



Published in final edited form as:

Expert Rev Proteomics. 2021 September ; 18(9): 767–780. doi:10.1080/14789450.2021.1992276.

An Overview of Multiplexed Analyses of CAR T-cell Therapies: Insights and Potential

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Abstract

Introduction: Cancer immunotherapy is a rapidly growing field with exponential advancement in engineered immune cell-based therapies. For instance, an engineered chimeric antigen receptor (CAR) can be introduced in T-cells or other immune cells and adoptively transferred to target and kill cancer cells in hematologic malignancies or solid tumors. The first CAR-T-cell (CAR-T) therapy has been developed against CD19, a B-cell marker expressed on lymphoma and lymphoblastic leukemia. To allow for personalized treatment, proteomics approaches could provide insights into biomarkers for CAR-T therapy efficacy and toxicity.

Areas Covered: We researched the most recent technology methods of biomarker evaluation used in the laboratory and clinical setting. Publications of CAR-T biomarkers were then systematically reviewed to provide a narrative of the most validated biomarkers of CAR-T efficacy and toxicity. Examples of biomarkers include CAR-T functionality and phenotype as well as interleukin-6 and other cytokines.

Expert Opinion: Biomarkers of CAR-T efficacy and toxicity have been identified, but still need to be validated and standardized across institutions. Moreover, few are used in the clinical setting due to limitations in real-time technology. Expansion of biomarker research could provide better understanding of patient response and risk of life-threatening side effects with potential for improved precision medicine.

Keywords

Adaptive transfer; Biomarker; Cancer; CAR-T cell therapy; Immunotherapy; T-cell therapy; Multiplexed analysis; precision medicine

Introduction

Cancer immunotherapy is a rapidly growing field with development of effective treatment in patients with prior refractory disease [1–5]. Immunotherapy manipulates the patients' own immune system to target and kill cancer cells [6]. This contrasts with conventional

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Reviewer declarations

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

chemotherapy which targets all rapidly dividing cells, leading to harsh side effects of the gastrointestinal, genitourinary, and integumentary systems.

Immunotherapy works by activating cytotoxic T-cells to target and kill tumor cells. Tumor associated antigens (TAA) are presented by dendritic cells (DC) in the context of major histocompatibility complex I (MHC I) to activate CD8⁺ cytolytic T cells (CTLs). Upon recognition, these cells release cytolytic granules such as granzyme and perforin resulting in cell death. TAAs are also presented by DCs in the context of MHC II complexes to activate CD4⁺ helper cells. These CD4⁺ T cells differentiate into specific effector cells which coordinate cytokine signalling for both downstream innate and adaptive immune responses. Additional immune cells involved in antitumor effects include macrophages, natural killer (NK) cells, NKT cells, and gamma-delta T cells [7].

In recent years, there has been an exponential increase in development of immunotherapies that are (i) active immunotherapies meaning they either a) overcome tumor-induced immune evasion mechanism such as checkpoint inhibitors against cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) or its ligand PDL-1 that work by blocking brakes on CTLs, or b) engage targeted T cells with bispecific T cell engagers (BiTEs), and (ii) passive immunotherapies involving adoptive transfer of “boosted highly cytolytic antigen-specific CTLs” or engineered immune cells [1, 8–10], Figure 1. These adoptive cellular therapies include viral mediated CTL’s, tumor infiltrating T-cell therapy, transgenic TCR modified T-cell therapy, NK-, NKT-cell therapy, $\gamma\delta$ T-cell therapy, and chimeric antigen receptor T-cell (CAR-T) therapy which is the primary focus of this review. [10, 11].

The chimeric antigen receptor (CAR) is an engineered molecule that includes a single chain variable fragment (scFv) against a specific antigen combined with an extracellular hinge domain, a transmembrane domain, and an intracellular T-cell signaling domain. Constructed for use with the T-cell, CAR-T therapy works by recognizing the malignant cell and binding to the scFV target. The CAR-T-cell is then activated and targeted cell killing is initiated. The costimulatory domain drives signal amplification and functional T-cell expansion on repeat exposure to the tumor antigen [9, 12, 13]. Given the success of CAR-T therapy in hematologic malignancies, additional immune effector cells such as NK cells, invariant NK T-cells, $\gamma\delta$ T-cells and even macrophages are being investigated for CAR engineering and therapeutic utility [8, 14].

CD19 was the first chosen target for the CAR-T cell due to its homogenous expression in B-cell malignancies, as well as specific on-target off-tumor effect [4, 12]. As CD19 is limited to cells of the B-cell lineage, off-target effect is primarily B-cell aplasia which can be mitigated with immunoglobulin repletion [9]. Success of the CD19 CAR-T molecule was initially proven to be effective in B-cell acute lymphoblastic leukemia (ALL) [15, 16] with use now expanded to include other B-cell malignancies, such as chronic lymphocytic leukaemia (CLL) [17, 18] and non-Hodgkin’s lymphoma (NHL) [4, 19]. Additional CAR-T targets include other markers seen in hematologic malignancies such as CD20, CD22, CD33, CD38, CD70, CD123 and BCMA [20]. Targets for solid tumor malignancies are also in various research stages, though CAR-T cells have not been as effective in these cancer

types [12]. This is presumed secondary to the lack of cancer-specific tumor targets [20] and immunosuppressive networks in the tumor microenvironment [21, 22].

Currently, there are five U.S. Food & Drug Administration (FDA) approved CAR-T products with indications and response rates as outlined in Table 1. Additional products await approval with over 500 open clinical trials worldwide [23]. These CAR-T products have been established as effective interventions in patients with a history of relapsed or refractory disease [15–17, 19, 24–27]. There is also evidence to support long lasting duration of action and persistence of CAR-T cells, which has been associated with relapse free survival among leukemia patients [17]. However, responses do vary between patients and reasons for efficacy or lack thereof is often not known. Further biomarker studies to evaluate CAR-T efficacy could facilitate risk stratification and personalized engineered cell therapy.

While CAR-T therapy remains a powerful treatment tool, adverse side effects frequently exist. Specific acute toxicities associated with CAR-T therapy include cytokine release syndrome (CRS) and immune effector cells associated neurotoxicity syndrome (ICANS). The FDA mandates reporting of these toxicities, but they do not require collection of patient samples. As such, biomarker research has been limited. If expanded, likelihood of side effects could be better understood. Herein, we will review the current literature on biomarkers of efficacy and toxicities focusing on CAR-T cells therapies as well propose future directions that may be taken.

