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Off the shelf T cell therapies for hematologic malignancies

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ABSTRACT

Adoptive transfer of autologous CAR-T cells can induce durable remissions in patients with relapsed/refractory hematologic malignancies. However, multiple challenges exist for manufacturing CAR-T cells from patients with advanced disease including inability to manufacture a product, disease progression or death while waiting for the CAR-T product to be available, and heterogeneity among autologous CAR-T products that contributes to unpredictable and variable clinical activity. Healthy donor T cells can provide a source for production of universal CAR-T cells when combined with gene editing to prevent expression of endogenous TCRs and avoid generation of GvHD in HLA mismatched recipients. Additional gene edits can be included to impart resistance to immunosuppression or improve trafficking to tumor sites. Recent advances in cell manufacturing and analytics technology can provide for consistent batch to batch manufacturing of gene edited allogeneic CAR-T cells in sufficient quantity to treat thousands of patients when needed as off the shelf products.

1. Autologous CAR-T therapies for treatment of hematologic malignancies

Chimeric antigen receptor (CAR) T cell therapies have produced significant and durable clinical responses in patients with refractory and relapsed hematologic malignancies. The majority of CAR-T therapies that have been studied in clinical trials and proven to provide a clinical benefit have been autologous products directed against the B cell antigen, CD19, in patients with B-cell acute lymphoblastic leukemia (B-ALL) [1–3], chronic lymphocytic leukemia (CLL) [4,5], and non-Hodgkin lymphoma (NHL) [6,7]. More recently CAR-T cells directed against B-cell maturation antigen (BCMA) [8,9] and CD123 [10] have shown activity in patients with relapsed or refractory multiple myeloma or acute myeloid leukemia, respectively. Manufacturing of autologous CAR-T therapies requires a leukapheresis procedure followed by activation and transduction of patient T cells using a lenti- or γ-retrovirus vector, or in some cases adenoviral vectors or a transposon/transposase system such as Sleeping Beauty [11] or PiggyBAC [12], to introduce a CAR transgene into the genome of the cell. Following transduction, T cells are expanded in culture, washed, resuspended in a solution compatible with i.v. administration to humans, and reinfused into the patient or cryopreserved for subsequent thawing and administration. Due to the advanced stage of disease and history of previous chemotherapies in patients with relapsed/refractory disease, it is sometimes not possible to produce an efficacious CAR-T product due to low T cell counts and/or poor quality of the T cells obtained from the patients at the time of leukapheresis. Several weeks are required from leukapheresis of the patient through manufacturing and shipping of autologous CAR-T product to the clinical site for infusion during which time patients may experience disease progression or die while waiting for their autologous product. In addition, in the case of autologous CAR-T therapies, a single manufacturing campaign generates a product that can be used to dose a single patient resulting in a significantly high cost of goods. The high costs associated with manufacturing of autologous CAR-T products places a burden on health care systems and restricts

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broad patient access to these novel therapies.

Autologous CAR-T products are inherently heterogenous due to differences in T cell quantity and phenotypes obtained from patients. In addition, the use of lenti- and retroviral vectors, which integrate semi-randomly within transduced T cells, introduces variability in CAR expression levels due to differences in copy number and expression of the CAR transgene within the genomes of the transduced cells. Turtle et al. [13] recently described autologous anti-CD19 CAR-T cells manufactured from defined $CD4^+$ and $CD8^+$ T cell subsets and administered in a defined CD4:CD8 composition to patients with B-ALL. The authors reported that this defined composition CAR-T product enabled identification of factors that correlated with CAR-T cell expansion, persistence and toxicity and facilitated determination of potential correlations between cell dose and efficacy or toxicity. Refinements in manufacturing procedures, such as pre-selection of T cell subsets, separate manufacturing streams for isolation, transduction, expansion, and remixing of $CD4^+$ and $CD8^+$ T cells can help to reduce the heterogeneity inherent in manufacturing of autologous CAR-T products. However, collection and shipping of leukapheresis products to a centralized manufacturing facility, and return of manufactured CAR-T product for patient infusion continue to represent logistical challenges that contribute to the complexity and costs associated with individualized manufacturing of autologous CAR-T products. Lastly, it is not possible to guaranty immediate availability of product when needed by the patient, some of whom cannot wait several weeks for the manufacture of an autologous CAR-T therapy.

