32]. At 14 days prior to leukapheresis, systemic chemotherapy and GVHD therapies [39], long-acting growth factors, tyrosine kinase inhibitors with known effects on T-cell function and proliferation (imatinib [40,41], dasatinib [42,43], ponatinib), inotuzumab and blinatumomab should be discontinued. Adherence to a washout period of at least 14 days for systemic GVHD therapies allows patients with chronic GVHD to undergo leukapheresis collection [39]. However, for patients with chronic GVHD receiving topical or inhaled steroids, care must be taken to ensure there is no active GVHD at leukapheresis, as this may affect CAR T-cell manufacturing. Donor lymphocyte infusion should be completed and pegylated asparaginase should not be given within 4 weeks of leukapheresis [7]. Therapy with clofarabine and T-cell lytic agents should be terminated 8 weeks before leukapheresis, and alloSCT should be completed at 12 weeks prior to leukapheresis as a general guideline. The half-life of polatuzumab may be too long to wait five half-lives for washout [44]; therefore, a washout period of at least 12 weeks is recommended. Bendamustine may have a prolonged effect on the suppression of hematopoiesis and depletion of lymphocytes [45,46] and, along with fludarabine therapy [47], should be discontinued at least 12 weeks before leukapheresis. Rituximab washout is not required if the patient's condition and disease status do not allow adequate washout, as the half-life may be too long to wait (22–32 days) [48]. Although polatuzumab, bendamustine, rituximab and fludarabine may have long-term effects on T cells, limited data are available in the context of CAR T-cell therapy [39]. In general, the use of CD19-targeted therapies should be avoided, as they may negatively impact CAR T-cell outcomes and increase the risk of CD19− clone selection. Tafasitamab has a long half-life (17 days) and is a CD19-targeted antibody. Thus far, *in vitro* studies have shown that the CD19 antigen (CAR T-cell therapy target) is still present after tafasitamab treatment [49]. Figure 2 provides guidance for leukapheresis washout periods, but the patient's disease status and overall condition should always be considered when determining the best time for drug washout and leukapheresis collection. Collectively, leukapheresis washout guidelines are constantly evolving as new agents become available and will require further evaluation as our understanding of their potential effects on T-cell function evolves.

AE management during leukapheresis

Leukapheresis has long been performed in the outpatient setting without serious complications, and many AEs can be prevented by thorough patient assessments prior to apheresis. Vasovagal/hypovolemic reactions, air embolus, extracorporeal hemolysis, citrate toxicity or tetany, hematoma and general mild reactions (e.g., headache, muscle cramping, irregular pulse, mild allergic reaction) are some of the AEs that may be reported during the leukapheresis process. AEs during the leukapheresis process are generally infrequent but are more common in younger patients and patients with comorbidities. The standard operating procedure of the University of Kansas Health System's Department of Pathology and Laboratory Medicine for managing these apheresis-related AEs depends on the nature of the reaction [50]. Typically, the health care professional will assess the patient and decide if the leukapheresis procedure should be discontinued. A rapid response team may be activated if the patient requires cardiopulmonary resuscitation or if there is a deterioration in the patient's condition that requires additional attention. Electrolyte management is essential during leukapheresis and includes ionized calcium and magnesium monitoring. The most common AEs include citrate reactions (hypocalcemia), post-procedure fatigue, syncope, transient loss of consciousness, hypotension, nausea, vomiting and diaphoresis [51,52] and can be prevented or managed with standard leukapheresis procedures. AEs reported during leukapheresis for CAR T-cell therapy are similar in type and frequency to those reported during stem cell apheresis for stem cell transplants [51,53]. Data on apheresis-associated AEs are currently being collected in CAR T-cell therapy clinical trials (e.g., CASSIOPEIA, COG AALL1721, ELARA, BELINDA) and may be valuable in further optimizing patient safety during collection. Acquisition of data from these clinical trials with regard to T-cell fitness and timing of collection is ongoing. Overall, AEs during leukapheresis are manageable and do not pose a barrier for CAR T-cell therapy.