1.0 Biomarker definitions and regulatory workflow

1.1 Definitions—It is recommended that biomarkers be defined as per the FDA Biomarkers, EndpointS and other Tools (BEST) Resource established by the FDA-National Institute of Health (NIH) joint leadership council in 2015 [28]. This resource was created to define and outline biomarker roles in biomedical research, clinical practice and medical / pharmaceutical product development [28]. Per the NIH, a biomarker is a “defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions [28].” Biomarkers are typically proteins or other biomolecular molecules further sub-classified based on respective role such as diagnostic, risk, prognostic, progressive or response / monitoring biomarker. These sub-class definitions are further expanded in Table 2.

1.2 Regulatory Workflow—Biomarker development consists of multiple phases from identification of candidate targets to integration in clinical use. The initial discovery phase identifies potential biomarkers within a pilot cohort and study often using state-of-the-art unbiased technologies. The candidate biomarkers are then evaluated for statistical significance. If they are statistically significant, they then require validation in at least one or more independent cohorts. Ideally these “large” cohorts will contain at least 100–300 patients that are all comers. Once validated, the biomarker is then verified in cohorts across multiple institutions. After verification, the biomarker can be advanced for use in prospective clinical trials. This workflow is summarized in Figure 2.

2.0 Proposed Sample Collection for evaluation of CAR-T cell therapy efficacy and toxicities

Clinical data at centers offering CAR-T is voluntarily collected through the Center for International Blood and Marrow Transplant Research (CIBMTR) Cellular Immunotherapy Data Resource (CIDR). Per the American Society of Hematology (ASH) workshop on biomarkers, it is recommended that patient samples also be collected and stored in a similar coordinated effort [29]. Primary sample collection would be best achieved from serum/plasma samples, and peripheral blood mononuclear cells (PBMCs). Additional sources of collection involve the cellular product, bone marrow aspirate or tumor biopsies. Timing of sample collection should be adapted to the specific therapy and include concurrent samples over time to trend intervention efficacy and side effect profiles. A suggested samples collection schema is presented in Table 3.

3.0 Methods of biomarker evaluation

3.1 Cytokine analysis—Cytokines are small proteins secreted by cells that play a pivotal role in intracellular pathways and communication within the immune system. Measurement of cytokine levels requires highly sensitive assays, given low levels within body fluids (picograms to nanograms per milliliter). Traditional cytokine analysis has utilized enzyme linked immunosorbent assays (ELISA) in plasma/serum and these are still standard of care. For PBMCs analysis, the enzyme linked immunosorbent spot (ELISpot) assay, and flow cytometry have been used. While these methods are still often employed, newer technologies such as the Isoplexis system for single cell cytokine profiling have since emerged.

The Isoplexis system uses control microvalves to isolate single cells in microchambers that contain capture antibodies in a barcode array. This barcode can quantify measurements for intracellular, transmembrane, or secreted proteins. Once proteins are captured, each microchamber's barcode array can then be quantitatively analysed using a surface bound immune sandwich assay [30]. This single cell barcode chip system allows for detection of sub-thousand protein copies and requires only a small sample size [31]. The system was validated with a human macrophage cell line and subsequently implemented clinically to assay the polyfunctionality of tumour antigen specific T-cells in a clinical trial for melanoma [32]. Success led to use across an array of cancer immunology fields [33–37] and was recently applied to uncover significant differences in allogeneic versus autologous CD19⁺ CAR-T transcriptional, phenotypic, functional, and metabolic profiles [38]. The Isoplexis polyfunctional strength index (PSI) has also been applied to pre-infusion CAR products in patients with non-Hodgkin's lymphoma (NHL) and associated with improved clinical outcomes [4]. As a result, the “proteomic barcode” used by Isoplexis was one of the top 10 innovations of 2020 by the Scientist magazine [39]. Currently, Isoplexis has three human immune panels, each identifying over 30 different cytokines. Non-human primate and mouse model barcodes are also available.

3.2 Immunoassays

3.2.1 Point of care (POC): Immunoassays have widespread use in both hospitals and the laboratory, measuring concentrations of molecules based on antigen-antibody reactions.

However, most conventional immunoassays are performed in established laboratories using bulky conventional equipment and well-trained staff, limiting widespread access. POC immunoassays are urgently needed to provide more time and cost-effective testing along with expanded access to healthcare facilities. At this time, CAR-T therapy biomarker POC testing is limited to the recently developed IL-6 specific lateral flow assays, also known as immunochromatographic assays [40]. Huang et al. used europium nanoparticles (Eu-np) as a label with the basis of a conventional sandwich immunoassay. Results were available in 15 minutes. The strips yielded adequate sensitivity at 0.37 pg/mL IL-6 and demonstrated significantly high ($p < 0.01$) correlation when compared to the traditional Siemens IL-6 ELISA kit [40]. Similar results using lateral flow assay for POC testing (Milenia QuickLine IL-6) were found by Schefold et al. and correlation with standard ELISA testing was also found to be significant ($p < 0.001$) [41]. Proxim Diagnostics has also created POC IL-6 testing but instead uses a handheld device with an indwelling ELISA cartridge [42].

3.2.2 Multiplex: Multiplex immunoassays allow for detection of multiple biomarkers and use less sample and labor compared to single assays [43]. However, translation into the acute clinical care setting has been challenging due to the time delay in results. Song et al. recently published a new technology platform for rapid single-molecule digital detection of protein biomarkers known as a pre-equilibrium digital enzyme-linked immunosorbent assay (PEdELISA) that can quantify results in an incubation time of 15–300 seconds over a 10^4 dynamic range [44]. The technique uses a pre-equilibrated 2 step sandwich assay quenched with single molecule binary counting to achieve an assay with minimal incubation time that still does not lose linearity [44]. Moreover, this near bedside approach can be performed at low cost and used for diagnosis of acute disease process and following longitudinally to assess disease response over time.

