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Alignment of practices for data harmonization across multi-center cell therapy trials: a report from the Consortium for Pediatric Cellular Immunotherapy

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ABSTRACT

Immune effector cell (IEC) therapies have revolutionized our approach to relapsed B-cell malignancies, and interest in the investigational use of IECs is rapidly expanding into other diseases. Current challenges in the analysis of IEC therapies include small sample sizes, limited access to clinical trials and a paucity of predictive biomarkers of efficacy and toxicity associated with IEC therapies. Retrospective and prospective multi-center cell therapy trials can assist in overcoming these barriers through harmonization of clinical endpoints and correlative assays for immune monitoring, allowing additional cross-trial analysis to identify biomarkers of failure and success. The Consortium for Pediatric Cellular Immunotherapy (CPCI) offers a unique platform to address the aforementioned challenges by delivering cutting-edge cell and gene therapies for children through multi-center clinical trials. Here the authors discuss some of the important pre-analytic variables, such as biospecimen collection and initial processing procedures, that affect biomarker assays commonly used in IEC trials across participating CPCI sites. The authors review the recent literature and provide data to support recommendations for alignment and standardization of practices that can affect flow cytometry assays measuring immune effector function as well as interpretation of cytokine/chemokine data. The authors also identify critical gaps that often make parallel comparisons between trials difficult or impossible.

Introduction

Cellular immunotherapy using autologous or allogeneic T cells genetically modified to express chimeric antigen receptors (CARs) or antigen-specific T-cell receptors has proven to be a transformative therapy for some cancer patients. Pivotal phase 1/2 pediatric clinical trials have led to approval of a CD19-directed CAR, tisagenlecleucel (Kymriah), as the first "living drug" for children with relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL) [1,2]. Although these trials provided a proof of concept for immune effector cell (IEC) efficacy and safety, the widespread clinical utilization of cellular therapies in pediatrics beyond B-cell malignancies continues to face a number of barriers, including a lack of predictive biomarkers for toxicity and efficacy, small patient cohorts and uniform access to trials [3–7]. As broadened application of immunotherapies occurs, there is a significant need for in-depth characterization of IECs and the immune landscape to identify mechanisms of resistance and to help guide development of the next generation of T-cell therapies [8–11].

Multi-center research extends increased access to cell therapy trials and provides larger sample sizes for more effective experimental and clinical analyses. However, the ability to compare correlative data across sites, within the same study or across studies remains a major challenge. In the context of hematopoietic cell transplant (HCT) studies, previously published work has emphasized the need to harmonize trial design, including selection of similar endpoints, immune monitoring strategies and time points, for collection of research samples [3,12–17]. Harmonization is critical to improve reproducibility in analyte measurement, reduce variability and ultimately yield greater confidence in correlative data and results. Robust datasets that accommodate side-by-side comparison in early-phase cell therapy trials are necessary to establish biomarkers that correlate with efficacy and clinical criteria for grading and treatment of immune effector-related toxicities [18,19]. Acquiring such datasets will require consistency in the handling of biologic samples as well as

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the use of analytic variables that can influence the generalizability of biomarkers across trials.

Evaluation of biologic samples collected from diverse sources, including peripheral blood, bone marrow, cerebrospinal fluid (CSF), tumor tissue and tumor-infiltrating lymphocytes, is key to identifying biomarkers of safety and efficacy in cell therapy trials. Common assays used to evaluate samples are (i) plasma or serum cytokine analysis, (ii) phenotypic and functional characterization of IECs by flow cytometry [20–25] and (iii) detection of persistence of IECs by polymerase chain reaction (PCR) [1]. Consistency and reproducibility of these assays can be affected by many factors: pre-analytic (patient- and sample-related), analytic (assay-related) and post-analytic (data-related) (Figure 1). Many recently published studies have highlighted the challenges of reducing analytic and post-analytic variability across multiple assay platforms, studies or sites [26–30]. One way to reduce analytic variability is to perform correlative experiments using batched samples at central sites with specialized instrumentation and expertise. However, pre-analytic variables such as biospecimen collection and processing procedures can also affect downstream results [31,32]. For example, phenotypic and functional markers of cell differentiation, activation and exhaustion are most sensitive to pre-analytic variables such as the choice of anticoagulant in blood collection tubes and the timing from collection to processing and cryopreservation [33–36]. Further, PCR detection assays are inhibited in DNA samples extracted from whole blood collected in heparin anticoagulant tubes [37]. Finally, the detection of many cytokines is affected by the choice of anticoagulant and processing procedures [38–40]. To enable data comparisons of correlative analysis across sites participating in multi-center studies as well as inter-study analysis, it is imperative that biospecimen collection be consistent and that guidance is provided to referring institutions where specimens are collected and shipped to participating study sites for testing using validated assays.

The authors set out to overcome some of the challenges of multi-center research by developing a committee of experts from four institutions participating in the Consortium for Pediatric Cellular Immunotherapy (CPCI). The CPCI institutions have significant experience with cellular immunotherapy research, conducting several trials testing CAR T cells directed against single- and multi-target antigens (e.g., NCT02028455, NCT03500991, NCT03638167, NCT03500991, NCT02311621, NCT03330691), non-gene-modified antigen-specific T cells (NCT01956084) and natural killer cells in combination with other immunotherapies (NCT02573896). The authors compared standard operating procedures (SOPs) at CPCI sites and reviewed published literature related to key pre-analytic variables, including specimen collection, shipping, processing and analysis of biologic samples in cytokine- and flow-based assays. The authors' objective was to identify commonalities and opportunities for alignment and provide best practice recommendations to promote consistency in sample handling across multi-center cell therapy trials. Here the authors provide an in-depth analysis of some of the pre-analytic variables that affect sample integrity in the context of cytokine analysis and immunophenotyping by flow cytometry (Figure 1). The authors further provide analytic recommendations for performing basic cytokine- and flow-based panels, which, when used uniformly across different cell therapy trials, could provide statistical power to interpret data across sites and between trials.

Methods

The reader is invited to refer to supplementary Table 3 for a comprehensive list of all reagents used as well as vendor information and catalog numbers.

CPCI correlative working group and gap analysis

The CPCI working group consisted of representatives from each participating site and included experts in clinical trial design, laboratory scientists and physician–scientists. The group met regularly to review and align practices across sites. This effort involved independent review of SOPs at each site, identification of similarities and gaps between SOPs, systematic review of the literature and analysis of relevant cell therapy data.