2. Gene editing for production of allogeneic CAR-T cells from unrelated healthy donor cells

Recent advances in gene editing technology allow for the manufacture of CAR-T cell therapies starting from healthy donor leukapheresis material in which the quantity and quality of T cells can be pre-selected. However, the use of unrelated healthy donor T cells for production of allogeneic CAR-T cell products requires the use of gene editing technology to prevent expression of endogenous T cell receptors (TCRs) in order to minimize the potential to cause graft-versus-host disease (GvHD) in HLA mismatched recipients. Several different gene editing technologies have been used to prevent expression of endogenous TCRs. Zinc-finger nucleases (ZFN) [14], CRISPR/Cas9 [15,16], transcription activator-like effector nucleases (TALEN) [17,18], and engineered homing endonucleases [19] have been described that target an exon within the TCRα constant (TRAC) or TCRβ constant 1 (TRBC1) or 2 (TRBC2) loci for genetic knockout thereby preventing expression of α/β TCRs on the cell surface. ZFN, CRISPR/Cas9, or TALEN are two component systems in which sequence-specific DNA targeting and DNA cleavage functions are provided by separate molecular entities. For gene editing using CRISPR/Cas9 the components are introduced into T cells via electroporation of mRNAs encoding guide RNA (gRNA) or Cas9 nuclease, although other methods such as the use of lipid nanoparticles are being developed to deliver the gRNA and Cas9 nuclease. In the case of ZFN and TALEN, T cells are electroporated with mRNA or plasmid DNA encoding chimeric proteins consisting of either a pair of Zn-finger binding domains, or a pair of TAL effector (TALE) DNA binding domains, linked to the DNA cleavage domain of FokI nuclease. The DNA cleavage domain of FokI and Cas9 nucleases introduce a DSB at the target site specified by the Zn-finger, TALE, or gRNA which, in the absence of a homology repair template, is repaired by nonhomologous end joining (NHEJ), an error prone cellular repair pathway that results in insertion or deletion of nucleotides at the cleavage site resulting in loss of functional gene expression [20,21]. In the case of the engineered homing endonuclease technology such as described by MacLeod et al. [19], a guide sequence is not required in order to target nuclease activity to the intended genomic DNA sequence. T cells are electroporated with an mRNA that encodes the engineered nuclease which directly binds the DNA target sequence and makes a staggered cut resulting in 4 bp 3′ overhangs, a feature that may favor targeted insertion of a transgene via homology-directed repair (HDR) in the presence of a homology repair template (described below).

Gene edited (TCR knockout) allogeneic CAR-T cells are often produced by isolating and activating healthy donor T cells, most often using soluble or bead-bound anti-CD3 and anti-CD28 antibodies followed by transduction of the activated cells with a lenti- or γ-retroviral vector to deliver the CAR transgene (or using a transposon incorporating the CAR transgene and a transposase) resulting in random integration of the CAR transgene at several sites within the genome of the T cell [18,22]. The transduced T cells are subsequently gene edited to prevent expression of endogenous TCRs. The cells are then expanded in culture, depleted of remaining TCR positive (i.e. unsuccessfully gene edited) cells, washed and frozen in cryopreservation medium. By starting with healthy donor T cells in which the number, ratio of CD4 and CD8, and percentage of cells with naïve and central memory phenotypes can be pre-selected, a CAR-T product with desirable properties for use in adoptive cellular immunotherapy can be manufactured provided that the process has been optimized to maintain the desired T cell phenotypes in the final product. The percentage of cells that express the CAR transgene but do not express α/β TCRs at the cell surface is dependent on the efficiency of transduction and gene editing. Transduction efficiency increases with the multiplicity of infection (MOI) used to transduce the cells with the viral vector. However, with increasing MOI comes cellular toxicity and increased number of randomly integrated copies of the viral genome, thereby increasing the potential for mutagenic events [23,24]. Gene editing efficiency is affected by the efficiency and toxicity associated with transfection of T cells and the efficiency and specificity (related to nuclease-associated toxicity) of the guide/nuclease. Efficiency and toxicity associated with transfection of large numbers of T cells in a single batch is a major challenge in the manufacturing of gene edited CAR-T products. In recent years a number of manufacturers have introduced electroporation devices that can efficiently transfect up to several billion T cells in less than 1 hour using a closed system that is amenable to GMP manufacturing but optimization of electroporation conditions is a challenging task that has high impact within the manufacturing process of gene edited CAR T cells (see below in Allogeneic CAR-T Manufacturing). Target specificity of guide/nuclease, or just the nuclease in the case of homing endonucleases, may impact the efficiency of gene editing by determining the amount of nuclease that is available and active at the intended target site as well as toxicity that may be associated with off-target nuclease activity. In addition to the contribution to gene editing efficiency, nuclease specificity for the intended target is of paramount importance as off-target DSBs can negatively impact CAR T cell phenotype and function, create the potential for translocations and/or result in undesirable genotoxicities that may

result in detrimental effects following administration of gene edited CAR-T cells. It is a very difficult to evaluate gene edited cells for unintended off-target effects in a global, non-biased manner. Technologies that are not based solely on testing for predicted off-target nuclease activity, such as GUIDEseq [25], have been used to search off-target nuclease activity.

3. Allogeneic CAR-T cells with targeted CAR insertion by homologous recombination

Gene edited allogeneic CAR-T products produced using methods that involve transduction with randomly integrating retroviruses may have varying degrees of heterogeneity in terms of potency due to differential transgene expression as a result of variation in the number and location of integrated CAR transgenes among the successfully transduced T cells. Recently, several groups have described production of gene edited allogeneic T cells containing a targeted insertion of a CAR transgene into the TRAC locus. Hale et al. [26] used a megaTAL (TALE covalently linked to a site-specific meganuclease) combined with a recombinant adeno-associated virus (rAAV) homology repair template to insert the CAR at the site of the DSB in TRAC by homologous recombination using cellular HDR machinery. Eyquem et al. [27] used CRISPR/Cas9 and an rAAV homology repair template to insert an anti-CD19 CAR into TRAC or the β_2-microglobulin (B2M) gene, and MacLeod et al. [19] describe targeted insertion of an anti-CD19 CAR into TRAC using an engineered homing endonuclease and an rAAV homology repair template. Targeted insertion of a CAR transgene into a defined site within the cellular genome allows for controlled and homogeneous expression of the CAR. MacLeod et al. [19] and Eyquem et al. [27] demonstrated high selectivity for integration of the CAR transgene within TRAC, absence of off-target integration "hot spots", and uniform levels of expression of the CAR among the population of TCR-, CAR + T cells in preparations from multiple donors.