3.3 Tandem mass spectrometry—Tandem mass spectrometry or MS/MS is a technique used for protein identification and characterization. This technology is often used as a discovery tool to identify novel proteins in an unbiased manner. In clinical proteomics, two fundamental strategies are employed: bottom-up and top-down [45]. In bottom-up proteomics, complex protein mixtures are subjected to proteolytic cleavage and the peptide products are analysed by MS/MS prior to proteolytic cleavage. These complex samples can be immunodepleted (of abundant plasma proteins) and / or separated by chromatography. However, this method has several disadvantages related to the early fragmentation, which may result in loss of identifiable protein regions or sequence variations. Top-down proteomics seeks to eliminate these problems by analyzing intact protein ions or large protein fragments subjected to gas-phase fragmentation for MS/MS analysis. The mass spectra are then matched to a sequence database to identify proteins. The Orbitrap Elite is an ultra-high resolution linear ion trap mass spectrometer that facilitates top-down LC-MS/MS [46]. It has revolutionized the field by improved ion transfer optics, increased dual cell linear ion trap speed that exceeds 12 Hz, and increased resolving power of the Orbitrap analyser which has doubled the observed frequencies [46]. Incorporation of an enhanced Fourier Transform algorithm has further increased the resolving power [46]. Due to the highly accurate identification of peptide mass and sequence, MS based proteomics play a key role in plasma discovery of biomarkers today. MS has also been recently used to analyse

CAR signalling in primary T cells to better understand the therapeutic mechanism of action [47, 48]. Ramello et al. was then further able to conclude that second generation CAR design was associated with increased downstream phosphorylation compared to the third generation CAR design [47]. This suggests activation of additional CD3 ζ pathways resulting in enhanced signal intensity and potential for superior anti-tumour efficacy [47].

3.4 Conventional and Spectral Flow Cytometry—Multiparameter flow cytometry allows for the measurement of multiple fluorescence parameters of individual cells or particles [49]. The term polychromatic applies specifically to systems that detect five or more markers simultaneously using varying fluorochromes [50]. This technology is used primarily to identify molecules expressed on the cell surface or within intracellular compartments and classify cells into subtypes based on specific characteristics. Some flow cytometers further provide cell sorting technologically, separating by bulk or individually [49]. Advances in flow cytometry have led to an increased number of fluorescent dyes and antibodies, as well as instrument progress with new lasers and optical filters [49]. These advances have enabled high-throughput and high-content flow cytometry which allow for multiple populations in complex samples to be analyzed simultaneously [51] playing an important role in both biomarker and drug discovery [51, 52]. New technology such as spectral flow cytometry favors high-throughput, in-depth analysis, at the single cell and population levels. In contrast to conventional flow cytometry, which primarily measures the peak emission of each fluorochrome, full spectrum flow cytometry measures the entire emission spectra for every fluorochrome. As a result, more information about each cell is collected allowing for highly multiparametric panels [53]. Flow cytometry is used for multiple applications in CAR-T therapy including quantification of antigen bearing cells in donor sample, functional assessment of circulating therapeutic cells, evaluation of tumor expressed molecules, monitoring of therapy response and more [54, 55]. Identification of specific cell populations and phenotype has provided prognostic information for CAR-T therapy success or failure both pre-infusion [18, 56, 57] and post-infusion [18, 57]. Following CAR-T infusion, flow cytometry can track CAR-T cell location and expansion, for example in bone marrow and cerebrospinal fluid in patients with acute leukaemia [16, 58]. The functional profile of the CAR-T cell can also be assessed to identify markers of activation, degranulation and exhaustion [55] which may shed light on factors that impact CAR-T therapy efficacy [59, 60]. Lastly, flow cytometry can be used for evaluation of longitudinal response in assessing minimal residual disease (MRD) [61, 62].

3.5 Mass cytometry or cytometry by time of flight (CyTOF)—Like spectral flow cytometry, mass cytometry, also called CyTOF, can assess high numbers of parameters (typically 40+). CyTOF is a technology that uses antibodies tagged with stable heavy metal ion isotopes (rather than fluorochromes) and provides readout using time of flight mass spectrometry [63]. Therefore, isotope labels have created high dimensional profiles to decipher proteins involved in cellular functioning using CyTOF at the single cell level [64, 65]. The main advantage of CyTOF is that it allows for the combination of more antibody specificities in a single sample without significant spill over between channels [45]. This technology is then combined with computational tools such as Citrix, PhenoGraph, SPADE, viSNE and X-shift that use various algorithms to analyse the complex datasets. CyTOF has

been used to evaluate expression of surface or intracellular proteins related to T-cell function demonstrated at pre and post CAR-T infusion times resulting in identification of specific populations of CD4⁺ CAR-T cells associated with clinical outcome at 6 months [66]. CAR-T therapies can also be monitored via CyTOF technology as evident in diffuse large B-cell lymphoma (DLBCL) and ALL patients treated with Tisagenlecleucel [67] and multiple myeloma patients treated with novel BCMA CAR-T therapy [68]. Future direction includes functional profiling of genetically engineered hematopoietic progenitor stem cells (HPSCs) to allow for antigen-specific immunotherapy with avoidance of on-target, off-tumor toxicity, though this has currently only been shown in animal models [69].

3.6 Multiplex bioimaging of single-cell spatial profiles—Multiplex bioimaging refers to the combination of analysis methods that detail proteomic, genomic, and metabolic profiles of human cancers at the single-cell level [70]. Solid or liquid tumor samples are evaluated by top-down or bottom-up approaches. The top-down approach targets intact biopsies which provide direct representation of tumor pathology while the bottom-up approach generates reproducible data from cells modified to resemble native tissue using microwell and microfluidic interfaces [70]. Immunohistochemistry (IHC), a colorimetric staining assay, has been classically used to study tumor pathology, but was limited by the ability to identify a single biomarker at a time. Quantum dots in immunofluorescence IHC increase the colour signal and multiplex IHC technologies detect up to eight markers, but IHC can still be limiting when attempting to analyze the complexity of the tumor microenvironment [70]. Immunofluorescence (IF) is another traditional method that can be used at the single cell level to detect multiple biomarkers using antibody sets conjugated to spectral dyes. However, IF is also limited by multiplex ability, though recent advances have used cyclic labelling and fluorescence imaging to detect up to 56 proteins in a tissue section [70]. Co-detection by indexing (CODEX) technology was developed by Akoya and measures over 50 parameters within a single tissue for high parameter, spatial profiling [71, 72]. Cyclic immunofluorescence (CyCIF) is a gentle process that uses a conventional epifluorescence microscope to perform highly multiplexed imaging [73, 74]. Multiplex immunofluorescence (MxIF) preserves tumor architecture and identifies spatial relationships between immune and tumor cells [75]. Immunostaining with signal amplification by exchange reaction (Immuno-Saber) utilizes DNA based multiplexing and an innovative amplification method based on primer exchange reactions [76, 77]. Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique used to identify and localize specific DNA or RNA targets in cells, circulating tumour cells or tissue samples. FISH has been used in combination with multiplexed error-robust FISH and sequential FISH to perform spatially resolved transcriptomics of up to 10,000 RNA gene targets [70]. Sequential FISH (SeqFISH+) specifically has been reported at the sub-diffraction limit resolution, the highest power in the realm of RNA profiling methods [78]. Multiplexed ion beam imaging (MIBI) uses secondary mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters [79–81]. It has since been utilized with time-of-flight mass spectrometry to increase channel multiplexing and decrease acquisition times (STAR method) resulting in large imaging fields at resolutions down to 260nm [79, 82]. Imaging mass cytometry (IMC) uses laser ablation, rather than analysing cells in suspension, to generate particles carried to a mass cytometer via inert gas stream and

produce high-dimensional images [83]. The Hyperion (Fluidigm) is an IMC system that combines CyTOF technology with precise laser technology at a resolution of 1 μm recently used to study patients with breast cancer [84], colorectal cancer [85] and squamous cell carcinoma [86]. Additional cancers are being investigated in the mouse model [87, 88].