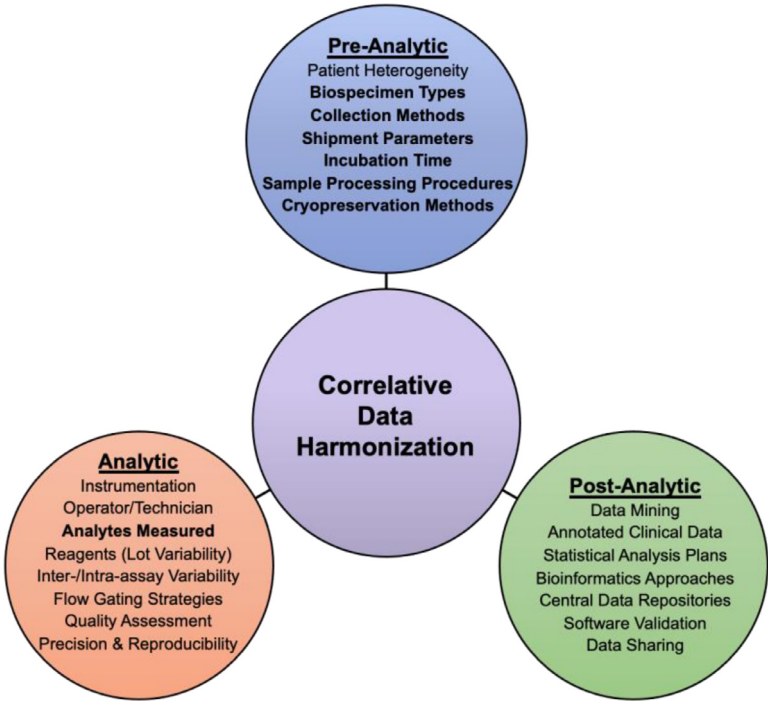

Fig. 1. Pre-analytic, analytic and post-analytic variables that impact correlative data harmonization efforts across sites participating in multi-center cell therapy trials. Pre-analytic (patient- and sample-related), analytic (assay-related) and post-analytic (data-related) factors can impact consistency and reproducibility of assays commonly used to evaluate IEC trial-related samples. An in-depth discussion of the variables highlighted in bold is included within the scope of this work, including pre-analytic variables related to biospecimen collection and processing, in addition to providing recommendations for analytes to be measured using cytokine- and flow-based assays. IEC, immune effector cell.

Cell thawing

Cryopreserved cell vials were thawed quickly in a 37°C water bath before slowly diluting the cells in R10 media consisting of Roswell Park Memorial Institute (Gibco) with 10% fetal bovine serum (Atlas) and 1% L-glutamine (Gibco). Cells were washed with R10 media before allocation to downstream assays. Cells were either stained immediately post-thaw or incubated in the relevant conditions for other analyses.

Whole specimen peripheral blood staining

A maximum of 2 mL of peripheral blood specimen collected in ethylenediaminetetraacetic acid (EDTA) was lysed with red blood cell (RBC) lysis buffer (Invitrogen) before counting the recovered number of white blood cells. For cell yields below the maximum allowable number of cells for staining assays, the entire recovered cell product was stained.

Recovery of mononuclear cells, CD34 and CD3 in cryopreserved versus freshly isolated samples

Percent recovery of cryopreserved and fresh peripheral blood mononuclear cell (PBMC) samples was compared; fresh samples were normalized to 100% (Figure 3A; also see supplementary Table 2). Mononuclear cell recovery was calculated by comparing mononuclear cell counts prior to freezing with live cell counts of the thawed cells for each sample. Similarly, CD3 and CD34 recovery was calculated by comparing flow cytometry expression between the fresh samples prior to cryopreservation and the subsequently thawed samples. Cell viability data were calculated by examining 7-aminoactinomycin D (7-AAD) expression of the thawed cells. Non-viable cells that had lost membrane integrity were identified by uptake of 7-AAD (i.e., 7-AAD-positive cells by flow cytometry) and were excluded from the analysis.

Flow staining

Samples were treated with viability dye before incubating with Fc receptor block (Miltenyi Biotec) to inhibit non-specific antibody binding. A cocktail of CD3 V450, CD4 BV605, CD8 PerCP-Cy5.5, CD36 fluorescein isothiocyanate (BD Biosciences) and truncated epidermal growth factor receptor (EGFRt) allophycocyanin and Her2tG-biotin (custom conjugates; BD Biosciences) diluted in Brilliant Stain Buffer (BD Biosciences) was used for surface staining. Antibody staining was performed at room temperature in the dark for 20 min. Cells were washed twice with phosphate-buffered saline (Gibco) between all staining and fixation steps. Secondary staining with streptavidin BUV395 (BD Biosciences) was performed in Brilliant Stain Buffer in the dark for 20 min at room temperature. Cells were fixed with 2% paraformaldehyde before acquisition on an LSRFortessa (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

CAR T-cell rapid expansion protocol

Cryopreserved T cells previously transduced to express CAR constructs were thawed and mixed at a standard ratio with irradiated target cells bearing the target antigen of interest and co-stimulatory molecules. Cells were cultured in R10 media supplemented with recombinant human IL-15 and IL-2 cytokines at 37°C for up to 21 days post-thaw. Cultures were split on a biweekly basis to allow for cell expansion and to replenish the cytokine supplements. Samples were removed from culture on a regular basis for immediate use in cell staining assays.

IL-2 resting post-thaw

Cryopreserved cells were thawed in R10 media. Following removal of an aliquot for staining, the remaining cells were divided and pelleted by centrifugation. Cell pellets were resuspended in either R10 media or R10 media supplemented with recombinant human IL-2 cytokine at 50 U/mL. Cells were incubated at 37°C in cell culture flasks for 2 days. Cells were removed from culture on day 1 and at the termination of culture on day 2 for immediate use in cell staining assays.

Results

Specimen type and sample collection methods affect downstream assays

Prior to analyzing samples from different sites in a multi-center cell therapy trial or across different trials, it is important that sites align regarding which biospecimen types to collect and how to process samples of interest. All CPCI sites had well-defined collection and isolation procedures for PBMCs, plasma or serum, and some sites also had practices for collecting bone marrow, CSF or tumor tissue, depending on the specific clinical trial requirements. Major gaps identified between CPCI site trials related to specimen collection and type were (i) time points of sample collection before and after IEC infusion, (ii) type of anticoagulant used in blood collection tubes and (iii) use of plasma versus serum to assess cytokines (Table 1). Collection time points varied based on the nature of the underlying disease, patient population and logistics of collection and were driven primarily by clinical trial aims. Sample types used for correlative assays and methods of collection and initial processing offered opportunities for alignment, as discussed in the following sections.

Sodium heparin and EDTA are common anticoagulants in most clinical flow cytometry assays. The authors found that three out of four CPCI sites isolated plasma instead of serum for cytokine analysis in cell therapy trials. Sodium heparin was the preferred anticoagulant for collection tubes compared with EDTA, especially when prioritizing plasma isolation, because sodium heparin permits plasma and PBMC isolation from a single collection tube, whereas serum is isolated in a red top tube lacking anticoagulant. Constraints caused by phlebotomy limits (i.e., typically 3 mL/kg with a maximum of 20–40 mL per collection) for safety in children favor sodium heparin tubes for plasma collections. Ultimately, the cytokine(s) being measured in downstream assays dictated the site's choice to isolate plasma versus serum. Interestingly, cytokine levels can be higher in serum compared with plasma (Table 2), possibly related to higher non-specific background found in serum [38,39]. For example, measurement of transforming growth factor beta (TGFβ), a key cytokine whose overexpression in solid tumors is associated with immunosuppression and resistance to CAR T-cell therapy [41–43], requires centrifuging samples at very high speeds to remove contaminating platelets that can aggregate, release TGFβ and confound results [44,45]. The authors suggest isolating plasma instead of serum to detect low-level changes in abundance, especially for cytokines that may exhibit transient systemic elevations in response to IEC infusion.