Achieving adequate cell counts with leukapheresis

Appropriate ALC and/or CD3+ cell counts in the peripheral blood prior to leukapheresis are recommended to avoid failure of T-cell collection for CAR T-cell production. In a small single-institution study (N = 23), tisagenlecleucel manufacturing was successful even in heavily pre-treated adult patients with r/r DLBCL who underwent leukapheresis and patients with very low ALC (<100/μL) [17]. In two separate trials (PLAT-02 and ENCIT-01) that had the same apheresis collection parameters, pediatric patients (N = 99) were required to have ALC >100/μL in the peripheral blood, platelet count >50 000 and hematocrit 25% regardless of whether blood prime was utilized for CAR T-cell production [14]. The researchers concluded that an adequate number of CD3+ T cells in the apheresis product can be obtained even in heavily pre-treated patients with low lymphocyte counts (ALC <500/μL) [14]. In a retrospective study across three trials, pediatric patients (N = 71) with ALC <1.5 × 10^3/μL yielded less than the target number of CD3+ cells, and researchers concluded that these patients may require processing of larger blood volumes [13]. Overall, the observed correlation between pre-collection CD3+ lymphocytes and successful leukapheresis has led some centers to set ALC thresholds between 100/μL and 500/μL and a minimum of 150/μL for CD3+ T cells [54].

In adult patients with DLBCL and young adults with ALL who are undergoing leukapheresis for CAR T-cell manufacturing, depending on ALC at the time of leukapheresis, two to four total blood volumes are typically adequate to achieve a successful leukapheresis collection. If ALC or peripheral CD3+ cell counts are low, it may be necessary to increase the total blood volume collected; alternatively, more than 1 day of collection may be required. Other parameters recommended to optimize leukapheresis collection in these patients include having a pre-leukapheresis hematocrit >26% and a platelet count >30 000/μL. In some cases involving both small pediatric patients and older DLBCL patients, an additional collection day may be required to obtain adequate leukapheresis product to achieve sufficient cell counts [22,23]. Likewise, when there are ≥90% blasts in the peripheral blood and/or low CD3 and ALC because of prior therapy, there may be a need for delayed collection until the patient exhibits a more stable clinical status. However, in patients with highly refractory disease, it may be advantageous to proceed with 2-day leukapheresis. Patients with GVHD are commonly lymphopenic, making peripheral blood draws challenging [55]; thus, steady-state peripheral blood draws have been recommended for GVHD patients.

Patients who undergo leukapheresis for CAR T-cell manufacturing must achieve designated collection yield requirements that can be different for different product manufacturers, varying from specific cell counts to more general requirements for a pre-specified volume of blood processed. Tisagenlecleucel requirements for leukapheresis product include specific collected cell counts for CD3+ lymphocytes (≥1 × 10$^{9\ \text{CD3+ cells}}$), TNCs (≥2 × 10^9) and TNC CD3+% (≥3%) (Table 2). In the authors'

Table 2
Leukapheresis specifications for CAR T-cell manufacturing.

Tisagenlecleucel[a]	
CD3 count	$\geq 1 \times 10^9$
TNC count	$\geq 2 \times 10^9$
CD3/TNC%	≥3%

General CAR T-cell leukapheresis guidance[b]

ALC	Total blood volume processed
100 cells/μL	Consider 2-day collection
>100 cells/μL but <300 cells/μL	3.5–4×
>300 cells/μL but <500 cells/μL	3×
>500 cells/μL but <1000 cells/μL	2.5×
>1000 cells/μL	1.5×

CAR, chimeric antigen receptor; CD, cluster of differentiation; TNC, total nucleated cell.
[a] Provided courtesy of Novartis Pharmaceuticals Corporation.
[b] Based on the authors' clinical experience.

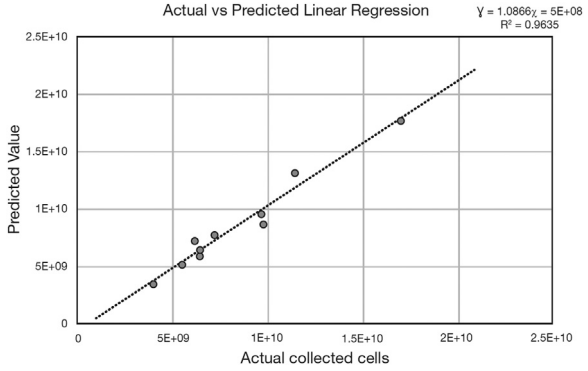

Fig. 3. Linear regression analysis of actual versus predicted leukapheresis collection. Relationship between actual yield of CD3+ cells collected during the data collection phase (10 procedures) and algorithm-predicted yield of CD3+ cells. The dashed line represents the linear regression between actual collected cells and predicted yield. Provided courtesy of Avera Medical Group, 2021.

experience, the measured cellular constituent counts are often rounded up in very small children, and this has not affected ALL CAR T-cell manufacturing outcomes, although an optimal cell count of 1.5×10^9 to 4×10^9 has been found to improve DLBCL CAR T-cell manufacturing outcomes. Alternatively, leukapheresis product requirements for other CAR T-cell therapies specify only blood volume to process.

