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Review

Factors Affecting the Potency and Safety of Chimeric Antigen Receptor T-Cells

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Abstract: Chimeric antigen receptor (CAR) T-cells have become a standard therapy for many hematologic malignancies. While many CAR T-cells are being produced commercially and are in clinical trials, more CAR T-cells are being developed, particularly for the treatment of patients with solid tumors. Unlike most other therapies used to treat cancer and hematologic malignancies, CAR T-cells are biological agents which are most often produced from a patient's own T-cells. A critical part of the development of new CAR T-cells is identifying the characteristics that are responsible for their potency, toxicity, and clinical effectiveness which often vary among patients. We describe factors responsible for CAR T-cell potency, ongoing work focused on understanding critical characteristics of CAR T-cells contributing to their potency and safety, and ways to improve their potency. Since the factors affecting CAR T-cell potency are dependent on their structure and manufacturing methods, the nature of CAR vectors and CAR T-cell manufacturing methods are also reviewed. When assessing CAR T-cell potency *in vitro* studies provide valuable information, but it is also important to use data obtained from early clinical trials of specific CAR T-cells to advance the understanding of factors and characteristics that contribute to CAR T-cell potency, safety and clinical effectiveness.

Keywords: chimeric antigen receptor T-cells; potency; cellular therapy; cancer immunotherapy

1. Introduction

Chimeric Antigen Receptor (CAR) T-cells have been used effectively to treat hematological malignancies for more than 15 years. Initially, anti-CD19 CAR T-cells were used to treat adults with Chronic Lymphocytic Leukemia (CLL) [1,2] and patients with B-cell lymphoma [3]. Since then, anti-CD19 CAR T-cells have been licensed for the treatment of adults with B-cell lymphoma and children with acute lymphoblastic leukemia (ALL) [4]. Anti-B-Cell Maturation Antigen (BCMA) CAR T-cells have also been licensed for the treatment of patients with multiple myeloma [4]. In addition to these approved products, several other CAR T-cells have been tested in clinical trials. CAR T-cells targeting CD20 and CD22 have been used to treat adults with B-cell lymphoma and those targeting CD38 and CD138 have been used to treat patients with multiple myeloma [5]. Children and young adults with ALL are being treated with anti-CD22 CAR T-cells [6] and the combination of anti-CD19 and anti-CD22 CAR T-cells [7]. Acute myeloid leukemia (AML) has also been treated with CD33 and CD123 CAR T-cells [5].

While CAR T-cells have been used clinically for many years the factors that are responsible for their clinical effectiveness are still not completely understood. This review discusses progress concerning the identification of factors responsible for CAR T-cell potency, ongoing work focused on understanding critical characteristics of CAR T-cells, and future directions. Since the factors affecting CAR T-cell potency are dependent on their structure

and methods used for manufacturing, the nature of the CAR vector and CAR T-cell manufacturing will also be briefly reviewed.

2. Structure and Manufacturing of CAR T-Cells

2.1. CAR T-Cell Structure

The structure of CAR T-cells has been well described [8]. They are T-cells that have been genetically engineered to express a transgene that encodes a single chain variable fragment (scFv) which recognizes a target antigen. The scFV signals through a CD3 zeta chain which is also encoded by the transgene. The CAR construct also includes a portion that encodes a T-cell costimulatory molecule and a transmembrane protein. The most commonly used costimulatory molecules are CD28 and 4-1BB.

Most CAR T-cell products are made from T-cells collected from the person who is to be treated and are known as autologous T-cells. Using autologous T-cells to make CAR T-cells helps prevent alloimmunization and rejection that often occurs when patients receive allogeneic cells. However, autologous CAR T-cells may be rejected due to alloimmunization to the proteins encoded by the CAR construct. This is more common if the scFv encoded by the CAR construct is derived from a mouse monoclonal antibody. Recipients of CAR T-cells with scFv of mouse origin can develop antibody or cellular immune mediated reactions to the CAR T-cells [9]. CAR T-cells with humanized scFv have been used in some clinical trials [10]. The fact that most CAR T-cells are made from autologous T-cells is important since the characteristics and function of T-cells from patients with hematologic malignancies may differ from those obtained from healthy subjects due to their underlying disease. Allogeneic CAR T-cells are being used in some clinical trials [11], however, typically these CAR T-cells undergo additional genetic engineering to minimize the risk of graft versus host disease [12] and alloimmunization [13]. Since these allogeneic CAR T-cells can be given to any patient with the targeted malignancy, they can be produced in large lots which reduces the cost of each individual dose given.