Another advantage of targeted insertion of the CAR transgene is the ability to control the level of expression of the CAR and to determine the impact of increasing or decreasing CAR expression on CAR-T cell activity. Our group observed a more homogeneous level of CAR expression using targeted insertion of an anti-CD19 CAR into TRAC [19] with either a weak or strong version of a synthetic promoter to drive CAR expression versus a highly variable level of expression among CAR-T cells produced from the same donor using the same CAR construct delivered by lentivirus transduction at low or high MOI (Fig. 1).

4. Additional gene edits to reduce immunogenicity and resist suppression within the tumor microenvironment

Multiple gene edits can be performed in order to impart specific properties to off-the-shelf CAR-T therapies depending upon the intended use of the product. For instance, in order to decrease the extent of allo-rejection that would be expected to occur in immunocompetent patients, gene editing to reduce or eliminate expression of HLA class I proteins can be performed. Ren et al. [28] recently described multiplexed gene editing of T cells that included knockout of the B2M gene combined with TCR knockout to prevent expression of α/β TCRs. In the absence of β_2-microglobulin (beta chain), class I HLA molecules do not form stable heterodimers at the cell surface. However, while the absence of HLA-I on the surface of CAR-T cells significantly reduces allogeneic attack by HLA-mismatched CD8 T cells (class-I restricted), it can result in increased destruction by NK cells due to "missing self" recognition. We observed this phenomenon in TCR/B2M double knockouts in which destruction by alloreactive T cells was significantly decreased but destruction by allo-NK cells was significantly increased. Therefore, we developed a targeted knock-in strategy in which a gene cassette containing a second generation anti-CD19 CAR driven by a synthetic promoter (RNA pol II target) and an anti-B2M short hairpin RNA (shRNA) driven by a U6 promoter (RNA pol III target) separated by a short intervening DNA sequence was knocked into TRAC. When compared with TCR/B2M double knockout CAR-T cells, the TCR knockout and anti-B2M shRNA knock down CAR-T cells showed significantly decreased destruction by both alloreactive T cells and NK cells (Fig. 2). It is possible that by significantly reducing the number of HLA-I molecules on the cell surface using the *B2M* knock down vs. a near

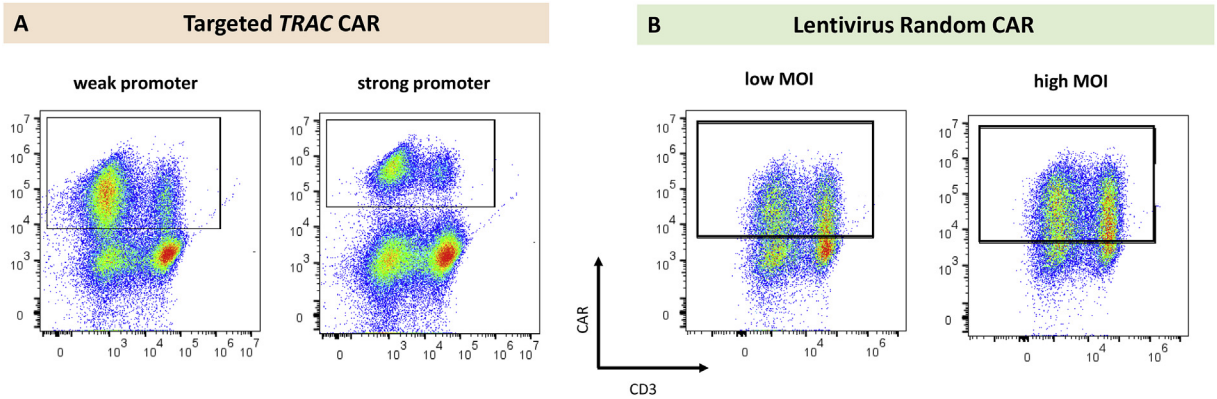

Fig. 1. CD3$^+$ T cells were isolated from a single donor and an anti-CD19 CAR was inserted into exon 1 of *TRAC* using ARCUS homing endonuclease and an rAAV vector (A). CAR T cells prepared from the same donor were transfected with ARCUS homing endonuclease to knockout expression of the TCR and transduced with a lentiviral vector engineered to express the CAR using the same promoter as the *TRAC* CAR (B). By targeting the insertion of the CAR transgene into a specific locus epigenetic position effects that affect CAR gene expression levels are eliminated. This results in a population of CAR T cells that are genetically identical and have more consistent behavior than lentivirus-derived CAR T cells.