At the University of Kansas Cancer Center, physicians have devised guidance for leukapheresis blood volume collections based on the patient's ALC (Table 2). Based on discussions with several leukapheresis centers, the use of defined cell counts is generally preferred to guarantee that a sufficient amount of material has been collected. An analysis of leukapheresis products that did not meet specifications found that they were associated with smaller blood volumes processed, shorter run times and smaller apheresis product volumes in patients with r/r DLBCL (N = 23) [17]. Overall, patients with low ALC and peripheral blood CD3+ cell counts were still able to obtain adequate CD3+ cell collection in the leukapheresis product [17]. Determining peripheral blood lymphocyte counts not more than 1 day prior to leukapheresis collection can aid in optimizing processed volumes and run times.

Methods for increasing the quality (fitness) and yield of T cells expanded from apheresis products of heavily pre-treated patients with r/r DLBCL and overall lower T cell counts should be explored. In support of this idea, an analysis of biomarker data from the ZUMA-1 trial of axicabtagene ciloleucel found that T-cell fitness in the final CAR T-cell product (estimated by global doubling time in culture) was associated with response [56]. Other studies have shown that prior therapy can affect T-cell fitness, and patients with DLBCL who received multiple rounds of chemotherapy had increased CD27 and CD28 senescence, resulting in lower T-cell expansion and viability when cultured with anti-CD3/CD28 beads [57]. Antagonism of certain pathways may have influenced the expansion of polyfunctional T cells, improving the leukapheresis product collected from heavily pre-treated patients. Antagonism of vasoactive intestinal peptide signaling in combination with inhibition of phosphatidylinositol 3-kinase delta decreased T-cell differentiation during bead-mediated expansion, resulting in a significant increase in the number of T cells (83.7%) cultured from lymphoma patients [57].

Improving leukapheresis collection efficiency

An established leukapheresis collection algorithm can improve collection success and prevent overcollection. Collection efficiency can be affected by several factors during leukapheresis, including higher platelet counts, high numbers of circulating malignant cells and the patient's age [58]. A retrospective analysis of leukapheresis outcomes from three clinical trials of CAR T-cell therapy found that the baseline peripheral blood CD3+ cell count can be used to estimate collection efficiency and customize the length of leukapheresis for each patient [13]. Determining the leukapheresis parameters for optimal collection efficiency is especially important for low-weight/pediatric patients. A retrospective analysis of heavily pre-treated pediatric patients with r/r ALL found that baseline peripheral blood CD3+ counts were variable and did not correlate with the patient's age or previous therapies [59]. Despite low T-cell collection efficiency, most patients achieved the target T-cell count needed for manufacture [59]. Another retrospective analysis evaluated collection efficiency in a cohort of heavily pre-treated pediatric patients with r/r ALL, lymphoma and neuroblastoma from two CAR T-cell therapy trials [14]. The planned collection volume was based on the total mononuclear cell count, and the researchers acknowledged that this method underestimated or overestimated the T-cell collection efficiency based on the wide range of CD3+ T-cell recovery observed (3–2623%). Despite the wide range in collection efficiency, an adequate number of T cells were able to be collected in these patients. Overall, these retrospective studies suggest that leukapheresis has been safe and effective despite highly variable collection efficiency; however, methods to improve collection efficiency using a prediction algorithm may be beneficial. Several groups have developed prediction algorithms to optimize collection efficiency and tailor the volume of blood processed to collect the desired target cell counts, with a goal of avoiding undercollection and preventing the need for additional collection days [60].

The Avera Medical Group developed a collection algorithm that routinely achieves 74% collection efficiency. Based on their experience, the group also devised a leukapheresis prediction model that demonstrated a high correlation between the predicted and actual number of cells collected (Figure 3). This suggests that this prediction model may be useful in determining which patients' T cells can be successfully collected and the amount of time needed for collection. The predictive collection algorithm was developed after performing 10 leukapheresis procedures using the processing volume recommended by the manufacturer. To better predict leukapheresis collection efficiency, validation for use in clinical practice must be further explored. The collection efficiency for each procedure was determined as follows:

$$\textit{Collection efficiency}(CE)(\%) = \frac{\textit{Total CD3 + lymphocyte count in leukapheresis material } CD3^+ \textit{ (cells)}}{\textit{Peripheral } CD3^+ \textit{ lymphocyte count } (\text{cells}/\mu\text{L}) \times 10^6 \mu\text{L}/L \times \textit{Total blood volume processed } (L)} \times 100$$