2.2. Manufacturing CAR T-Cells

Manufacturing CAR T-cells is relatively easy compared to some other cell therapies. T-cells are collected, isolated, stimulated, cultured, transduced and expanded [14,15]. The process takes 7 to 10 days. Typically, the T-cells used to manufacture CAR T-cells are collected by apheresis, but a method to produce CAR T-cells using T-cells isolated from a whole blood collection has recently been described [16]. To make CAR T-cells the T-cell rich peripheral blood mononuclear cells (PBMCs) that have been collected by apheresis or whole blood collected by phlebotomy are enriched for T-cells. Several different methods are available [17], but the most effective methods involve T-cell selection with monoclonal antibodies. T-cells may be selected directly using anti-CD4 and anti-CD8 monoclonal antibodies or they can be isolated by negative selection which involves the removal of unwanted cell by the use of a mixture of monoclonal antibodies directed to other leukocytes such as B-cells, monocytes, NK-cells and granulocytes [17].

The isolated T-cells are placed in culture, stimulated with cytokines such as IL-2 or the combination of IL-7 and IL-15 and are expanded. CAR T-cells expanded in IL-7 and IL-15 have increased expansion and persistence in mouse models compared to those expanded in IL-2. In addition, those expanded in IL-2 contained more T-reg cells and expressed higher levels of PD-1 [14,18]. Agents that activate T-cell costimulatory molecules are also added. A typically reagent used to activate T-cells is made up of anti-CD3 and anti-CD28 which are immobilized on a solid surface such as a bead [15], however, other matrices have been used including DNA-based T-cell activator consisting of a single stranded DNA platform and oligo nucleotide conjugated anti-CD3 and anti-CD28 [19].

After a few days in culture the T-cells are transduced with the CAR construct. They are then expanded in culture for approximately 5 to 7 days. A wide variety of systems have been used to culture and expand CAR T-cells. They can be cultured in bags, gas permeable G-rex flasks [20] or a variety of bioreactors such at the Cytiva Xuri Cell Expansion System [15], Lonza Cocoon, Miltenyi Prodigy and Terumo Quantum [21]. At the end of the culture period, the CAR T-cells are harvested, tested and packaged. They may be cryopreserved and later thawed and infused or they may be infused fresh shortly after they are harvested.

The first CAR T-cell products were administered immediately after manufacturing was completed. However, to allow for better scheduling of patient treatments, optimization of manufacturing resources, and the completion of final product sterility testing, many centers now cryopreserve CAR T-cell products at the end of the manufacturing process. Comparison of the clinical outcomes of patients receiving fresh or cryopreserved CD22 and CD19/CD22 bispecific CAR T-cells found that fresh and cryopreserved CAR T-cells had similar *in vivo* expansion, and patients receiving these products had similar clinical disease response and toxicity, but cryopreserved CAR T-cells had slightly less *in vivo* persistence [2,22].

2.3. Factors Affecting CAR T-Cell Potency

Several factors affect the potency of CAR T-cells (Figure 1). One of the most important factors is the CAR construct. The effectiveness of the binding of the scFv is an important CAR T-cell characteristic as are the costimulatory molecules. If the binding of the scFv to the target antigen is weak, the ability of the CAR T-cells to bind and kill target cells may be low and clinical outcomes may be poor. However, excessive scFV affinity may lead to excess CAR T-cell stimulation and exhaustion [9] and off target toxicity [23]. The density of the expression of the antigen targeted by the CAR T-cell is also important. If the antigen density is not great enough, the CAR T-cells will not kill the targeted cells.