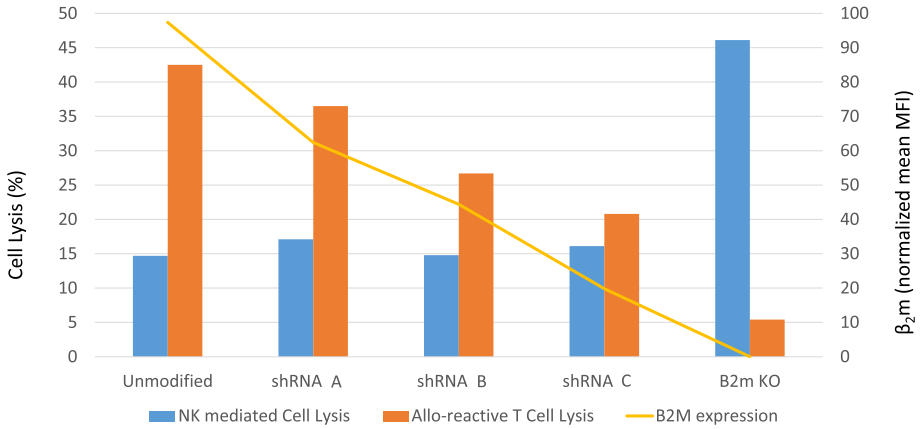

Fig. 2. Allogeneic CAR T cells are efficiently targeted for destruction by primed HLA-mismatched CD8 T cells. Knockout of *B2M* significantly reduces destruction of allogeneic CAR T cells by HLA-mismatched CD8 T cells but results in significant destruction by allo-NK cells. A gene cassette consisting of anti-CD19 CAR driven a JeT promoter and including a 3′ poly-A sequence combined with an anti-*B2M* shRNA under control of a U6 promoter in opposite orientation to the transcriptional direction of the CAR gene was knocked into TRAC. Knock-down of β_2-microglobulin production via anti-B2M shRNA, results in reduction of the level of HLA-I expressed on the surface of allogeneic CAR-T cells. Selecting a vector that expresses a desired level of anti-B2M shRNA allows for production of CAR-T cells that express a level of HLA-I proteins on the surface that results in reduced destruction by alloreactive CD8 T cells as well as reduced killing by allo-NK cells.

complete lack of surface HLA-I following B2M knock out, the extent of killing by HLA-I restricted CD8 T cells as well as killing by NK cells due to "missing self" is reduced. Specific killing of CD19 $^{+}$ target cells by the anti-CD19/B2MshRNA targeted knock-in CAR T cells was equivalent to the anti-CD19 only targeted knock-in CAR T cells (data not shown) indicating that CAR-directed cytotoxic killing is not negatively impacted by B2M knock down. This approach may represent a strategy to improve in vivo persistence of allogeneic CAR-T cells via decreased allo-rejection in immunocompetent patients.

In addition to knocking out expression of endogenous TCRs in order to minimize the potential for GvHD, additional gene edits can be performed in order to reduce susceptibility of allogeneic CAR-T cells to immunosuppression within the tumor microenvironment and improve activity of CAR-T cell products against solid tumors. CAR-T cells containing multiple gene edits that include knockout of inhibitory signaling receptors such as PD-1 (PDCD1) [29,30], LAG-3 [31], or knock-in of a dominant negative TGFβ2R (TGFBR2) [32] have been described and are expected to enter into clinical trials in the near future.

5. Allogeneic CAR-T cell manufacturing

The remarkable progress over the past decade in Chimeric Antigen Receptor T cell (CAR-T) translational and clinical research has vastly increased the understanding of the cell processing techniques necessary to reliably deliver a cellular therapeutic product that is well characterized and potent. Peripheral blood leukapheresis collections have proven to be the most widely utilized source of T-lymphocytes in current CAR-T clinical trials, whether autologous or allogeneic. The cellular manufacturing unit operations that define autologous and allogeneic CAR-T manufacturing typically include the following: separation of the T cells of interest from the bulk leukapheresis collection; T cell activation; transduction with a viral vector to integrate the CAR-construct into the T cell genome, ex vivo cell expansion, washing and concentration of the culture-harvested cells, and fill and finish of the washed and concentrated cells into the final drug product packaging, followed by cryopreservation. Allogeneic CAR-T cell manufacturing generally includes two extra manufacturing unit operations that are not present in a typical autologous CAR-T cell manufacturing schema: a genome editing step to render the T cell receptor nonfunctional, and the depletion of any cells which were not successfully genome edited (Fig. 3). Similar to many autologous CAR-T manufacturing processes [22,33,34], most allogeneic CAR-T cell manufacturing exists as a primarily functionally closed process that utilizes a mix of manual, semi-automated, and fully automated equipment for each unit operation.

5.1. Peripheral blood leukapheresis

As is the case for autologous CAR-T cell therapies, non-mobilized peripheral blood leukapheresis collections are the primary starting material for allogeneic CAR-T cell manufacturing. These collections are currently performed on normal, healthy adult donor volunteers on either the COBE Spectra or Spectra Optia (Terumo BCT, Lakewood, CO) or Amicus (Fresenius Kabi, Lake Zurich, IL) therapeutic apheresis systems utilizing approved mononuclear cell collection protocols developed by the device manufacturers. The COBE Spectra System will be replaced by the Spectra Optia worldwide by 2019. These leukapheresis products may either be cryopreserved and processed into CAR-T therapeutics at a later date, or alternatively shipped fresh overnight from a collection center at either ambient conditions or 2–8 °C to a manufacturing site for immediate initiation of bioprocessing. Donor criteria vary between allogeneic CAR-T cell developers, but generally a young, healthy, infectious disease-free male donor is sought due to the belief that