Shipment considerations for biologic samples collected in cell and gene therapy trials

With multi-site trials, local participating institutions are required to ship samples, either fresh or frozen, to a centralized laboratory for advanced analysis (e.g., NCT02028455, NCT03330691, NCT03684889). Consideration should be given to packaging material, shipping temperature and integrity of the shipping container upon arrival at a central site for processing and analysis. If possible, the recipient laboratory should monitor factors affecting sample

Table 1
Gap analysis of general collection, shipment, PBMC isolation and cryopreservation practices across CPCI sites.

	Site A	Site B	Site C	Site D	Gap identified
Blood collection tubes					
Plasma isolation	EDTA	Sodium heparin	Sodium heparin	Sodium heparin	X
Serum isolation	Red top	–	–	–	X
Shipment of Correlative Samples					
Blood/bone marrow temperature	Ambient	Ambient	Ambient	Ambient	
CSF temperature	4°C	4°C	4°C	4°C	
Shipment container	Temperature-controlled	Temperature-controlled	Temperature-controlled	Temperature-controlled	
Qualified courier	Local courier	Local courier	Local courier	Local courier	
Density gradient centrifugation					
Whole blood lysis step to remove RBCs	Yes	Yes	Yes	Yes	
Tube type	50-mL SepMate	50-mL SepMate	50-mL conical	50-mL Leucosep	X
Speed, × g	830	1200	400	800	X
Time, min	20	10	30	15	X
PBMC centrifugation					
Speed, × g	250	400	400	500	X
Time, min	10	5	10	10	X
Cryopreservation					
Cell freezing	Media + 10% DMSO	Media + 10% DMSO	Media + 10% DMSO	Media + 10% DMSO	
Average cell number cryopreserved/vial	$5–10 \times 10^6$	$5–10 \times 10^6$	$5–10 \times 10^6$	$5–10 \times 10^6$	

CSF, cerebrospinal fluid; RBCs, red blood cells; PBMC, peripheral blood mononuclear cells; DMSO, dimethyl sulfoxide.

Table 2
Serum/plasma mean fluorescence intensity of cytokines commonly assessed in cell therapy trials.

Cytokine	Representative cytokine clinical trials	Ratio of serum: plasma MFI
GM-CSF	NCT02933333	2.615
	NCT04408092	
	NCT01495637	
	NCT03769844	
	NCT02502786	
IFNα	NCT04534634	1.206
IFNγ	NCT01965327	1.584
	NCT02593773	
	NCT03548818	
	NCT02797080	
	NCT03378102	
	NCT03063632	
IL-1α	N/A	1.491
IL-1β	N/A	2.225
IL-1RA	NCT04169022	1.226
	NCT02780583	
IL-2	NCT03138889	2.259
	NCT02983045	
	NCT03282344	
	NCT03435640	
	NCT02983045	
	NCT02350673	
	NCT02627274	
	NCT03386721	
	NCT03063762	
	NCT03063762	
IL-4	N/A	2.134
IL-6	N/A	4.058
IL-8	NCT03400332	1.280
IL-10	NCT02923921	1.433
IL-15	NCT02989844	2.868
	NCT01875601	
	NCT02465957	
	NCT01385423	
	NCT01369888	
	NCT02689453	
	NCT02384954	
TNFα	NCT03293784	1.986
TGFβ	NCT02423343	2.506
	NCT02734160	
	NCT02581787	
	NCT03451773	
	NCT03451773	
VEGF	NCT01984242	3.393

Representative NCT numbers were sourced from Berranondo et al. [39] and clinicaltrials.gov. Ratio of MFI values were referenced from Rosenberg-Hasson et al. [38]. IFNα, interferon alpha; NCT, National Clinical Trial; MFI, mean fluorescence intensity; VEGF, vascular endothelial growth factor.

integrity, such as the temperature at which the sample was shipped/received, the accuracy of patient identifiers and the collection tube type and expiration and document any deviations prior to processing. A recommended checklist for biospecimen receipt criteria is shown in supplementary Table 1.

A comparison of shipping practices between CPCI sites revealed that all groups ship and receive blood and bone marrow at ambient temperature, preferably in insulated containers that limit temperature fluctuation (e.g., ClinPak) (Table 1). Some specimen types, such as CSF, required special pre-processing at the clinical trial site prior to shipment to a central laboratory. More specifically, immediately after collection, fresh CSF was transported on ice and centrifuged as soon as possible at 4°C. The supernatant portion intended for cytokine analysis was isolated, aliquoted and shipped frozen on dry ice to ensure high sample integrity for future batched analysis. CSF cell pellets generated by centrifugation were intended mainly for flow analysis—for example, to assess immune cell infiltration or IEC persistence. Cell pellets were diluted in media and shipped at 4°C on ice or in a temperature-controlled shipping container (e.g., NanoCool). Regardless of specimen type, biologic samples for cell therapy trials ideally should be shipped the same day as collection to preserve sample integrity. All CPCI sites used a shipping courier with the ability to deliver the day after pickup.

Incubation time between sample collection and processing must be validated and standardized for follow-up time points

As local referring institutions become more involved in collecting and shipping follow-up samples to central sites for analysis, the incubation time between sample collection and processing lengthens. Although this workflow aims to increase patient access to clinical studies and eliminate their burden to travel to study sites for long-term follow-up, extended incubation time may compromise sample integrity [46]. Phenotypic and functional cell surface markers can be rapidly downregulated or cleaved over time, and fluctuations in cytokine concentrations in blood and CSF can occur after sample collection [36,47]. If shipping fresh samples, the impact of incubation time between collection and processing must be investigated and quantified for each type of specimen (e.g., blood, bone marrow, CSF) and assay, and the stability of key biomarkers after collection must be defined.

The authors analyzed validation data generated by a CPCI site to determine the maximum incubation time between blood collection and processing prior to signal loss. To determine post-collection stability standards for T- and B-cell surface markers commonly identified in

	Range				Assay Precision Range			
Post-Collection Timepoint	D2	D3	D4	D7	D2	D3	D4	D7
CD3+	0	0	1	2	0	0	1	2
CD4+	0	0	0	1	0	0	2	2
CD8+	0	1	2	1	0	2	2	2
CD3-	1	0	3	4	0	0	1	3
CD19+CD22+	2	2	3	3	2	2	3	3
Total Number of Flags	3	3	9	14	2	4	9	12
Out-of-Range Rate (%)	15	15	45	55	10	20	45	60

Fig. 2. Maximum incubation time (3 days) between blood collection in EDTA tubes and initial processing prior to loss of common T- and B-cell surface markers.