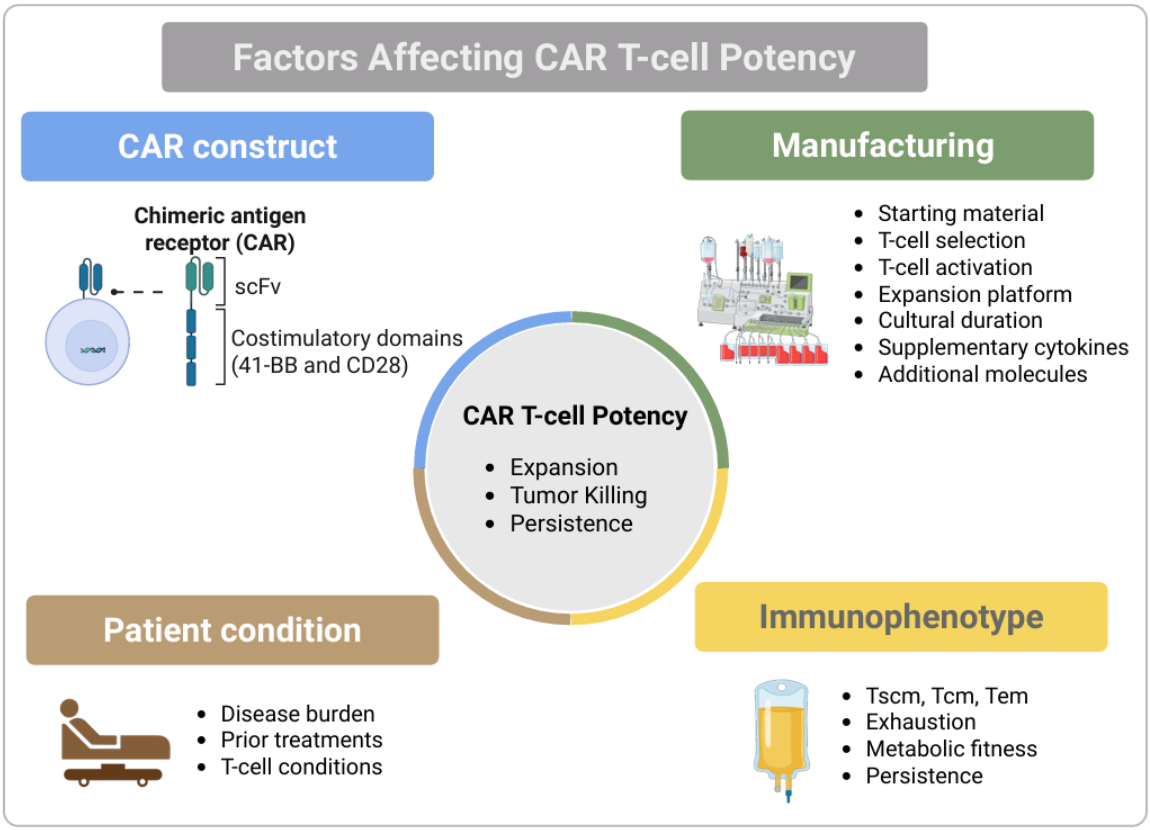

Figure 1. Factors affecting CAR T-cell potency. Key determinants influencing CAR T-cell potency, categorized into four major aspects. (**Top left**) CAR construct: Structural components of the chimeric antigen receptor, including the antigen-binding domain (scFv) and costimulatory domains (e.g., 4-1BB, CD28), which influence activation, signaling strength, and persistence. (**Top right**) Manufacturing: Manufacturing-related variables that impact CAR T-cell quality, including starting material, T-cell selection and activation methods, expansion platforms, culture duration, and use of supplementary cytokines or additional molecules. (**Bottom left**) Patient condition: Patient-specific factors such as disease burden, prior treatments, and baseline T-cell fitness that can affect CAR T-cell function and therapeutic outcomes. (**Bottom right**) Immunophenotype: Phenotypic characteristics of apheresis and infusion products, including differentiation states (e.g., T_{scm}, T_{cm}, T_{em}, etc.), exhaustion status, metabolic fitness, and persistence, which are associated with functional potency.

The costimulatory molecules encoded by the CAR construct also affect the characteristics of CAR T-cells. Clinically effective CAR T-cells expand and persist *in vivo* for several days. The costimulatory molecule encoded by the CAR construct can affect CAR T-cell expansion and persistence. CAR T-cells with CD28 as a co-stimulatory molecule typically demonstrate greater expansion than those with 4-1BB, but CAR T-cells with 4-1BB as a costimulatory molecule show better persistence [9]. In clinical trials neurological toxicities have been greater in CAR T-cells with CD28 co-stimulatory molecules, but this likely due to multiple factors [24].