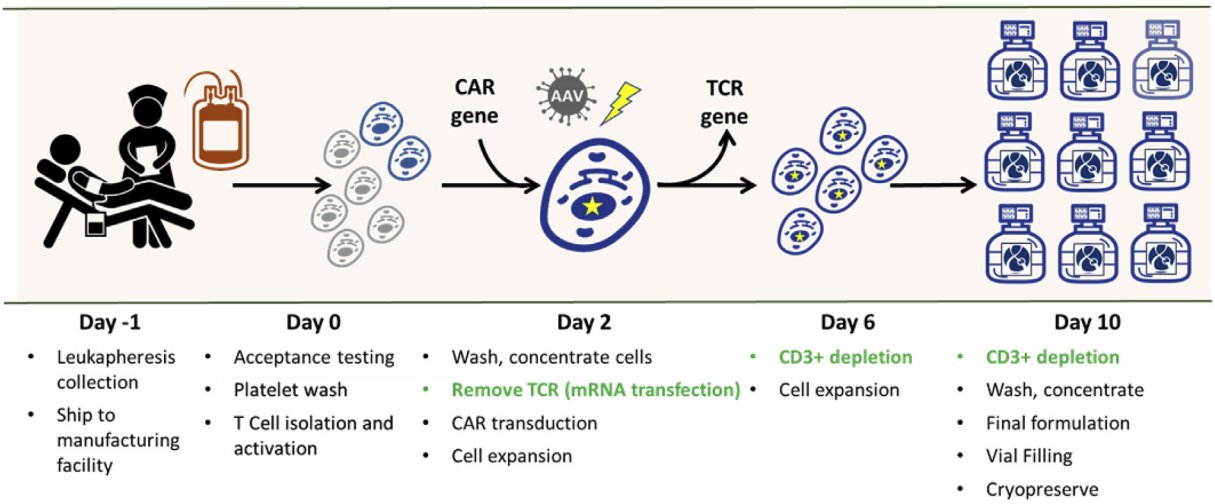

Fig. 3. Manufacturing unit operations for the production of clinical-grade allogeneic CAR-T cells. Unit operations that are unique to allogeneic CAR-T cell manufacturing are highlighted in green.

generally larger leukapheresis collections may be obtained from this population. Additionally, certain ratios of cellular phenotypes, such as CD4 to CD8 ratios may also be desired and included in donor acceptance criteria. A healthy donor leukapheresis collection generally processes 6–12 L of peripheral blood, yielding between 200-450 mL of collected fluid volume containing anywhere from 8-20 billion total nucleated cells (TNC). This starting material therefore defines the scale at which subsequent bioprocessing may occur. Research into utilization of iPSC or cord-blood derived progenitor cells as starting materials for allogeneic CAR-T cell therapeutics points to intriguing possibilities for future scale-up of allogeneic CAR-T cell manufacturing, as does the potential to pool leukapheresis collections from a single healthy donor from collections obtained and frozen over the course of several months or even years.

5.2. Isolation of T-lymphocytes

Upon receipt of a fresh or frozen leukapheresis product at a cellular manufacturing site, the next step in the bioprocessing of an allogeneic CAR-T cell product is the isolation of cells of interest, generally $CD3^+$, or $CD4^+$ and $CD8^+$ T cells, and the oftentimes concomitant depletion of other mononuclear cell types that are not of interest. This can be achieved in a number of ways, utilizing manual or automated methods, or a mix of the two modalities.

A first step in the isolation process is oftentimes a gross removal of platelets or the bulk enrichment of lymphocytes. The Biosafe Sepax 2 (GE Healthcare Life Sciences, Marlborough, MA) and the LOVO Cell Processing System (Fresenius Kabi) are both functionally closed and automated devices than are used for platelet reduction of leukapheresis products prior to isolation of cellular subsets. The Sepax utilizes a spinning syringe technology while the LOVO utilizes spinning membrane technology to reduce platelets. Centrifugation with large-volume centrifuge containers (250 mL or 500 mL) or centrifugation of the leukapheresis collection bag itself and expression of unwanted components utilizing a plasma press are also commonly utilized for manual platelet removal. The Elutra System (Terumo BCT) may also be deployed for both platelet reduction and lymphocyte concentration in one step, whereby platelets, monocytes, and granulocytes may be separated from lymphocytes through counter-flow elutriation. A major advantage of allogenenic CAR-T cell therapies is seen during this unit operation, where the presence of contaminating blasts in the leukapheresis collection is not an issue with healthy donor products. Blast contamination with autologous leukapheresis products collected from patients with hematologic malignancies is a significant manufacturing challenge for developers of autologous CAR-T's, as it is extremely difficult to remove blasts utilizing currently available bulk separation technologies.

After the initial removal of platelets or lymphocyte concentration, fine separation of the T cells of interest is achieved through antibody-coupled magnetic bead isolation. Three commercially available systems are able to enable this unit operation: Dynabeads (Thermo Fisher Scientific, Waltham, MA), CliniMACS Reagents (Miltenyi Biotec, Bergisch Gladbach, Germany), and EasySep Reagents (StemCell Technologies, Vancouver, Canada), respectively. Both Dynabeads and CliniMACS Reagents are currently available in GMP-grade for clinical manufacturing; the EasySep Reagents are currently only available as Research-grade reagents, however, may be made commercially available as GMP-grade reagents in the near future. Each magnetic bead isolation reagent technology is coupled with its own magnetic separation platform device capable of processing a full leukapheresis collection. The DynaMag CTS Platform (Thermo Fisher) allows for the functionally closed isolation of T cell populations, however, it is a manual system where an operator must control the flow rates independently. The CliniMACS System (Miltenyi) is an automated system where software protocols driving a series of pumps and valves control the magnetic isolation process which takes place in a single-use disposable set. Finally, EasySep reagents (StemCell Technologies) may be utilized with the LabSep System; however, this system is not functionally closed although it is semi-automated.