For baseline analysis (left side), a range of −20% to +20% of the day 1 value for all samples and populations was used for quantitative analysis. Subsequent time point population values were examined and flagged if out of range. The out-of-range rate was calculated at each time point by the number of flagged populations out of the 20 total parameters examined per time point (five populations for each of the four samples per time point). Total number of flags and out of D1 range rates are shown on the left. For inter-assay precision analysis (right side), the %CV for each population at each time point compared with the D1 value was determined. Values were flagged if out of range. Total number of flags and out-of-inter-assay-range rates are shown on the right. D1, day 1; %CV, percent coefficient of variation.

cellular immunotherapy, peripheral blood from four healthy donors was collected in EDTA tubes and stored at room temperature until processing on day 1, day 2, day 3, day 4 and day 7 post-collection (day 0 = day of collection). Quantitative stability analysis was performed by determining the last time point at which the observed five cell surface markers were within a ± 20% range of the day 1 baseline value or a minimum of 80% of the observed markers were within the inter-assay precision range from day 1 [48]. The authors concluded that beyond day 3, specimen quality was degraded such that benchmark assay levels were unacceptable (Figure 2). These data highlight the need for sites to establish acceptability criteria for fresh sample incubation time that are consistently applied across multi-center clinical sites.

Impact of fresh versus cryopreserved samples on cell therapy trial correlative studies

The authors first compared standard practices for isolating PBMCs across all four CPCI sites regardless of whether freshly isolated or frozen samples were used for analysis. PBMC isolation by Ficoll gradient centrifugation of diluted whole blood at room temperature was performed at all four sites. Although centrifugation lengths, speeds and tube types used for Ficoll purification differed between labs (Table 1), all CPCI sites washed isolated PBMCs with a buffered salt solution, and a whole blood lysis step was performed to remove RBCs prior to flow analysis. Isolated PBMCs were then used either fresh or frozen in downstream correlative assays. Given the limited amount of whole blood that can be drawn from pediatric patients, an important consideration is anticipating low abundance of cells of interest (e.g., engineered cells, target cells). In cases where collection volume is low, the authors recommend performing Ficoll in a smaller tube format (e.g., 15-mL tube versus the typical 50-mL tube) and eliminating the RBC lysis step because Ficoll gradient centrifugation may be sufficient to remove contaminating RBCs from low-volume samples. Additionally, for downstream functional assays using PBMCs isolated from low-volume samples, the authors recommend expanding PBMCs *ex vivo* to achieve a higher frequency of effector cells prior to analysis. *Ex vivo* PBMC expansion prior to analysis may affect the phenotype and functional profile of the expanded cells; therefore, if performed, this expansion should be implemented consistently across samples in the study.

Frozen sample collections facilitate batched sample runs at a central laboratory and can be performed after all clinical endpoints are satisfied. Batched analysis aims to minimize experimental variability and allows for direct comparison between post-drug product infusion time points. Although there is no definitive evidence to support using fresh versus frozen samples for analysis in CAR T-cell or HCT studies, some previously published work assessing T- and B-cell subsets in human PBMC samples has reported that there are no observed differences between fresh versus frozen samples for some surface and intracellular markers [49,50]. Conversely, other groups have shown that cryopreservation can introduce variables that impact sample integrity, including viable cell recovery and stability of cell populations between freeze–thaw [34,51–53]. The authors measured recovery of CD34 and CD3 expression in participant-matched fresh versus frozen human PBMCs and found that although there was no difference in CD34 recovery (paired *t*-test, $P = 0.0752$), there was a significant reduction in CD3 detection in frozen samples compared with fresh (paired *t*-test, $P = 0.0004$) (Figure 3A; also see supplementary Table 2).

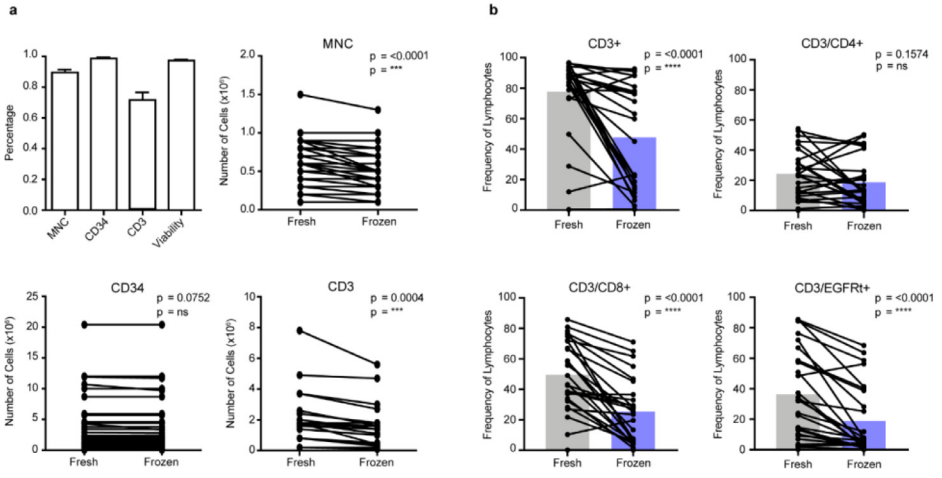

Fig. 3. Recovery of MNCs and CD34$^+$ and CD3$^+$ cells in freshly isolated versus cryopreserved samples and CD3 and CAR marker detection in fresh versus cryopreserved matched pairs. (A) Comparison of percent recovery of cryopreserved and fresh participant-matched PBMC samples; fresh samples normalized to 100%. MNC recovery was calculated by comparing MNC cell counts prior to freezing with live cell counts of the thawed cells for each sample (n = 60). Percentages of expression of each antigen are derived from lymphocytes of CD45$^+$ events. Similarly, CD3$^+$ (n = 20) and CD34$^+$ (n = 46) recovery was calculated by comparing flow cytometry expression between the fresh and thawed samples. Cell viability data (n = 62) were calculated by examining 7-AAD expression of the thawed cells. Significance was determined using paired *t*-test. (B) Target population recovery/stability was evaluated for CAR T cells by examining the frequency of lymphocytes expressing markers of interest in participant-matched fresh and cryopreserved cells. Fresh samples (n = 26) were obtained from peripheral blood specimens treated with RBC lysis prior to staining, whereas frozen samples (n = 26) underwent MNC isolation by Ficoll density gradient separation prior to cryopreservation. Gating strategy included viable singlet non-myeloid cell isolation prior to selection of the lymphocyte population of interest. Significance was determined using the Wilcoxon matched pairs signed-rank test. MNC, mononuclear cell. ns, not significant. ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