3.7 Single cell RNA Sequencing and Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)—High throughput single cell RNA sequencing has led to significant advance in describing heterogenous cell populations but is limited in scale and lacks phenotypic information. CITE-seq is a method that combines highly multiplexed protein markers with unbiased transcriptome profiling for thousands of single cells [89]. Essentially, oligonucleotide labelled antibodies are used to assimilate cellular protein and transcriptome measurements into a single cell readout [89]. This can provide multimodal single cell analysis and has been shown to be fully compatible with commercially available single cell platforms. Cite-seq has been used to identify specific signalling of molecular function associated with both long-term CAR-T cell persistence as well as lack thereof [90]. It has also been used for multiomics characterization of healthy patient versus acute lymphoblastic leukemia (ALL) donor CAR-T cells. Donor cells had more pronounced activation compared to patient cells, which could lead to improvement and increased use of “off the shelf products” [38]. Transcriptional programs have also been discovered using CITE-seq technology, related to the performance of CAR-T cells after infusion [91].

4.0 Most validated biomarkers of CAR-T Efficacy

4.1 Peak levels of CAR T cells—Several studies have found that increased in vivo expansion of CAR⁺ T cells is associated with disease response. This was initially published in 2015 in a small cohort of 14 patients with relapsed refractory CLL who received CD19 CAR-T infusions. Peak CAR⁺ T cell expansion was found to be significantly greater in patients who achieved continuous remission (CR) compared to those who achieved partial remission (PR) or had progressive disease (PD) ($p=0.013$) [17]. Similar results were found in 2017 in the [NCT00924326](#) study conducted by the National Cancer Institute (NCI) which looked at 22 patients with advanced stage lymphoma treated with CD19 CAR T cells. Again, the peak blood CAR⁺ cell numbers were higher (median 98/uL CAR⁺ cells) in patients who achieved lymphoma responses of CR or PR compared with those who had stable disease (SD) or PD (median 15/uL CAR⁺ cells), ($p=0.027$) [92]. Both of these studies utilized flow cytometry for measurement of serum CAR+ levels. The phase 2 clinical trial of ZUMA-1 also suggested higher CAR-T levels in the blood were associated with disease response upon evaluation of 111 patients with large B-cell lymphoma who received CD19 CAR-T therapy. Expansion of the CAR-T cells was measured via ELISA and significantly associated with response ($p<0.001$) when objectively comparing responders vs non-responders [3]. All of these findings occurred irrespective of CAR-T infusion dose.

4.2 CAR-T cell polyfunctionality and polyfunctional strength index—T-cells deploy a broad spectrum of immune programs [93], but it is unclear which are specifically utilized by CAR-T. Rossi et al. hypothesized that CAR T cells deploy multiple immune programs that functionally complement one another [4]. They tested this hypothesis on

22 patients with DLBCL receiving CD19 CAR-T therapy. Evaluation was performed via a high-content single cell multiplex cytokine analysis [94, 95]. Polyfunctional T-cells were defined as “cells co-secreting at least 2 proteins from the specified panel per cell coupled with the amount of each protein produced [4].” Results showed CAR products did contain polyfunctional T-cell subsets capable of deploying multiple immune programs represented by effector cells, cytokines, and chemokines. Moreover, within the total product cell population, association with clinical outcomes were greater with polyfunctional CD4⁺ T cells (p=0.0023) compared with CD8⁺ T cells (p=0.0507) [4]. A prespecified T-cell polyfunctionality strength index (PSI) was then applied to the pre-infusion CAR product. The PSI was defined as “the percentage of polyfunctional cells multiplied by mean fluorescence intensity of the proteins secreted by those cells [4].” The median PSI was found to be twice as high in responders vs. non-responders and was significantly associated with objective response (OR) (p=0.019). Combined with CAR-T expansion or pre-treatment serum IL-15 (correlated with CAR-T expansion), additional significance was conferred (p=0.0046).

4.3 Defined CD4/CD8 Ratio—Previous work has shown human CD4⁺ and CD8⁺ T cells comprise functionally and transcriptionally distinct subsets that differ in their capacities to proliferate, persist in vivo, and mediate anti-tumor effect after in vitro expansion and adoptive transfer [19, 96–99]. However, early CAR infusions were composed of heterogeneous T cell subsets which may have contributed to varying results of efficacy. Thus, in two different studies by Turtle et al., a defined ratio of 1:1 CD4⁺ and CD8⁺ CD19 CAR-T infusion was created using the CliniMACS system and used for patients with B-ALL and NHL. Both studies reported feasibility and success of product manufacturing and when administered after Cyclophosphamide / Fludarabine conditioning at maximum tolerated doses saw marked disease regression [19, 99]. Furthermore, this was seen at CAR-T cell doses 5- to 100- fold lower than those used to treat patients in other trials [5, 16, 25, 27]. Consistency within CAR-T product will also allow for improved systematic comparison and titration of CD4⁺/CD8⁺ dosing moving forward that optimizes efficacy and minimizes toxicity.

4.4 Stem Cell Phenotype—T cells can be categorized according to the stage of cell differentiation. Naïve T cells (T_n), stem cell memory T cells (T_{scm}) and central memory T cells (T_{cm}) have been shown to correlate with therapeutic efficacy and in vivo persistence while memory effector T cells (T_{em}) and effector T cells (T_{ef}) have not [18, 99, 100]. Xu et al. first showed that in vivo expansion of CD19 CAR T cells correlated with the frequency of CD8⁺CD45RA⁺CCR7⁺ cells infused, a phenotype closest to T_{scm} in a cohort of 14 patients with B-cell malignancies [100]. The phenotype was defined by flow cytometry with evaluation of CAR-T levels measured via quantitative polymerase chain reaction assays. Turtle et al. then similarly concluded CAR-T products with defined ratios of CD8⁺ T_{cm} cells could provide effective therapy at significantly lower cell doses compared to prior clinical trials in patients with B-ALL [99]. Moreover, in patients with relapsed or refractory CLL treated with CD19 CAR-T, sustained remission was associated with increased frequency of lymphocytes possessing memory like characteristics (defined by CD8⁺CD27⁺CD45RO⁻ cells) [18]. Transcriptome profiling of complete responding patients

also showed enrichment in memory related genes compared to non-responders who had up-regulation of effector differentiation [18]. Following transcriptome profiling, results were verified by computational analysis of flow cytometry. This data fueled the generation of a clinical grade CD19 specific CAR-modified T_{scm} product with improved metabolic fitness and sustained anti-tumor effect against systemic ALL xenografts [101].