5.3. T cell activation

T cell activation following T cell isolation is generally achieved utilizing either bead-based reagents or soluble factors and can be performed in closed, static, cell cultureware such as VueLife fluorinated ethylene propylene (FEP) closed cell culture bags (St. Gobain Life Sciences, Gaithersburg, MD) which are handled manually, or in GRex culture devices (Wilson Wolf Corporation, St. Paul, MN) which can be filled and emptied semi-automatically with an accompanying fluid transfer pump unit. Generally, 500 mL-1L size culture vessels are utilized for this activation unit operation where the media is generally a T cell media supplemented with IL-2 and either 5–10% FBS or Human AB Serum. CD3/CD28 Dynabeads (Thermo Fisher) which are used for cell isolation also activate T cells, although monocyte engulfment of these 4.5 micron-sized beads can sometimes pose a bioprocessing challenge with leukapheresis products that are high in monocyte concentration, thereby necessitating the use of monocyte reduction technology such as the Elutra System (Terumo BCT) when utilizing high-monocyte leukapheresis products, prior to incubation of the leukapheresis product with CD3/CD28 Dynabeads. This phenomenon is not generally seen with activation with either TransAct Reagents (Miltenyi) which is based on a polymeric nanomatrix conjugated to CD3/CD28 antibodies or Immunocult Activator Reagents which are soluble tetrameric antibody complexes that bind CD2/CD3/CD28 or CD3/CD28 (StemCell Technologies). TransAcT Reagents are currently available in GMP-grade for clinical manufacturing; the Immunocult Reagents are currently only available as Research-grade reagents, however, as is the case with the EasySep Reagents, may be made commercially available as GMP-grade reagents in the near future.

5.4. Genome editing

This unit operation can occur before or after the CAR transduction, depending on the whether the transduction is achieved through targeted gene knock-in, whereby transduction will occur after the genome edit, or in the case of non-targeted insertion where the transduction unit operation can be performed before or after the genome edit. Genome editing nucleases are generally delivered to cells utilizing transfection of mRNA. Electroporation is the primary method of choice to deliver the one or more mRNA's necessary to perform a genome edit. Although numerous electroporation instruments exist for research-scale electroporation of a few thousand to a few million cells at a time, only one large volume electroporation device suitable for allogeneic CAR-T bioprocessing is on the market at this time. The GT Scalable Transfection System (MaxCyte, Gaithersburg, MD) is a clinical-scale automated electroporation system that is capable of transfecting up to 20 billion cells in up to 100 mL of total fluid volume in one single functionally closed disposable set. This scale accommodates allogeneic CAR-T transfection requirements whereby typically between 2-10 billion activated T cells will be available for transfection from any one donor leukapheresis starting product. Two additional commercially available devices are capable of electroporating up to 1 billion cells at a time, however, in order to electroporate cell numbers higher than this, more than one device must be run in parallel or else batches of 1 billion cells must be run sequentially, utilizing a new cartridge/disposable set for each run. The automated 4D Nucleofector LV Unit (Lonza, Basel, Switzerland) can electroporate up to 1 billion cells in 20 mL total fluid volume, utilizing a disposable cartridge and tubing set system, and the AgilePulse MAX (BTX/Harvard Apparatus, Holliston, MA) is able to electroporate the same cell numbers in a 10 mL total fluid volume utilizing a syringe-loaded cuvette system which is not functionally closed.

5.5. CAR transduction

Transduction of the CAR transgene into genome-edited allogeneic CAR-T cells utilizing non-targeted integrating vectors such as gamma retroviruses, lentiviruses, or transposons occurs via the same methodologies as utilized for the transduction of autologous CAR-T cells [22,35,36]. This transduction unit operation generally takes place immediately before the transfection unit operation or immediately afterwards. In the case of targeted CAR insertion utilizing non-integrating vectors such as AAV, the transduction step will always follow the genome editing transfection unit operation, oftentimes occurring in such temporal proximity, on the order of minutes, that the transfection and transduction unit operations may be described as simultaneous or considered a single manufacturing unit operation as opposed to their own discrete manufacturing unit operations.

5.6. Cell expansion

Post transfection/transduction expansion of allogeneic CAR-T cells takes place with similar cell culture methods utilized for cellular activation in terms of media, cytokines, and supplements, but without the use of activators such as CD3/CD28 beads or soluble factors. Additional cytokines utilized during this unit operation may be IL-7, IL-15, and IL-21, with the exact composition and relative concentrations in the cytokine cocktail determined by preference to maintain a particular T cell phenotype or to enhance the proliferation of a particular subset of the T cell population. The culture platforms deployed in the unit operation include VueLife fluorinated ethylene propylene (FEP) closed cell culture bags (St. Gobain Life Sciences), GRex culture devices (Wilson Wolf Corporation), or the automated rocking wave bag-based perfusion bioreactor Xuri System (GE Healthcare Life Sciences). Theoretically, allogeneic CAR-T cells may be culture-expanded for a longer period of time and to greater total maximum cell numbers than has been the case with autologous CAR-T cells therapies where the shortest feasible manufacturing timeframe to produce adequate product to dose oftentimes critically ill patients, is of vital importance. Practically, this possibility to manufacture much larger batch sizes of allogeneic CAR-T cells must be balanced with the requirement to maintain or enhance desired cellular phenotypic characteristics, as well as limit cellular exhaustion and senescence.