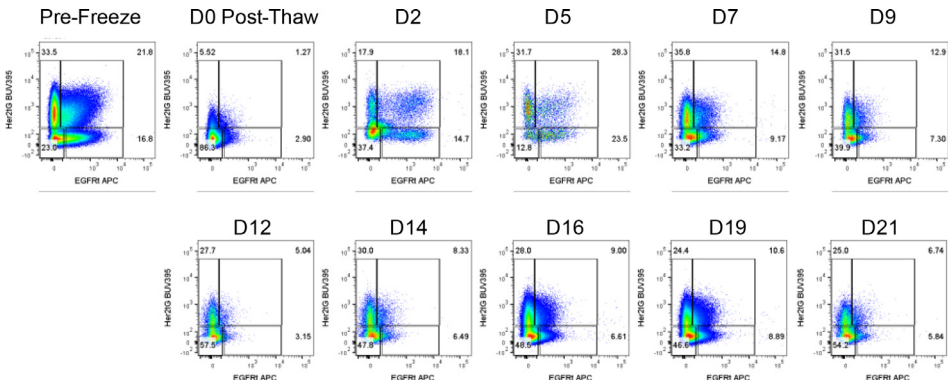

Fig. 4. Time course of CAR T-cell transduction marker detection upon co-culture with target cells. Healthy donor T cells underwent transduction with two CAR constructs expressing either the EGFRt or Her2tG reporter molecule. Following the post-transduction expansion period, an aliquot was removed for flow cytometry staining and the remaining culture was cryopreserved. Cells were later thawed for stimulation and culture expansion via CAR T-cell REP, with periodic sampling for flow cytometry analysis of the CAR reporter molecules. Although detection of EGFRt and Her2tG CAR markers dramatically decreased immediately post-thaw, Her2tG expression and detection recovered to pre-freeze levels during the course of the culture. APC, allophycocyanin; D, day; REP, rapid expansion protocol.

The authors further confirmed the reduction in CD3 enumeration in frozen samples in a separate analysis comparing cell marker detection in 26 participant-matched fresh versus frozen PBMC samples from pediatric subjects who received CD19-directed CAR T cells (NCT02028455) (Figure 3B). The percentages of $CD3^+$ and $CD8^+$ cells were significantly decreased in frozen samples compared with fresh (Wilcoxon matched pairs test, $P < 0.0001$), whereas the percentage of $CD4^+$ cells was not significantly different ($P = 0.1574$). Similarly, the authors examined the effect of cryopreservation on EGFRt, which is used as a cell surface marker tag to detect transduced CAR T cells [24,54–56]. Interestingly, detection of CAR T cells was also significantly decreased in frozen PBMC samples compared with fresh (Wilcoxon matched pairs test CD3/$EGFRt^+$, $P < 0.0001$). Together, these data show that cryopreservation can affect detection of cell populations of interest in cell and gene therapy trials. They also highlight the benefit of analyzing freshly isolated samples to accurately monitor the status of cell populations in real-time in subjects receiving CAR T-cell therapy.

Cell and gene therapy products are typically cryopreserved at the end of manufacturing and then thawed at the bedside for infusion [57,58]. The authors next examined the effect of cryopreservation on CAR T-cell marker detection using a dual-transduced, bispecific CAR product (Figure 4). The product was stained pre-freeze and post-thaw with cetuximab and trastuzumab antibodies to detect the CAR transduction markers EGFRt and Her2tG, respectively. Immediately upon thaw, cells were co-cultured with target cells expressing CAR-specific antigens. Compared with pre-freeze, CAR T cells were not readily detectable post-thaw (day 0) via flow cytometry, and the product appeared predominantly CAR-negative. The ability to detect CAR T cells was rescued by day 2 of culture, and percentages continued to increase between day 2 and day 5. It is unclear if the recovery of CAR T cells was due to improved stability or detection of the CAR markers or whether co-culture with antigen-expressing target cells promoted expansion of the CAR T cells over time. Both factors may have affected CAR positivity of the product post-thaw. By day 7 and onward, EGFRt detection declined, whereas Her2tG detection remained relatively consistent over time.

Together, these data show that results from thawed PBMC samples and cell products may not accurately reflect pre-freeze characteristics. They further emphasize the need to consider the stability of the phenotypic and transduction markers used to identify cell populations in fresh versus frozen cell therapy samples and products and their persistence in peripheral blood over time. The authors recommend fresh analysis when possible to monitor toxicities in real-time and to eliminate pre-analytic variables introduced by cryopreservation.

Resting effector cells in cytokine-containing media may improve the recovery of cell markers affected by cryopreservation

Although analysis of fresh samples is preferred, this workflow may not optimize adherence to follow-up sample collection schedules at referring institutions, especially when local providers may be far from participating study sites. If samples cannot be processed, shipped and analyzed in real-time, cryopreservation likely will be necessary for expanding patient access and utilization of cellular therapies. A comparison of cryopreservation methods across CPCI sites revealed that all sites were consistent in their practices, using freezing media containing 10% dimethyl sulfoxide and initiating freezing in a commercial freezing container (e.g., Nalgene Mr. Frosty) followed by long-term storage in liquid nitrogen (Table 1). Samples were cryopreserved in aliquots to prevent repeat freeze–thaw cycles, and the size of aliquots for plasma and PBMCs or bone marrow mononuclear cells was validated by each site per their SOPs.

Related to the effect of cryopreservation highlighted previously, there is evidence to suggest that resting of cryopreserved samples post-thaw can promote recovery of T-cell function and restore several phenotypic and functional markers [59,60]. Using a frozen cell therapy product as an example, the authors sought to determine the impact of thawing with or without resting on the ability to detect CAR T-cell populations. A dual-transduced, bispecific CAR product was thawed and then stained with cetuximab and trastuzumab antibodies to detect EGFRt-tagged and Her2tG-tagged CAR T cells, respectively. The product was stained immediately post-thaw (day 0), after 24 h of rest and after 48 h of rest (Figure 5). Cells were rested with or without the addition of IL-2 to the media. Similar to that shown in Figure 4, the authors found that the ability to detect surface expression of CAR T-cell markers, especially EGFRt, was suboptimal immediately post-thaw compared with detection in fresh samples. In the absence of IL-2, 24- or 48-h resting was able to improve detection of the Her2tG tag but not the EGFRt tag. Interestingly, supplementing media with IL-2 during the 24-h rest markedly improved detection of EGFRt from 1.96% post-thaw to 24.2% compared with resting in media without IL-2, which had a slight increase in detection to only 4.5%. No further increase in EGFRt was detected after 48 h of rest compared with 24 h in IL-2-supplemented media. These data show that stability and detection of CAR transduction markers are impacted by freeze–thaw. If cryopreservation is unavoidable, however, these data support incorporation of a resting step with IL-2 upon thaw to promote recovery of CAR T-cell markers of interest prior to downstream assays.