The phenotype of a CAR T-cell also affects their cytotoxicity and persistence. CAR T-cells express T-cell surface makers and at the end of the culture period CAR T-cells can display a wide variety of phenotypes ranging from stem memory to effector T-cell. CAR T-cells with a more naïve T-cell phenotype are more persistent and more potent [25].

The nature of the manufacturing process can affect the CAR T-cell phenotype (Figure 1). The characteristics of the same type of CAR T-cells produced using different methods vary [21]. As mentioned previously, the type of cytokines used in the culture media can also affect CAR T-cell characteristics and potency. In addition, the duration of time in culture affects the phenotype of CAR T-cells. Longer duration of culture produces CAR T-cells with a more differentiated T-cell phenotype [26]. Some CAR T-cell manufacturing protocols include the addition of reagents that suppress T cell exhaustion and terminal differentiation by metabolic inhibition [14,27]. AKT inhibitors can increase the proportion of CAR T-cells that express a less differentiated phenotype [28].

3. Measuring CAR T-Cell Potency

3.1. Laboratory Evaluation

When a new CAR T-cell is being developed, its potency is often initially evaluated *in vitro* using cytotoxicity assays targeting a cell line that expresses the target antigen (Figure 2). If the new CAR T-cells demonstrates promising results in an *in vitro* cytotoxicity assay, it is generally tested in a mouse model that makes use of malignant cells that express the target antigen. The ability of the CAR T-cell to eliminate the malignant cells is assessed as a measure of the CAR T-cell's potency (Figure 2).

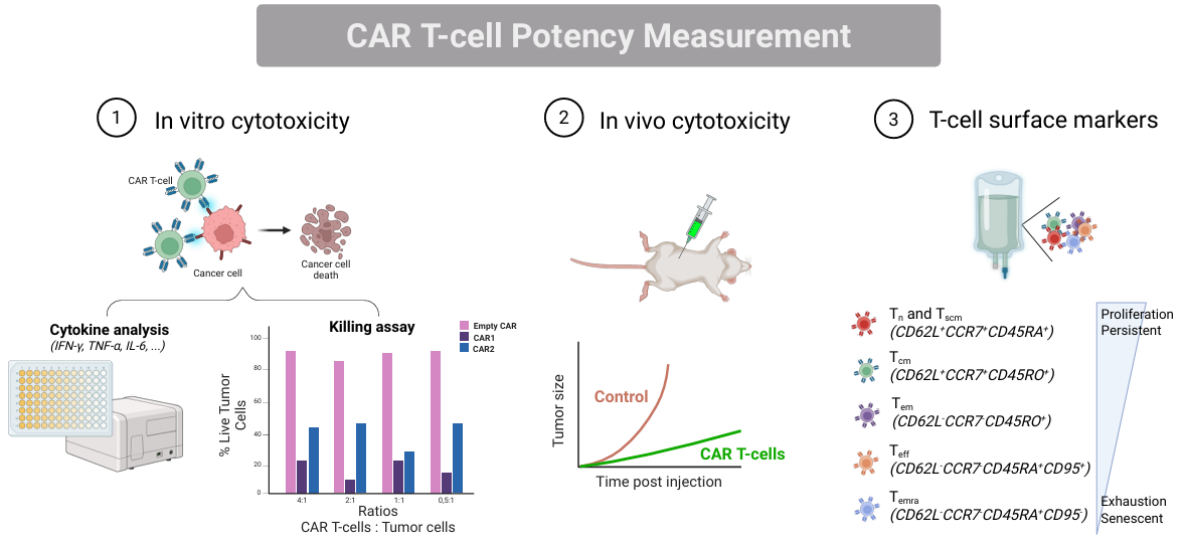

Figure 2. CAR T-cell potency measurement. Key functional assays used to evaluate CAR T-cell potency, categorized into three main aspects. (**Left**): *In vitro* cytotoxicity. Assessment of immediate effector function following co-culture with tumor cells, measured by cytokine release (e.g., IFN-γ, TNF-α, IL-6) and tumor cell killing efficiency. (**Middle**): *In vivo* cytotoxicity. Evaluation of CAR T-cell antitumor activity in animal models by monitoring tumor growth over time after CAR T-cell administration. (**Right**): T cell surface markers. Characterization of CAR T-cell phenotypes by flow cytometry, including markers associated with proliferation, persistence, and differentiation states.