5.7. Cell depletion

Depletion of T cells which were not successfully genome edited to prevent expression of the TCR is necessary to achieve adequate product purity for an allogeneic CAR-T cell product in order to minimize the risk for GVHD. This unit operation employs similar bioprocessing technologies as in the earlier lymphocyte isolation unit operation, namely fine separation of $CD3^+$ T cells with antibody-coupled magnetic bead isolation. Generally, positive selection is deployed whereby the $CD3^+$ T cells are retained on the magnetic column and the CD3- T cells which comprise the allogeneic drug substance, are collected in the negative fraction and retained for final formulation. A second round of cell expansion followed by a second round of cell depletion may be utilized in allogeneic CAR-T cell manufacturing in order both increase total cell yield as well as to increase the purity of the final cellular product.

5.8. Final formulation, filling, and cryopreservation

Washing and concentration of the post-depletion allogeneic CAR-T cell drug substance is important to remove manufacturing residuals and exchange cell depletion buffer for the final formulation solution. As in the initial platelet wash step, the Biosafe Sepax 2 (GE Healthcare Life Sciences) or the LOVO Cell Processing System (Fresenius Kabi) can be deployed for this unit operation. Other automated cell washing devices including the COBE2991 (Terumo BCT) and the Sefia Cell Wash and Concentration System (GE Healthcare Life Sciences) are also suitable for this step.

As an off-the-shelf therapeutic, allogeneic CAR-T cells are always cryopreserved. Cryostor Solution (BioLife Solutions, Bothell, WA) containing 5–10% DMSO, human serum albumin, and saline or PBS is a robust cryopreservation solution that is also suitable for infusion without the need to wash the cellular drug product after thawing and before infusion. Doses of allogeneic CAR-T cells can be expediently and accurately filled into cryovials utilizing closed system processing with either 2–10 mL AT Closed Vials and the M1 Filling Station (Aseptic Technologies SA, Gembloers, Belgium) or 2–5 mL CellSeal Vials and Filling Station (Cook Regentec, Indianapolis, IN). These drug product vials are then placed in controlled rate freezers and finally into vapor-phase liquid nitrogen for long term storage.

6. Allogeneic CAR-T cell analytical methods

Levine et al. [22]described potential approaches for the global manufacturing of autologous CAR-T cells in which a quality target product profile and critical quality attributes must be established for the final product as this relates to the successful development of a CAR-T cell manufacturing process. Target-specific, highly potent T cells that are capable of robust expansion and long-term persistence in vivo were defined as the fundamental quality target product profile. Based on this quality target product profile, critical quality attributes must be defined, understood and controlled including cell number, transduction efficiency, growth rate, cellular phenotype, and functional analysis. Of particular importance to developers of allogeneic CAR T cells is the ability to ensure a consistent manufacturing process and control strategy to guarantee product uniformity.

Separating T cell or certain T cell subsets as early in the biomanufacturing process as possible can improve the outcome of subsequent downstream processing. The starting cell population used for all CAR-T cell therapies consists of CD4 and CD8 T cells at the ratio present in the peripheral blood of the patient at the time of leukapheresis. The in vivo proliferative capacity depends on the composition of the T cells in the infused product. Administration of autologous CAR-T cells in a fixed CD4:CD8 ratio has been shown to provide for more consistent and predictable activity in patients with ALL and non-Hodgkins lymphoma [37,38]. Long-term persistence and function are provided by central memory phenotype T cells that retain longer telomeres and higher proliferation compared to the more differentiated effector T cell populations [39]. Thus, ideally, a final CAR T cell product should consist of both CD4 and CD8 T cells expressing early memory phenotypes with the ability to expand in the blood of patients and generate long-term memory. Analytical development for CAR-T manufacturing supports the quality control of incoming material, monitors defined critical quality attributes in-process to identify and support crucial decision points, as well as enables the characterization of the final product through the utilization of well established, robust analytical approaches.

6.1. Viability and cell count

Although manual viable cell counting with a hemocytometer is still considered as a gold standard, automated cell counting instruments provide the possibility of analyzing a bigger number of samples in a shorter time in addition to the reduction of variability associated with human error. Currently, two main types of automated cell counters are available, image-based counters that are equipped with a digital camera to obtain microscope images and counters that utilize microfluidic systems to count cells passing through a flow cell, as in flow cytometry or Coulter systems. Some image-based counters operate in the brightfield like the Luna™ (Logos Biosystem, Seoul, South Korea), Countess®Automated Cell Counter (Thermo Fisher) or Cellometer™ Auto T4 Cell Viability Counter (Nexcelom Bioscience, Lawrence, MA), whereas others utilize fluorescence imaging like the Cellometer Vision CBA (Nexcelom Bioscience) or instruments of the NucleoCounter family (ChemoMetec, Copenhagen, Denmark). When choosing a counter, aside from instrument price, cost per a sample, and a simple user interface, accuracy should be the key consideration.