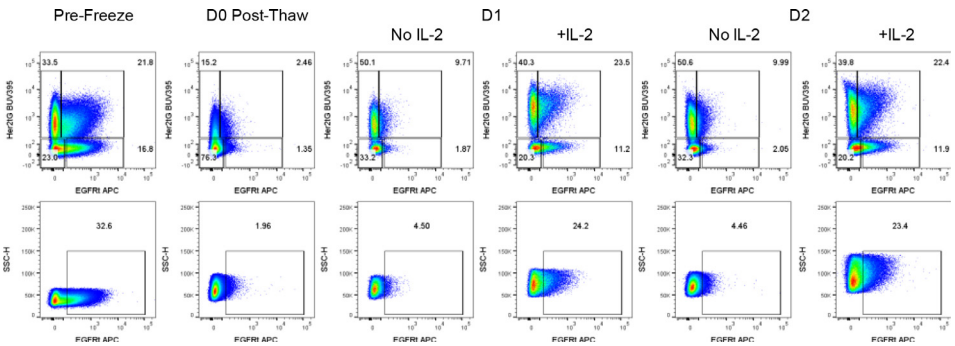

Fig. 5. CAR T-cell transduction marker detection with or without rest in media with or without IL-2. Enhanced CAR transduction marker recovery in cryopreserved cells was attempted by the addition of a rest period post-thaw with or without the addition of IL-2. Transduced CAR T cells were examined pre- and post-cryopreservation as described in Figure 3. Following thaw, cells were resuspended in either R10 media or R10 media supplemented with 50 U/mL of rhIL-2 cytokine. Cells were incubated at 37°C and evaluated by flow cytometry staining on D1 and D2 post-thaw. The addition of IL-2 showed enhanced CAR T-cell transduction marker staining compared with D0 (immediately post-thaw) and the unsupplemented culture. Extending the rest period beyond 1 day did not result in any further increase in reporter molecule detection in either culture. APC, allophycocyanin; D, day; rhIL-2, recombinant human IL-2.

Key cytokines to measure in correlative samples for cell therapy trials

After careful consideration of pre-analytic variables that adversely affect correlative data results, the authors also compared markers used at the respective CPCI sites for cytokine profiling to develop a consensus panel for pediatric cell and gene therapy samples. Table 3 shows a list of recommended cytokines to analyze in participant-derived plasma. All of the cytokines included have established roles in immune effector-related toxicities associated with IEC therapy, including cytokine release syndrome (CRS) and IEC-associated neurotoxicity syndrome. For example, interferon gamma (IFNγ), IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) and C-C motif chemokine ligand 2 (also known as monocyte chemoattractant protein 1 [MCP-1]) have all been implicated in CRS onset and severity as well as neurotoxicity [21,56,61–64]. Additionally, hemophagocytic lymphohistiocytosis, a syndrome of cytokine-driven immune activation that results in multisystem organ dysfunction and failure, has largely been associated with high levels of IL-1β, IL-6, IFNγ and tumor necrosis factor alpha (TNFα) [20,65,66].

The cytokines listed in Table 3 have roles in potentiating the immune response (e.g., interferon alpha, IL-2, IL-10, IL-12, IL-15, IL-21, GM-CSF) [67,68] or inhibiting immunosuppressive activity (e.g., IL-1, TNFα, TGFβ, colony-stimulating factor 1) [69–72] and are therapeutically targeted in clinical trials [39]. In particular, IL-1 blockers, such as anakinra and canakinumab, are approved by the Food and Drug Administration and widely used for the treatment of autoimmune and autoinflammatory diseases (e.g., NCT02179853, NCT04656184, NCT02780583) and are currently being tested in preclinical and human clinical trials for cellular immunotherapy (e.g., NCT04148430) [73–75]. Importantly, the authors include cytokines that may be produced by genetically modified therapeutic cells themselves or by other cell types within the microenvironment (e.g., myeloid cells, tumor cells). Analyzing cytokines produced by multiple cell types maximizes data generated from pediatric samples while improving understanding of the immune landscape.

Methods of analyzing cytokine biomarkers in biologic samples differed across CPCI sites. Some sites measured cytokines in plasma using standard Luminex or enzyme-linked immunosorbent assay-based analyses, whereas others detected intracellular cytokine expression and mean fluorescence intensity via flow cytometry. Given the technical limitations of each assay platform, it may not be possible to assay all of the cytokines listed in Table 3 simultaneously. At a minimum, the authors suggest analyzing IFNγ, IL-1, IL-2, IL-6, IL-15 and TNFα in a basic cytokine panel because of the potential clinical utility of these cytokines as well as their wide use in current pediatric cellular immunotherapy clinical trials.

Table 3
Recommended cytokines to measure in cell therapy trials.

Cytokine	Toxicity	Role
CSF-1		Regulates monocyte/macrophage differentiation [99]
GM-CSF	CRS, HLH and neurotoxicity	Drives CRS and neuroinflammation [63]
IFNα		MSC production [100] and cytokine delivery; induces expression of tumor suppressor proteins [101]
IFNγ[a]	CRS and HLH	Contributes to immunotherapy, tumor suppression and the efficacy of immune checkpoint blockade [102]
IL-1[a]	CRS	Innate immunity [103]
IL-2[a]	CRS, HLH and neurotoxicity	Promotes expansion of T and NK cells
IL-4		Promotes B-cell proliferation; mediates inflammation [104]
IL-5		Promotes B-cell proliferation [105]
IL-6[a]	CRS and HLH	Associated with CRS onset and severity
IL-8	CRS	May be predictive of resistance to ICIs [106]
IL-10	CRS	
IL-12		Activation and regulation of macrophages, T and NK cells [107]
IL-13		Promotes B-cell proliferation; mediates inflammation [104]
IL-15[a]	CRS	Induces proliferation of CD8 memory and NK cells, cytotoxicity and release of other cytokines (e.g., IFNγ) [108]
IL-17		Pro-inflammatory cytokine [109]
IL-21		Activates STAT3 signaling in T- and B-cell differentiation [110]
TGFβ		Promotes cancer progression [111]
TNFα[a]	CRS and HLH	Mediates inflammation, anti-tumor response and infection [112]

Cytokines are listed with associated roles in immune effector-related toxicities.
CSF-1, colony-stimulating factor 1; HLH, hemophagocytic lymphohistiocytosis; ICIs, immune checkpoint inhibitors; IFNα, interferon alpha; MSC, mesenchymal stem cell; NK, natural killer; STAT3, signal transducer and activator of transcription 3.
[a] Indicates cytokine should be included in a basic minimum panel.

Table 4
Recommended markers to evaluate IEC activation or exhaustion status as a part of cell therapy trials.

Marker	Role
CD3	Lineage
CD4	Lineage
CD8	Lineage
CD25	Activation
TIM-3 (CD366)	Exhaustion
CTLA-4 (CD152)	Exhaustion
LAG-3 (CD223)	Exhaustion
PD-1 (CD279)	Exhaustion
Perforin or granzyme B[a]	Function
IFNγ[a]	Function
TNFα[a]	Function
IL-2[a]	Function

CTLA-4, cytotoxic T lymphocyte-associated protein 4; LAG-3, lymphocyte-activation gene 3; PD-1, programmed cell death protein 1.
[a] Indicates intracellular staining required.