4.5 Absence of CAR-T Exhaustion—CAR-T exhaustion results in diminished function and reduced efficacy by way of transcription and epigenetic alterations that lead to overexpression of immune inhibitory proteins [102]. It is defined by poor effector function, sustained expression of inhibitory receptors and a transcriptional state different from that of functional effector or memory T cells [103, 104]. Major mechanisms of exhaustion include the AP-1 related bZIP-IRF families [105], NFAT/NR4A axis [106, 107] and TOX transcription factor [108]. Disruption of these pathways could improve therapeutic efficacy of CAR-T cells. Inhibitory markers PD1, TIM3 and LAG3 are identified as signs of T-cell exhaustion and have been targeted to reinvigorate CAR-T cells in specific populations. For example, strategies include PD-1 checkpoint blockade [109], modification of CAR T cells to secrete PD1 blocking scFv [110] and disruption of PD1 by gene editing via clustered regularly interspaced short palindromic repeats (CRISPR) technology [111]. Likewise, CAR-T cells have been engineered with downregulated PD1, TIM3 and LAG3 and demonstrated increased tumor infiltration and enhanced tumor growth control [112]. Recent investigation by Weber et al. also looked at the utility of transient cessation of receptor signaling or rest, in CAR-T therapy and showed enhanced CAR-T efficacy by preventing or reversing exhaustion utilizing flow cytometry, mass cytometry and RNA sequencing. [102].

5.0 Biomarkers of CAR-T Toxicity

5.1 Cytokine Release Syndrome (CRS)—CRS is the most common life-threatening toxicity associated with CAR-T therapy (approximately a third of patients affected) [5, 16, 17, 25, 58, 113]. Research is ongoing to better understand the underlying disease process and identify biomarkers that could improve CRS recognition, treatment, and risk stratification. Clinically, CRS presents as early as 36 hours post CAR-T infusion, typically peaking in correlation to in vivo CAR-T expansion [114]. Symptoms commonly include fever, hypotension, respiratory distress, and capillary leak, but can ultimately progress to end organ damage, disseminated intravascular coagulopathy (DIC) and severe inflammatory response [like macrophage activation syndrome (MAS) or hemophagocytic lymphohistiocytosis (HLH)]. Associated neurologic symptoms are further explained below. Diagnosis and severity grading has been established based on input by both the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) and the American Society for Transplantation and Cellular Therapy (ASTCT) [115, 116]. CRS is triggered by the activation of T cells when CARs engage with targeted antigens on tumor cells, resulting in CAR-T expansion and release of cytokines and chemokines. Activation of other bystander immune cells such as monocytes, macrophages, and dendritic cells are also common [117]. The most frequently reported cytokine elevations include interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin (IL-10), interferon gamma (IFN- γ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-beta

(MIP-1 β) and tumor necrosis factor-alpha (TNF- α) [5, 16, 19, 99, 113, 117], though many new biomarker studies are underway. Of the potential biomarkers studied to date, IL-6 is the most established and thus the primary target for current CRS treatment with tocilizumab, an anti-IL-6 receptor antibody approved by the FDA in 2017 [118].

5.2 IL-6 as a biomarker for CRS—IL-6 is a cytokine secreted by a multitude of various cell types in response to inflammatory, infectious or malignant processes [119] that has been well established as a biomarker for CRS following CAR-T infusion (Table 4). IL-6 is involved in classical and trans-signaling leading to activation of the cytokine and coagulation cascade, which is manifested by vascular leakage and disseminated intravascular coagulation [120, 121]. Moreover, IL-6 has also been suggested to be involved in CRS associated cardiomyopathy, although the exact pathophysiology is not known [116]. It is also directly linked to neuro-inflammatory processes as discussed further below [122]. IL-6 levels can become elevated within 0–36 hours of CAR-T cell infusion which may occur prior to onset of clinical symptoms of CRS [114]. Peak levels typically occur 2–5 days post infusion [114] and can be measured in a variety of ways based on the laboratory or clinical setting. Administration of tocilizumab is given to prevent progression of CRS and functions by binding to both cell associated and soluble forms of the IL-6 receptor. It has also been recently studied as a prophylactic measure but has only been shown to reduce the incidence of severe (grade 3–4) CRS [123].

5.3 Other CRS Biomarkers—Interleukins are a type of cytokine that play a key role in activation and differentiation of immune cells with both anti- and pro- inflammatory properties. Interleukins have been studied in CRS to identify their role as biomarkers and enhance potential for alternative targeted therapies. Teachey et al found peak levels of 24 different cytokines amongst 51 patients with CRS in the month following infusion, which were increased in patients with severe CRS (grade 4–5) compared to those with mild disease (grade 0–3) [113]. Besides IL-6, findings included IL-8 as well as IFN- γ , soluble Interleukin-2 receptor alpha (sIL2R α), soluble glycoprotein 130 (gp130), soluble IL-6 receptor (sIL6R), MCP-1, macrophage inflammatory protein 1-alpha (MIP1- α), MIP1- β , and granulocyte macrophage colony stimulating factor (GM-CSF) [58]. Hay et al. also found elevations in markers of endothelial cell activation such as Von Willebrand Factor and Angiopoietin-2 amongst 60 patients with CRS, which were increased in patients with severe (grade 4) CRS versus those with mild disease [114]. Both studies utilized the 14 and 30-plex Luminex immunoassay to measure cytokine concentrations. There has also been suggestion of the role of IL-1 and potential for CRS treatment with IL-1 blockade, however this has only been evaluated in the mouse model [124].