6.2. Cellular phenotype

Traditional multicolor flow cytometry remains the only technology that allows the precise analysis of cellular subsets in heterogeneous cell populations or the performance of extended phenotyping on the cell populations of interest. Implementation into a GMP environment of FACS-based methods can be a time-consuming and onerous procedure due to the complexity of instrument set up, method execution, data analysis, and data release. Although there is no current suitable alternative to multi-color flow cytometry to analyze donor cellular material at the initiation of bioprocessing and to fully characterize the manufactured cellular product at release, in-process sample analysis usually does not require multi-parametric approaches to identify cells of interest e.g. concentration of $CD3^+$ cells before and after the T cell enrichment unit operation or CD4:CD8 ratio during T cell expansion. Imaging fluorescent cytometers developed for Point-of-Care (POC) testing potentially may be a good alternative to traditional flow cytometry. These instruments are designed to operate with disposable cartridges preloaded with all the reagents required for the test. Some customization will be required to ensure that these POC systems are applicable for cell therapy manufacturing needs. The entire analytics workflow from the sample loading step to receiving printed results takes about 20 min for the BD FACSPresto (BD Biosciences, San Jose, CA), and includes $CD4^+$ enumeration, and 30 min for the Accellix (LeukoDx, Jerusalem, Israel) using a cartridge designed for T cells to detect and count viable CD45, CD3, CD4 and CD8 subpopulations of a sample. Operation of POC instruments does not require highly specialized knowledge and skills as is the case for conventional flow cytometry, and can be executed in the GMP manufacturing facility by manufacturing operators.

6.3. Functional analysis

Traditionally, functional response of any T cell to antigenic challenges is estimated by cytokine production, cytotoxic activity, and T cell proliferation. Interferon gamma is a classical target to analyze T cell potency and is utilized as indicative of the biological functionality of manufactured T cell products. Conventional ELISA is a time-consuming procedure which requires well trained personnel or additional expenses for the automatization of the entire procedure. The Microfluidic Simple Plex cartridge-based assay on the ELLA instrument (ProteinSimple, San Jose, CA) may be a viable alternative to traditional ELISA. The entire procedure is hands-free and take approximately 90 min for readout. The cytotoxic activity of manufactured CAR T cells has been traditionally analyzed with Chromium-51 release assays. This procedure requires a certified “hot-room”, trained personnel, and additional expenses associated with the proper handling, storage, and disposal of radioactive waste material. Fluorescent cell viability assays e.g. calcein-acetyoxymethyl (calcein AM), have become a more popular alternative to Chromium-51 release assays. Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of calcein AM by intracellular esterases produces calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. Target cells preloaded with calcein AM upon killing by effector cells release calcein into the culture media, and this supernatant can subsequently be analyzed on a fluorescence plate reader. Preloading of the target cells during execution of the assay may introduce undesirable test-to-test variability. Target standardization can be achieved through customizing target cell lines to express a reporter gene, e.g. luciferase, that can be measured in the supernatant from target cells expressing the cognate antigen that have been killed by CAR-T cells.

7. Summary

Manufacturing of off-the-shelf CAR-T therapies derived from unrelated healthy donors and utilizing gene editing technology to render the final product safe for use in HLA-mismatched recipients can avoid some of the pitfalls associated with manufacturing of autologous CAR T products such as unpredictable pharmacology due to heterogeneity of individual products and guaranteed availability of drug product when needed. Production of CAR T cells containing targeted insertion of a CAR transgene into a cellular genomic target allows for controlled and homogeneous CAR expression and production of a CAR-T drug product that may provide for predictable dose-related efficacy and toxicity. Multiple gene edits can be performed to impart additional desirable properties such as resistance to immunosuppression that may provide for better efficacy of CAR-T products against solid tumors. New technology and equipment are being introduced for large scale manufacturing of allogeneic gene edited CAR T cells that enable GMP-compliant manufacturing and qualification of CAR-T therapies that can be made available as frozen, off-the-shelf products.

Conflicts of interest

The authors disclose that they are employees of, and receive salary and stock options from Precision BioSciences, Inc.

Practice Points

- Off-the-Shelf CAR T cell products can be created from unrelated healthy donor T cells provided that gene editing to prevent expression of endogenous T cell receptors is used in order to prevent or significantly reduce the potential for Graft vs. Host Disease in HLA-mismatched recipients.
- Knockout of TCR expression via targeted knock-in of CAR transgenes delivered using rAAV vectors as a homology repair template allows for production of CAR T cells that are more homogenous with respect to level of CAR expression.
- Reduction in immunogenicity of allogeneic CAR T cells can be achieved through genetic knockout or knock down of β2-microglobulin.

Healthy donors of leukapheresis products to serve as starting material for production of allogeneic gene-edited CAR T cells should pre-screened for presence of transmissible infectious agents and selected based upon total T cell number, ratio of CD4 to CD8 T cells (higher ratio is better), and percentage of T cells with naïve and central memory phenotypes vs. effector and effector memory phenotypes (naïve and central memory are preferred).
Gene edits that may be considered to improve CAR T cell activity in solid tumor microenvironments include genetic knockout of inhibitory signaling receptors (e.g. PD-1, LAG- 3, TGFBR2)
GMP manufacturing of allogeneic gene-edited CAR T cells at large scale requires careful selection and qualification of equipment and conditions for each unit operation and analytical methods that can ensure product identity, purity, potency, and stability.

Research Agenda

Further research into production of allogeneic gene-edited CAR T cells using gene editing platforms with high specificity and low toxicity coupled with advances in equipment suitable for cell therapy manufacturing in a GMP environment are needed to advance the field.

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