Evaluation of function and exhaustion of IECs by flow cytometry

The authors next compared basic flow staining practices, gating strategies and panels across participating CPCI sites. The authors found that flow analysis of biologic samples for cell therapy trials mainly aimed to monitor IEC engraftment, persistence and effector function and the immune landscape of pediatric malignancies. All CPCI sites used basic immunophenotyping and memory/differentiation markers to distinguish T- and B-cell subsets, natural killer cells and monocytes as previously described in the context of cell-based therapies in HCT [3]. However, pediatric cell and gene therapy trials require a more in-depth analysis of genetically modified immune cells to discover new biomarkers of functional or dysfunctional responses. It is important to correlate activation, exhaustion and functional status of adoptively transferred cells with toxicity data and critical endpoints such as loss of persistence, relapse or clinical outcome.

The authors recommend the markers shown in Table 4 to assess activation, exhaustion and functional status of IECs. All CPCI sites analyze these markers as part of the exploratory objectives of clinical trials, with the goal of linking them to other correlative findings, including cytokine/chemokine data. All of these markers are commercially available with staining kits, making them even more accessible for sites to explore as a part of multi-center research.

Discussion

Correlative studies contribute significantly to our understanding of the *in vivo* activity, safety and performance of adoptive cellular therapies. Most cell and gene therapy pediatric clinical trials include collection of biologic samples for short- and long-term monitoring during and after treatment. These correlates are necessary to detect and study inherent risks related to factors such as site of vector integration, persistence of the gene product, replication competence of the vector and associated immunogenicity-related reactions. CAR T-cell clinical trials have also shown that severe CRS and IEC-associated neurotoxicity syndrome/neurotoxicity are associated with early increases (<3 days post-T-cell infusion) in predictive biomarkers such as IFNγ, IL-2, IL-6, IL-10, GM-CSF, MCP-1 and vascular endothelial growth factor [21,76–78]. Furthermore, the initial expansion and long-term persistence of CAR T cells after infusion are two of the key correlates of long-lasting clinical remission [79–81]. The authors report consensus recommendations from the CPCI sites leading cellular immunotherapy trials for pediatric disease to align practices and improve the reliability and reproducibility of correlative assays for key biomarkers in cellular immunotherapy.

The authors identified the choice of blood collection tubes, incubation time between sample collection and processing and choice of plasma or serum for cytokine measurement as important variables of correlative studies relevant to cell therapy trials. The anticoagulant used in collection tubes can impact the recovery of IECs and their phenotypic and functional attributes and can also impact the feasibility of obtaining plasma prior to isolation of PBMCs or bone marrow mononuclear cells [47,82]. Plasma is preferred over serum for cytokine analysis because of the ease of collection in a single sodium heparin tube, especially for low-level blood draws from children, and the ability to detect low-level changes in cytokine abundance. The authors' review also assessed the impact of cryopreservation on markers used to identify and characterize IECs and cellular products in correlative assays. Assays that require detection of specific cell markers that are altered by cryopreservation should be performed on freshly isolated samples if at all possible, although a combination of fresh and frozen samples may be required to promote broader study participation. The authors recommend validating and using the same sample preparation within individual trials for consistency and reproducibility.

Although the authors' results highlight the effects of cryopreservation and thawing on CAR T-cell products and markers expressed on PBMCs, the findings can be extended to other sample types. Flow-based detection of IECs or rare immune cell subsets in cryopreserved-then-thawed samples may be even more challenging compared with cellular products that are enriched for IECs. For example, phlebotomy volume limits to ensure safety in pediatric patients can also impact the ability to detect CAR T cells post-infusion in bone marrow or CSF samples in real-time. This limitation is exacerbated by relying on cryopreservation of leftover samples in these patients. Although CAR transduction marker tags (e.g., EGFRt, Her2tG) can be useful for identifying engineered cells in fresh products or samples, the authors recommend avoiding use of these markers for flow-based detection of cryopreserved and banked samples. Instead, the authors suggest using direct single-chain variable fragment–fluorochrome conjugates, protein L or other strategies for flow-based CAR detection [25]. Alternatively, where cryopreservation or biobanking of leftover samples for future research is preferred, molecular-based approaches such as quantitative PCR or digital droplet PCR to detect CAR T-cell DNA may be more sensitive than flow-based strategies for detecting cells. PCR or other molecular-based approaches may also be advantageous in cases where there are delays in receiving follow-up samples from referring institutions to participating study sites.

In addition to peripheral blood, bone marrow and CSF samples, in recent years, more studies have begun using information from biofluid samples, such as dialysate and urine, as non-invasive tests to evaluate treatment response and resistance to cancer immunotherapy [83]. As utilization of biofluids shows promise in toxicity assessments of IEC therapy, future work is needed to standardize collection and processing methods for pediatric patients.

Only a single CPCI site had guidance related to solid tissue collection and processing. Tumor biopsies and resection tissues from children are particularly rare, and future work will be necessary to develop best practices for collecting, processing and biobanking solid tissue as a part of multi-center trials. Clinical trials evaluating cell and gene therapies targeting the most common types of pediatric solid tumors, such as brain tumors, neuroblastoma and osteosarcoma, are becoming increasingly prevalent (e.g., NCT03294954, NCT02311621, NCT03618381, NCT04483778, NCT03500991, NCT03638167, NCT04185038, NCT02789228, NCT02573896, NCT01326104), but there are numerous challenges to using cellular therapies in the context of solid tumors [84]. Correlative analysis of fresh or frozen tissue will be essential to gain insight into mechanisms of action of cell therapies within the context of solid tumor microenvironments. Guidance for developing correlative studies for pediatric solid tumor trials is severely lacking and will be an important focus of future CPCI work.

Comprehensive cytokine profiling post-infusion of IECs informs mechanisms of action of cell therapies and immune effector-related toxicities, highlighting the importance of establishing a base panel of cytokine analyses to be incorporated across trials. Many of the cytokines in Table 3 are often measured in peripheral blood and bone marrow samples. However, cytokines of interest to evaluate in CSF for cell therapy trials are not well established. Potential biomarkers of neuroinflammation and neurotoxicity associated with CD19-directed CAR T-cell infusion in patients with B-ALL have been explored [19,54,76]; however, tumor heterogeneity and the complexity of the central nervous system environment contribute to the variability and inconsistencies in many previous studies. For example, a study evaluating the clinical utility of C-C motif chemokine ligand 2/MCP-1, C-X-C motif chemokine ligand (CXCL) 8/IL-8, CXCL10, CXCL13 and IL-6 found that all of these cytokines were detectable in the CSF of symptomatic patients, but compared with the combination of biomarkers commonly assessed in CSF, the majority of these cytokines had decreased sensitivity and specificity for confirming neuroinflammation (e.g., white blood cell counts, oligoclonal bands, total protein levels, CSF:serum albumin ratios) [85]. Future work is needed to align sites regarding which cytokines to explore in CSF as part of correlative studies. The chosen cytokines will likely vary depending on trial objectives and tumor type—for example, studies assessing CSF biomarkers of neurotoxicity in leukemia patients versus biomarkers of inflammatory response in pediatric patients with central nervous system tumors.