5.4 Immune effector cell associated neurotoxicity syndrome

(ICANS): Neurotoxicity is the second most common side effect associated with CAR-T therapy. It was previously referred to as CAR-T cells related encephalopathy syndrome [125] but now is termed immune effector cell associated neurotoxicity syndrome (ICANS) [115]. Clinically, ICANS can present as early as the day after or as late as the fourth week after CAR-T infusion [117, 126]. It is associated with higher CAR-T doses, fever and severe CRS [127]. Symptoms may include altered mental status, depressed consciousness, seizure,

headache, focal neurological changes, expressive aphasia or increased intracranial pressure / cerebral edema [125, 126]. Like CRS, diagnosis and severity grading also relies on both the NCI CTCAE and ASTCT guidelines [115, 116]. Pathogenesis of neurotoxicity is less clear than CRS. There has been evidence of similar cytokine elevation as seen in CRS, including markers of endothelial activation: VWF, Ang1 and Ang 2 [127]. Moreover, high concentrations of cytokines have been found in the cerebrospinal fluid (CSF) in patients with ICANS, proving the blood brain barrier does not protect against infiltration of these inflammatory molecules [126, 127]. The Luminex immunoassay was used for all cytokines in serum and CSF samples with the exception of Ang1 and Ang2 which required the immunoassay based Meso Scale Discovery Quickplex. Treatment for severe neurotoxicity has included tocilizumab with limited success, in theory because it does not cross the blood brain barrier. Dexamethasone, which does permeate the central nervous system, has often been given in tandem with tocilizumab with improvement in neurological symptoms. However, there are risks associated with steroid therapy and thus recommended as a last line agent. As of today, ICANS typically have a self-limited course with limited case reports endorsing permanent symptoms.

Conclusions and Future directions

Advancement in engineered immune T-cell based therapies, specifically CAR-T therapy, has resulted in significant improvement in outcomes for patients with a history of relapsed or refractory malignant hematologic disease. However, progress needs to continue to develop cost efficient, non-invasive, and timely assays which can be used to identify biomarkers indicative of risk, diagnosis and / or response to treatment. Moreover, if the biomarker is involved in the pathogenesis of the disease process, targeted therapies can be utilized to mitigate or prevent toxicity. Innovation in proteomics technology has resulted in the discovery and validation of biomarkers for efficacy and toxicity of CAR-T therapy. Technology utilized includes expansion of prior traditional analyses methods (ELISA, flow cytometry and mass spectrometry) to develop state of the art methodologies such as single cell cytokine analysis, CyTOF, multiplex imaging of single cell spatial profiles, and CITE-seq.

Future directions will include incorporation of CAR-T biomarkers in clinical trials that integrate diverse patient populations with sample collection, analysis and data sharing as per the American Society of Hematology (ASH) Taskforce for Immunotherapies workshop on biomarkers for CAR-T cell and BiTE therapies toxicity and efficacy [29]. Harmonized and validated biomarker assays should be established amongst all clinical centres / open clinical trials with subsequently standardized widespread delivery of materials and instruction [29]. Investigation could then be expanded to include assessment of risk, confirm diagnosis, evaluate response to treatment and track associated toxicities. In collaboration, further discovery of candidate biomarkers and preclinical testing of biomarker-based interventions will be enabled [29].

Expert Opinion

Proteomics has revolutionized the field of biomarker discovery and validation, compared to traditional hypothesis driven methods. Moreover, the identification of these biomarkers has provided insight into pathophysiology following CAR-T infusion and associated efficacy and toxicity. However, biomarker validation and standardization across institutions is still needed. To start, biological samples from CAR-T patients should be collected and reported in a standardized manner in the current way in which clinical data is reported. Network development for collaboration between clinicians, academia, pharmaceuticals and health authorities is also vital with focus on centralized biorepositories with standardized sample storage and data analysis. By expanding the number of validated biomarkers, researchers could design prospective, randomized studies leading to an increase in clinical trials. The ASH Taskforce for Immunotherapies workshop on biomarker development recently published an outlined consensus detailing the efforts outlined above [29], with the goal for extension to international registries and plan for future meetings.

When developing biomarker based clinical trials, one of the current limitations involves the lack of real-time technology. While POC IL-6 testing and the PedELISA have been created for use at the bedside, other technologies remain limited to the laboratory setting and do not produce rapid results. Technology is also limited by lack of standardized biomarker assays and need for reagents to specifically detect CAR-T cells by flow cytometry and polymerase chain reaction (PCR). Improvements are needed to secure funding, increase product availability and regulate staff training for integration into the clinical setting.

Biomarkers of CAR-T efficacy and toxicity have been identified but expanded research could provide a better understanding of mechanistic factors and identify alternative therapeutic targets. Currently, Tocilizumab is the only FDA approved drug for the treatment of CRS and steroid therapy the mainstay for management of ICANS. With increased validation of biomarkers, additional targeted drug therapies could be developed.

Over the past 5 years, proteomics technology has developed at rapid speed and lead to substantial progress in the identification and validation of biomarkers in CAR-T therapy. In the upcoming 5 years, it is anticipated that significant improvement will be made in standardized collection of biomarkers in a centralized repository available for large-scale data analysis with the goal of international collaboration. Technology will also continue to advance, with a focus on developing real-time testing for use at the clinical bedside and thus incorporated into clinical trial. These trials will allow for identification of patient response and risk of life-threatening side effects with potential for improved precision medicine.

Funding

This paper was funded by the U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute, grant: R01CA168814; and the U.S. Department of Health and Human Services, National Institutes of Health, National Heart, Lung, and Blood Institute, grant: R21HL139934.

Declaration of interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes

employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Article highlights:

- CAR-T is a powerful engineered T-cell based therapy; however, companion biomarkers remain underexplored.
- New methods for biomarker evaluation are revolutionizing the field of biomarker and will allow for risk stratification in clinical setting.
- Current most validated biomarkers of CAR-T efficacy and toxicity are reviewed and direction for future advancement proposed.