Once a specific CAR T-cell becomes a clinical therapy potency testing of each clinical product prior to its infusion into a patient is more challenging. Since CAR T-cells may be given as a "fresh" product immediately after manufacturing is complete or they may be cryopreserved and given after a few days of storage, potency testing of clinical CAR T-cells must be completed within a limited time frame. Using animal models or cytotoxicity assays to measure potency of clinical CAR T-cells is not practical because these assays typically require several days or weeks to complete.

Assays that measure CAR T-cell release or production of cytokines or enzymes can be used to measure the potency of clinical CAR T-cell products. The CAR T-cells can be incubated with cells expressing the target antigen and after a few hours of incubation, the production or release or cytokines by the CAR T-cells can be measured. These assays may assess the production or release of tumor necrosis factor alpha, interferon gamma, granzyme B or other factors [29].

Another important method of assessing CAR T-cell potency involves analysis of surface antigen expression (Figure 2). CAR T-cells can express surface markers characteristic of T-cells at different states of maturation such as naïve, stem memory, memory, effector memory or effector T-cells. The proportion of CAR T-cells with each of these phenotypes varies among CAR T-cells from different patients. Using animal models and clinical studies

involving tumor infiltrating lymphocytes, Restifo and Gattinoni showed that T-cells with more immature phenotype are more proliferative and more persistent and more potent [25]. As previously mentioned, others have found this is also true for CAR T-cells.

The effectiveness of CAR T-cell therapy is also dependent on the number of cells administered. Consequently, the viability of the CAR T-cells and the proportion of T-cell expressing the CAR construct are also a reflection of their potency.

3.2. Using Clinical Data to Identify Factors Effecting CAR T-Cell Potency

While CAR T-cells have been shown to be effective in patients who have failed multiple other therapies, some patients do not response to CAR T-cell therapy. The clinical response rate of CAR T-cell therapy depends on the patient's disease and CAR T-cell type, but typically approximately 50% to 70% of patients experience a complete clinical response.

Several factors affect the likelihood that patients will experience a clinical response to a specific CAR T-cell therapy. Like any cancer therapy, effectiveness is dependent on patient's condition. Patients with more advanced disease are less likely to respond. The characteristics of the CAR T-cell product also contribute to the patient's clinical outcome. Since CAR T-cells are biologic therapies that are made from the patient's own cells, there is variability in CAR T-cells characteristics among patients. This variability among CAR T-cells from different patients contributes to the variability in clinical outcomes among patients.

Many studies have compared CAR T-cells from patients who experienced a clinical response with those that did not in order to identify factors that contribute to CAR T-cell effectiveness. These types of studies have found that *in vivo* expansion and persistence of CAR T-cells is associated with clinical responses to CAR T-cell therapy [30,31]. When CAR T-cells demonstrate greater *in vivo* expansion and persistence patients are more likely to experience a better clinical outcome.

Another similar study also found that CAR T-cells from patients with clinical responses are more likely to express characteristics of naïve and stem memory T cells then CAR T-cells from patients without clinical responses [32]. In addition, CAR T-cells from patients who don't have a clinical response are more likely to have characteristics of effector T-cells than CAR T-cells from patients with clinical responses [26,33,34].

CAR T-cells are frequently used to treat patients with hematologic malignancies, and the underlying disease can affect the characteristics of the patients T-cells. Since autologous T-cells are used to produce these CAR T-cells, the quality of the autologous T-cells is an important factor which may affect the characteristics of the CAR T-cell product.