There is increasing evidence that exhaustion and senescence of engineered cells affect their *in vivo* proliferative capacity, long-term persistence and anti-tumor function, which in turn impacts the efficacy of CAR responses and ability to achieve durable remissions [86]. The authors recommend the inclusion of activation, exhaustion and functional markers in the basic immunophenotyping flow panels that have been previously described [3]. Finney *et al.* [55] have recently shown that including additional exhaustion and functional markers in correlative studies has the potential benefit of identifying cellular products or patients that may be capable of achieving sustained remissions or at risk of therapeutic failure. It is also important to note that expression of a single inhibitory receptor alone, such as programmed cell death protein 1, T-cell immunoglobulin mucin 3 (TIM-3) (CD366) or lymphocyte-activation gene 3, does not always indicate exhaustion. For example, TIM-3 is associated with both co-stimulatory and inhibitory functions, depending on the cell type, so it is important to measure TIM-3 expression in combination with other markers of T-cell dysfunction [87,88]. Terminally exhausted T cells exhibit a loss of effector cytokine production and co-express multiple inhibitory receptors, with the number of co-expressed receptors directly correlating with the severity of exhaustion [89,90]. The authors therefore recommend measuring co-expression of at least three inhibitory receptors in addition to other functional markers. Significantly, the insight gained from the evaluation of exhaustion markers immediately following IEC infusion could provide guidance for the utilization of checkpoint blockade to enhance the function and persistence of the infused product [91,92]. This insight can also guide development of next-generation cell therapies using gene editing approaches to knock out checkpoints that can improvise the engineered product [93–97] or the manufacture of "armored" CAR T cells, especially in the setting of solid tumors [98].

Future discovery of new biomarkers, and the study of next-generation cell and gene therapy products in multi-center trials, will require alignment of sample collection time points so data can be compared across sites. In addition to the type of sample and methodologies used to evaluate biomarkers, the timing of correlative sample collections can vary greatly depending on clinical trial design, resources available at each site and whether the participants are undergoing inpatient or outpatient care. For instance, the timing of evaluation of pre-infusion bone marrow disease burden for pediatric B-ALL differs not only between trials but also in clinical practice. Some sites collect bone marrow prior to lymphodepletion before IEC infusion, whereas other sites collect after lymphodepletion, and thus a subject may already be considered to be in a minimum residual disease-negative status prior to infusion. This variation could lead to difficulty in interpreting the optimal disease burden required for adequate antigen stimulation or identifying the pre-existing or emerging resistant clones. Similarly, for optimal detection of early *in vivo* changes, such as engraftment of IECs, cytokine levels or toxicity monitoring, most clinical trials collect two or three time points within the first week of treatment followed by weekly draws for the first 30–35 days and monthly draws for the first 6 months [21,76]. Although these considerations are not included in the authors' current review, future research may need to assess the impact of timing of other correlative sample collections on study conclusions.

Increasing patient access to cellular immunotherapy warrants a focus on basic assays that can be performed at multiple participating sites. However, it is important to note that the cell and gene therapy field offers several advanced molecular assay platforms for the evaluation of correlative samples. For example, next-generation sequencing, single-cell profiling of RNA/DNA and T-cell receptor immunosequencing can provide a comprehensive look at the T-cell receptor repertoire of IECs infused into patients. Given the complexity of these assays and their cost, it will be challenging to implement them widely across centers, necessitating analysis at a centralized site. Recommendations for sample collection for and processing and shipping of these assays will be an important future direction.

Key considerations need to be taken into account when designing correlative studies across multi-center cell therapy trials (Table 5). Increased standardization of current practices will improve sensitivity and specificity of data generated in early-phase IEC trials and of comparisons of data between different trials. Standardization will have the greatest impact on the study of pediatric diseases where correlative studies are limited by small subject numbers and sample

Table 5
Alignment considerations for sites participating in multi-center cell therapy trials.

Parameter	Key considerations
Experimental design	• What type of correlative data are necessary for the clinical trial? • What sample types and collection time points are required? • What analytes are to be measured in biologic samples? • What instruments and detection parameters are required? • Will samples be cryopreserved or banked for future research?
Site evaluation	• What instruments are available at each participating site? • What assays can be performed at each participating site?
Assignment versus centralization	• Will any assays be performed locally or will samples be shipped to a central lab? • Has assay validation been performed at sites using fresh or frozen samples? • Consider determining acceptable ranges for measurements of key analytes across sites? • Partner with an external quality assurance organization or program to broaden validation and harmonization efforts? • Are centralized SOPs created to facilitate staff training on correlative sample collection, processing and analysis?

collection volumes of biospecimens. When standardizing practices across sites, consultation and participation with external quality assessment programs are highly encouraged. There are numerous organizations that work toward establishing best practices globally. The scope of assessment can range from pathology testing (e.g., College of American Pathologists, UK National External Quality Assessment Scheme) to technologies that are relevant to IEC trials, such as flow cytometry (e.g., International Society for Advancement of Cytometry). Accreditation from an external party can also ease harmonization efforts, as participating sites will already have met similar standards.

Once SOPs have been established and standardized across sites participating in multi-center cell therapy trials, initial validation testing should be conducted to compare instrument performance and processing standards across sites. During initial testing, baseline and threshold parameters should be established for each procedure and acceptable ranges should be determined. To ensure continuous harmonization, periodic reviews should be performed to ensure uniformity and reproducibility. In one example study, standardization procedures between instruments were repeated every 3–6 months [27]. The time points between harmonization efforts may depend on the length of a particular study and the types of correlative studies. After initial validation testing, sites should regularly monitor instrument performance and quality control to establish guidelines for periodic review. Proficiency samples with known performance ranges should be tested regularly for each analyte of interest at participating sites to confirm continued performance within range across sites. Maintaining SOPs and providing training to study site staff related to correlative studies and validation testing are key to ensuring process and data harmonization. Open discussion of correlative study strategies during clinical trial development, consideration of quality control criteria and boundaries for acceptable measurements of key analytes and consistency in pre-analytic sample handling are key steps to ensuring reliable and high-quality correlative data across sites.

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Declaration of Competing Interest

HD has served on an advisory board for Pfizer.

Author Contributions

Conception and design of the study: HA, HD, KJ and ALW. Acquisition of data: HA, HD, KJ, ALW, SR and AL. Analysis and interpretation of data: HA, HD, KJ, ALW, SR and AL. Drafting or revising the manuscript: HA, HD, KJ and ALW. All authors have approved the final article.

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Supplementary materials

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