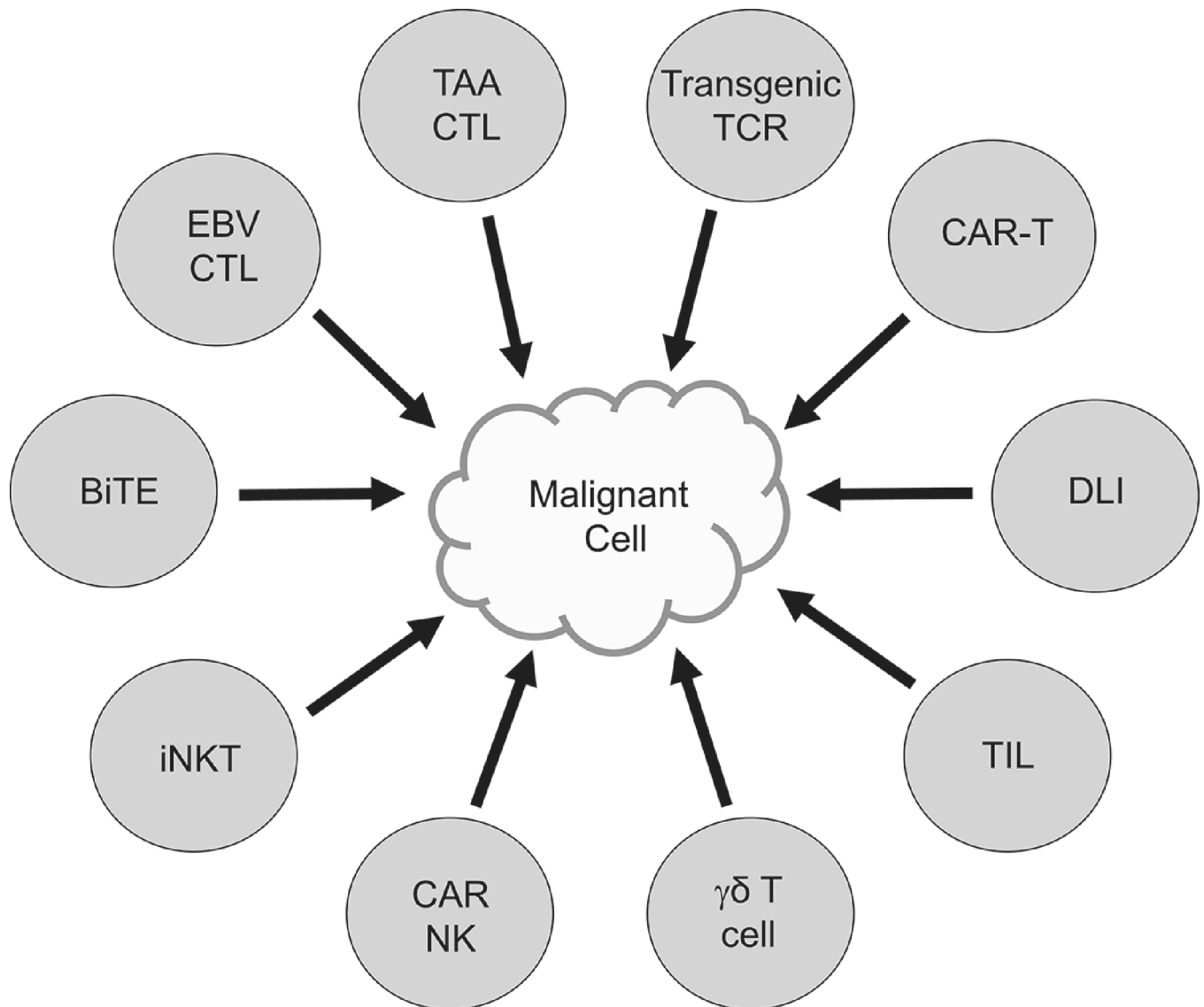


Figure 1 –.

Adaptive passive immune-cell therapies used to target malignant diseases (both liquid and solid). $\gamma\delta$ T cell (gamma delta T cell), BiTE (bi-specific T-cell engager), CAR-T (chimeric antigen receptor T cell), CAR-NK (chimeric antigen receptor natural killer cell), DLI (donor lymphocyte injection following allogeneic hematopoietic cell transplantation), EBV CTL (Epstein-Barr virus cytotoxic T-lymphocyte), iNKT (invariant natural killer T cell), Tumour antigen associated cytotoxic T lymphocyte (TAA CTL), Transgenic TCR (transgenic T-cell receptor) and TIL (tumour-infiltrating T cell that have been extracted from the autologous tumour and expanded in IL-2).

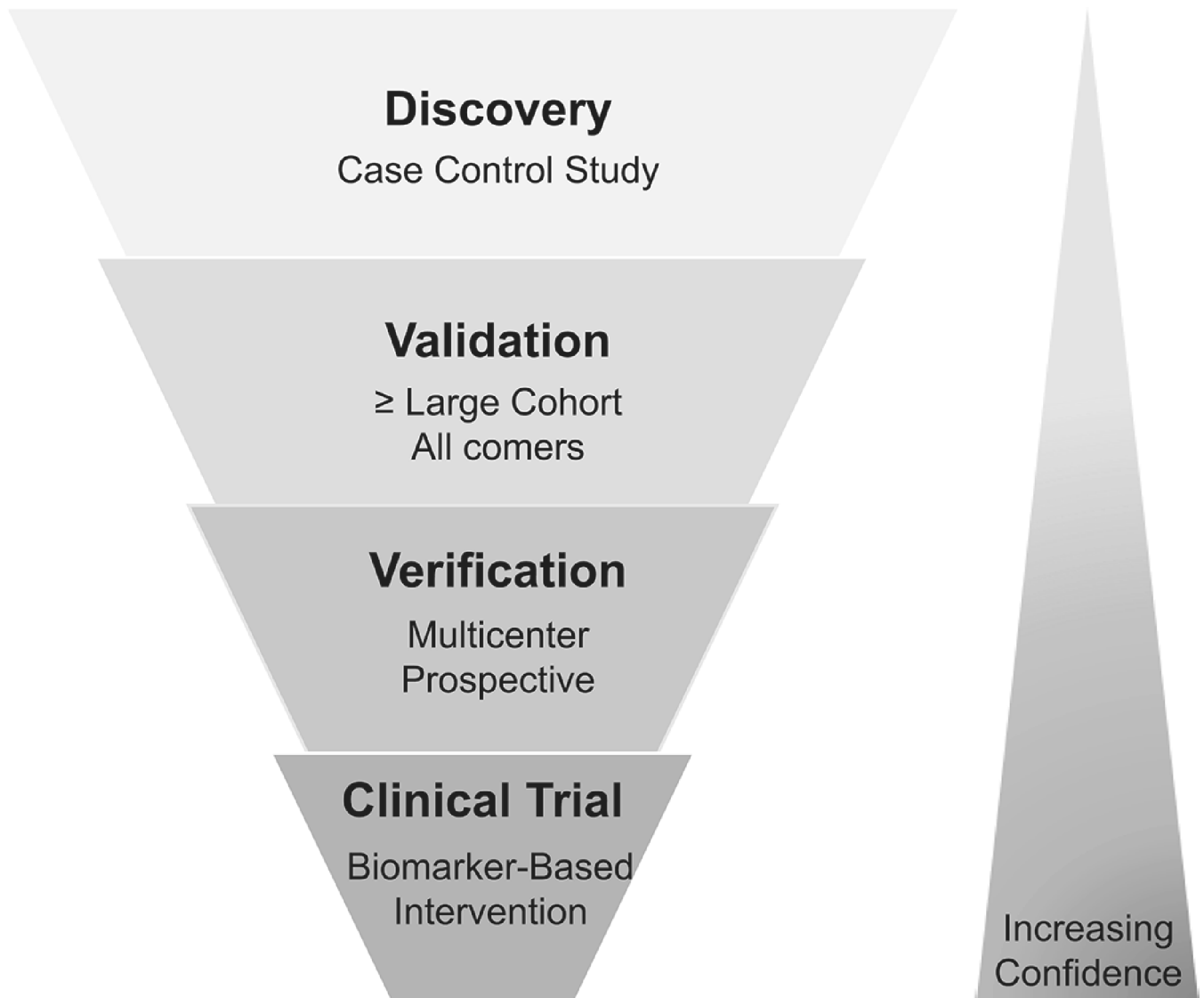


Figure 2.