The mean collection efficiency of the 10 procedures was calculated to be 74%. The target CD3+ yield was set to 4×10^9 cells—higher than the recommendation for tisagenlecleucel ($\geq 1 \times 10^9$)—to allow for a margin of error. The estimated blood volume needed to process the target CD3+ yield was calculated as follows:

$$\text{Blood volume } (L) = \frac{\text{Target CD3}^+ \text{ yield (cells)}}{\text{Peripheral CD3}^+ \text{ lymphocyte count } (\text{cells}/\mu\text{L}) \times 10^6 \mu L/L \times \text{Mean CE } (\%)}$$

Following 10 collections, the leukapheresis prediction algorithm was used to compare the actual CD3+ yields with predicted CD3+ yields. The predicted yield for each collection was calculated by substituting the actual total blood volume (L), peripheral CD3+ lymphocyte count (cells/μL) and mean collection efficiency (%). A strong linear correlation (R^2 = 0.9635) between the collected CD3+ cells and the predicted CD3+ value was observed (Figure 3). Based on the strong linear correlation, the authors were able to successfully use the prediction algorithm in subsequent leukapheresis collection procedures.

Excess leukapheresis product

It is possible for a robust leukapheresis collection to result in collection of more cells than are needed for manufacturing. There are several theoretical options that may be available for patients with excess leukapheresis product, including the potential to provide consent to donate excess cells to research or to store excess cells for potential future use. Storage of excess cryopreserved leukapheresis product may be possible at some treatment centers or manufacturers, and in some cases it may be possible to store cells at independent storage cell banks. The ability to store excess leukapheresis product at a treatment center or manufacturer is largely dependent on the practices of the treatment center or manufacturer and the availability of storage space. There may be costs associated with the storage of cells, and in some cases the willingness of patients to pay for long-term storage may be a factor.

Discussion

Herein the authors have provided guidance for improved leukapheresis for CAR T-cell manufacturing based on our clinical experience. Optimization of the leukapheresis process is clearly important for achieving a high rate of CAR T-cell manufacturing success. A basic requirement for CAR T-cell manufacturing is the ability to collect a sufficient number of viable T cells during leukapheresis. Leukapheresis collection may be optimized in several ways to achieve high CAR T-cell manufacturing success, including via appropriate staff training, alignment of collection days with manufacturing availability, potential collection of patient cells earlier during the course of the disease, appropriate washout of prior therapies to minimize potential negative effects on T cells, utilization of a prediction algorithm to improve leukapheresis collection efficiency, same-day shipment or cryopreservation of the leukapheresis product following collection and observation of recommendations for collection from pediatric patients with low body weight (Table 3).

Conclusions

A deeper understanding of the potential influence of prior therapies on leukapheresis collection and CAR T-cell therapy outcomes is needed. Future studies that evaluate the phenotype of T cells in peripheral blood prior to leukapheresis and determine the immunomodulatory effects of chemotherapy on T cells will be beneficial in further optimizing leukapheresis collection for CAR T-cell therapy. As our understanding grows through knowledge gained from clinical trials and real-world experience, the timing and mechanics of leukapheresis and processing of the leukapheresis product will likely be further optimized to achieve the best possible outcomes for our patients.

Table 3
Key considerations for leukapheresis collection to ensure optimal T-cell fitness and promote CAR T-cell manufacturing success.

Timing	Considerations
Before leukapheresis	• Consider leukapheresis and cryopreservation early in the course of disease for patients with aggressive/high-risk disease. • Appropriately timed washout of chemotherapy, medications and other agents prior to leukapheresis collection is important to optimize T-cell fitness. • Provide therapy to reduce the number of circulating blasts prior to leukapheresis for patients with high disease burden (when possible). • Verify adequate ALC and/or CD3+ cell count day prior to leukapheresis. • Evaluate the need for a second day of collection using prediction algorithm. • Take into account patient-specific considerations (e.g., infants).
After leukapheresis	• Cryopreservation of leukapheresis material the same day as collection can improve post-thaw cell viability and manufacturing outcomes. • Verify that post-collection leukapheresis specifications for CAR T-cell manufacturing have been met.

Declaration of Competing Interest

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Drafting or revising the manuscript: MQ, JPM, GDM, VP, EKW, PH, MR, LFC and JW. All authors have approved the final article.

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