Analysis of T-cells from patients with CLL that were used to manufacture the CD19 CAR T-cells found that T-cells from patients who did not response to therapy were enriched in genes involved with T effector cell differentiation, exhaustion, apoptosis and glycolysis [34]. Further comparison of T-cells from responders and non-responders found T-cells from CLL patients with clinical responses were enriched for stem memory T-cells and CD8+ T-cells which were $CD45RO^{-}CD27^{+}$ [34]. Analysis of the CAR-T cells from these same patients with CCL found that the CD19 CAR T-cells from patients with complete clinical response were enriched in T memory cell genes and CAR-T from patients who did not achieve a complete response were enriched for genes involved effector T-cell differentiation, apoptosis, aerobic glycolysis and regulation of the late memory T-cell phenotype [34].

A study of children and young adults with ALL who received autologous CD22-CAR T-cells found that the characteristics of T-cells isolated with anti-CD4 and anti-CD8 that were used to begin the manufacturing process effected the characteristics of the CAR T-cells and the patients' clinical outcomes [33]. A comparison of the T-cells from patients in this study who experienced clinical responses with those that did not found that T-cells from clinical responders demonstrated a greater proportion of T-cells with an early memory phenotype and those from non-responders had a greater proportion of T-cells with an effector memory phenotype. When the CD22 CAR T-cells given to patients who experienced clinical responses was compared to those from patients who did not, the CAR T-cells from non-responding patients had a more differentiated T-cell phenotype and greater expression of genes associated with T-cell exhaustion and cytotoxicity.

3.3. CAR T-Cell Manufacturing and Potency

There are a wide variety of methods that can be used to manufacture CAR T-cells, and several studies have demonstrated that CAR T-cell characteristics are dependent on the methods used to manufacture the cells. Factors shown to affect CAR T-cell characteristics include the composition of the cellular materials to begin manufacturing, nature of the methods to enrich the concentration of T-cells in the cellular starting materials, the

nature of anti-CD3 and anti-CD28 complexes used to stimulate T-cells, and the type of system used to culture and expand the cells.

Typically, CAR T-cells are manufactured using T-cells obtained from mononuclear cell concentrates collected by apheresis. These mononuclear cell concentrates are rich in lymphocytes, but they also contain a considerable quality of monocytes, granulocytes, red blood cells and platelets. One of the initial steps in the manufacturing process is enriching the autologous PBMCs collect by apheresis for T-cells. The type of enrichment method used can affect that final CAR T-cell product. Some enrichment methods may not remove all the monocytes and granulocytes from the starting material and if too many of these myeloid cells remain in the T-cell rich material used to manufacture the CAR T-cells, cell expansion and vector transduction may be poor [35]. The use of a robust T-cell selection process which yields a highly enriched T-cell population to start CAR T-cell manufacturing eliminates this problem. However, changing the T-cell selection method from a less specific to a more specific method can change the characteristics of the CAR T-cells [17,36].

Many different methods can be used to isolate T-cells, but positive selection of T-cells with anti-CD4 and anti-CD8 bound to paramagnetic particles is often used and is very effective. One clinical trial found that when the PBMC products used to manufacture CD22 CAR T-cells contained a high concentration of monocytes or granulocytes, the CAR T-cells failed to expand during manufacturing. The manufacturing process was modified to include a step to enrich the PBMCs for T-cell using an anti-CD4 and anti-CD8 selection method. The use of these highly enriched T-cells to begin CD22 CAR T-cell manufacturing resolved the problem of failure of the CAR T-cells to expand, but the potency and toxicity of the CAR T-cells changed [36]. CD4/CD8 cell selection resulted in increased inflammatory toxicities.

An alternative to positive selection of T-cells with anti-CD4 and anti-CD8 is negative selection. This involves the removal of contaminating cells from the mononuclear cell concentrate using antibodies directed to granulocytes, monocytes, B-cells and NK cells. One issue with positive antibody selection of T-cells is that antibodies directed to T-cells can induce changes or activate the T-cells. One study compared CD22 CAR T-cells produced from T-cells obtained from the same apheresis concentrate by positive antibody selection and by negative antibody selection [17]. They found that when the final CD22 CAR T-cell product was stimulated the CD22 CAR T-cells produced from positively selected T-cells released higher levels of interferon-gamma and IL-2 secretion than negatively selected T-cells, but there was no difference in their ability to eradicate leukemia in NSG mice.