Major Phases of Biomarker Development. First, the Discovery phase compares 20 to 40 cases and controls to identify potential candidate biomarkers, defined by area under the curve (AUC) of the receiver operating characteristic (ROC) >0.70 . Next, the validation phase is performed usually via high throughput immunoassay typically in a retrospective cohort from multiple centres. The verification phase then uses the same testing as the validation phase but in a prospective study from multiple centres. This phase also includes the goal of defining high and low cut-offs for a specific outcome. Finally, the biomarker can be used in clinical trial. If the biomarker impacts patient outcomes, it is likely that it will become standard of practice.

Table 1.

FDA approved CAR-T products

Product	Generic Name	Target	Indication	Response	Reference
ABECMA	idecabtagene vicleucel	BCMA	<ul style="list-style-type: none"> • Adult patients with relapsed or refractory multiple myeloma after four or more prior lines of therapy 	<ul style="list-style-type: none"> • 73% 	Munshi [128]
BREYANZI	lisocabtagene maraleucel	CD19	<ul style="list-style-type: none"> • Diffuse large B cell lymphoma (DLBCL) • High-grade B-cell lymphoma • Primary mediastinal large B-cell lymphoma • Follicular lymphoma grade 3B 	<ul style="list-style-type: none"> • 68%–81%* • 76% • 79% • 84% 	Abramson [129], Chow [130]
TECARTUS	brexucabtagene autoleucel	CD19	<ul style="list-style-type: none"> • Relapsed or refractory mantle cell lymphoma 	<ul style="list-style-type: none"> • 93% 	Wang [29]
KYMRIAHA	tisagenlecleucel	CD19	<ul style="list-style-type: none"> • Adult patients with relapsed or refractory DLBCL • Young adult patients up to age 25 with relapsed or refractory acute lymphoblastic Leukaemia 	<ul style="list-style-type: none"> • 52–61% • 81% 	Schuster [131], Sesques, [132] Maude [133],
YESCARTA	axicabtagene ciloleucel	CD19	<ul style="list-style-type: none"> • DLBCL • Primary mediastinal B-cell lymphoma • High grade B-cell lymphoma • DLBCL that results from follicular lymphoma • Follicular lymphoma 	<ul style="list-style-type: none"> • 64–92% ** 	Locke [24], Neelapu [3] Neelapu [134], Sesques [132]

* Included group of DLBCL patients, subtype not otherwise specified.

** Included group of DLBCL patients, subtype either grouped with refractory DLBCL versus Primary mediastinal B-cell lymphoma combined with DLBCL that resulted from follicular lymphoma or did not otherwise specify subtype.

Table 2.

Biomarker Definitions as per NIH BEST Resource [28]

Biomarker Type	Definition
Diagnostic	An assay used to confirm the presence of the disease
Risk	An assay that indicates the potential for developing the disease in individuals who do not have clinically apparent disease
Prognostic	An assay used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease
Predictive	An assay used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product (before treatment is received)
Response/monitoring	An assay used to show that a biological response has occurred in an individual who has been exposed to a medical product (after treatment is received)

Table 3.

Recommended Biomarker Patient Sample Collection

Sample Type	Sample Aliquot Volume & Quantity	Calendar Driven Collection										Event Driven Collection			
		Apheresis Product	Pre-lympho depleting Rx	CT product	Day -1/0	Day 2	Day 4	Day 7	Day 10	Day 12	Day 14	Day 21	Day 90	Day 360	At Relapse
Plasma (EDTA)	0.25ml × up to 10 aliquots	x	x		x	x	x	x	x	x	x	x	x	x	x
Buffy Coat Cells	Buffy coat cells isolated from 5ml tube for plasma processing.	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Viable PBMC	5×10 ⁶ cells/1ml, 1–7 aliquots	x	x	x											x
Bone Marrow	5 ml aspirate														x
Tumor	FFPE slides 10×5µm														x

Table 4:

Publications examining IL-6 as a biomarker of CAR-T toxicity

Title	Year
Efficacy and Safety of Humanized anti-CD19-CAR-T Therapy Following Intensive Lymphodepleting Chemotherapy for Refractory/Relapsed B Acute Lymphoblastic Leukemia [135]	2019
Clinical and Biological Correlates of Neurotoxicity Associated with CAR T-cell Therapy in Patients with B-cell Acute Lymphoblastic Leukemia [126]	2018
The Clinical Efficacy of First-Generation Carcinoembryonic Antigen (CEACAM5)-Specific CAR T-cells is Limited by Poor Persistence and Transient Pre-conditioning-Dependent Respiratory Toxicity [136]	2017
Axicabtagene Ciloleucef CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma [3]	2017
Endothelial Activation and Blood–Brain Barrier Disruption in Neurotoxicity after Adoptive Immunotherapy with CD19 CAR-T Cells [127]	2017
CD19 CAR–T cells of Defined CD4+·CD8+ Composition in Adult B cell ALL Patients [99]	2016
Identification of Predictive Biomarkers for Cytokine Release Syndrome after Chimeric Antigen Receptor T-cell Therapy for Acute Lymphoblastic Leukemia [113]	2016
Cytokine Release Syndrome After Chimeric Antigen Receptor T Cell Therapy for Acute Lymphoblastic Leukemia [137]	2016
Chimeric Antigen Receptor T-cells Persist and Induce Sustained Remissions in Relapsed Refractory Chronic Lymphocytic Leukemia [17]	2015
T-cells Expressing CD19 Chimeric Antigen Receptors for Acute Lymphoblastic Leukemia in Children and Young Adults: A Phase 1 Dose-Escalation Trial [25]	2015
Chimeric Antigen Receptor T-Cells for Sustained Remissions in Leukemia [16]	2014