Various reagents can be used to stimulate T-cell proliferation during the manufacturing process. Typically, T-cells are stimulated with IL-2 and anti-CD3 and CD28 antibodies. The anti-CD3 and CD28 antibodies are usually conjugated to magnetic beads or other types of particles. One study compared the effects of using four different activators with IL-2 on the characteristics of CAR T-cells [37]. The activators tested included anti-CD3 and anti-CD28 bound to magnetic microspheres (Dynal), bond to polymeric nanomatrix (TransAct), alginate hydrogel (Cloudz) and lipid membrane containing perfluorocarbon gas (Microbubbles) [37]. The bivalent anti-CD19/anti-CD20 CAR T-cells produced had different characteristics related to the type of activator used. Anti-CD3 and anti-CD28 bound to magnetic microspheres produced CAR T-cells with a larger subset of cells expressing naïve T-cell phenotypes and the alginate hydrogel produced CAR T-cells that were more likely to CD8+ and contained a larger subset of effector and effector memory T-cells [37].

When CAR T-cells were first produced more than 14 years ago, the cells were cultured and expanded in cell culture bags. However, as the use of CAR T-cells grew new devices and instruments became available for CAR T-cell culture and expansion including automated bioreactors and gas permeable flasks. As previously mentioned, some investigators have found that the presence of myeloid cells in the T-cells used to manufacture CAR T-cells can affect T-cell growth and expansion [36] suggesting that the manufacturing process can affect the characteristics of the final CAR T-cell product.

The type of cell culture method used to produce CAR T-cells can also affect the characteristics of the final CAR T-cell production. A study by Song and colleagues compared CAR T-cells manufactured with the same reagents expect that they were produced in four different expansion platforms: gas permeable bags, gas permeable G-Rex flasks, Xuri W25 rocking platform bioreactor and the CliniMACS Prodigy bioreactor [21]. They found that while the CAR T-cells produced in gas permeable bags, gas permeable flasks and the Xuri bioreactor were similar but those produced in the Prodigy bioreactor were enriched for CCR7+CD45RA+ naïve/stem central memory-like cells and were less cytotoxic and less exhausted cells [21].

Among the four cell culture devices in this study there were considerable differences in the density of CAR T-cells and culture media oxygen tension levels at the end of the culture period. Further comparisons by Song and colleagues of CAR T-cell culture conditions found the cell density was greatest in the Prodigy bioreactor, and the oxygen levels were the lowest. To determine if either high cell density or low oxygen levels affect CAR T-cell characteristics, the same type of CAR T-cells were produced in bags and in gas permeable flasks under conditions

of various cell densities and oxygen levels. While the density of cells in culture had no effect on CAR T-cell characteristics, growth in low oxygen tension levels produced increased proportions of CAR T-cells with a T naïve/stem central memory phenotype [21].

3.4. Making CAR T-Cells More Potent

While the characteristics of CAR T-cells can be changed to increase their potency by modifying the manufacturing process, this may not be advisable since it may also increase toxicities associated with CAR T-cells such as cytokine release syndrome or hemophagocytic lymphohistiocytosis (HLH) [36].

Ongoing studies suggest that the variability in the autologous T-cells used to begin the manufacturing process contributes to variability in the final CAR T-cells products [33]. If specific T-cells markers can be identified that are associated with T-cells that make less potent CAR T-cells, it may be possible to tailor the manufacturing process when such starting populations are used. In these cases, selecting or modifying manufacturing methods to enhance *in vivo* expansion, persistence, or functional capacity could help generate CAR T-cell products with improved potency.

4. Summary and Conclusions

CAR T-cells have become an important part of cellular cancer immunotherapy particularly for the treatment of patients with hematological malignancies. While many CAR T-cells are being produced commercially and are in clinical trials, more CAR T-cells will likely be developed, particularly for the treatment of patients with solid tumors. A critical part of developing new CAR T-cells is identifying the characteristics that are responsible for their potency and effectiveness. While *in vitro* studies of CAR T-cells provide valuable information, it's important to use data obtained from their use in early clinical trials to further understand factors and characteristics contributing to their clinical effectiveness.

Author Contributions

D.F.S., L.S. and J.P. developed the concept of this review. D.F.S. drafted the manuscript. L.S. prepared the figures and L.S. and J.P. reviewed and revised the manuscript. All authors have approved the submitted version and agree to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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