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Review

Toward an Optimized Process for Clinical Manufacturing of CAR-Treg Cell Therapy

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Chimeric antigen receptor (CAR) technology and its application to regulatory T cells (Tregs) has garnered interest among researchers in the field of cell and gene therapy. Merging the benefits of CAR technology with Tregs offers a novel and promising therapeutic option for durable reshaping of undesired immune responses following solid organ or hematopoietic stem cell transplantation, as well as in immune-related disorders. However, major challenges remain for developing a standardized and robust good manufacturing practice (GMP)-compliant manufacturing process for CAR-Treg cells. We review current progress in the field and recommend ways to improve CAR-Treg manufacturing processes based on lessons learned from first-generation Treg therapeutics as well as from anticancer CAR-T cell development.

Highlights

Advances in the field of cell therapy have led to promising novel approaches to treat malignancies and other debilitating diseases. Redirecting the target antigen specificity of CAR-Treg cells represents one such promising approach.

The clinical success of anticancer CAR-T cells will, in all likelihood, accelerate the clinical translation of CAR-Treg therapeutics aimed at reshaping undesired immune responses following solid organ or hematopoietic stem cell transplantation as well as in immune-related disorders.

Current manufacturing processes for CAR-Tregs demonstrate challenges in cell purification, yield, expansion, CAR selection and gene delivery, supply chain, and quality control/product release testing.

We propose a GMP-compatible manufacturing framework to enhance the CAR-Treg production process for clinical application.

The New Era of Cell and Gene Therapy

One recent and exciting example of progress in the field of cell and gene therapy has been the ongoing development of **chimeric antigen receptor** (CAR; see Glossary) technology and its application to **regulatory T cells** (Tregs) [1–3]. There is a high medical need for more effective and sustainable therapies targeting immune-related diseases as a result of an increase in their prevalence (>10% of chronic diseases) and the high socioeconomic burden associated with managing long-term illness and chronic conditions (e.g., >100 billion €/year in the EU). **Adoptive cell transfer** (ACT) of Tregs is a promising therapeutic option to reshape immune balance toward tolerance with long-lasting efficacy. First-in-human (FIH) trials with first-generation thymus Treg cell products have yielded promising data regarding safety and efficacy in immune-related disorders [2,3].

Advances in the development of genetically modified **CAR-T cells** targeting malignancies will, in all likelihood, also accelerate the clinical translation of **CAR-Treg cells** [4]. The introduction of costimulatory domains such as **cluster of differentiation** (CD) molecules CD28 and CD137 has allowed the engineered CAR-T cells to persist and remain active within the body [5]. CAR-T cell therapies have been successfully investigated in a multitude of Phase I and II trials where autologous or allogeneic tumor-specific CAR-T cells were applied. By the end of 2017, these efforts led to marketing approval of two CD19-directed CAR-T cell therapy products, tisagenlecleucel (Kymriah®, Novartis) and axicabtagene ciloleucel (Yescarta®, Kite Pharma/Gilead) in the USA and subsequently in the EU by mid-2018, as well as in several other countries.

Since their description as a distinct subset of $CD4^+$ T cells in 1995, natural thymus-derived Tregs have been studied as an instrumental therapeutic approach for controlling undesired immune responses and for reshaping the host immune system toward tolerance [6]. Merging the benefits of CAR technology (redirecting antigen specificity) with Tregs offers a novel and promising therapeutic option for tolerance induction in the context of solid organ or hematopoietic stem cell transplantation (HSCT), as well as other immune-related disorders. However, major challenges remain for developing a standardized and robust **good manufacturing practice** (GMP)-compliant

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manufacturing process. We review current progress in the field and recommend ways to improve the current CAR-Treg manufacturing processes based on lessons learned from our experience in developing Tregs and current progress in CAR-T cells for cancer.

Therapeutic Potential of Tregs and the Application of CAR Technology

Tregs possess immunosuppressive properties that are essential for the maintenance of immune homeostasis. Box 1 summarizes the phenotypic and immunological characteristics of Tregs. A decade after the first Treg product was administered in graft-versus-host disease (GvHD) patients [7], various early-phase trials, primarily in solid organ transplantation/GvHD and autoimmunity, have provided evidence for the safety and feasibility, as well as initial hints on efficacy of administered Treg products [3]. Our own data have shown that, in 73% of living-donor kidney transplant patients who received a single dose of polyclonal Tregs 1 week after transplantation, a stable switch from standard dual/triple-drug **immunosuppression** to low-dose monotherapy was achieved [8]. Despite these promising initial results, only tapering of immunosuppression is currently feasible, and further refinement of the therapeutic concept will be necessary to reach the goal of complete weaning.

The evolving landscape of CAR-Treg technology can be traced back to 2016 when MacDonald and colleagues reported the successful transduction of human Tregs with a CAR targeting the **human leukocyte antigen** (HLA) class I molecule A2 (A2-CAR) [9]. These findings were confirmed by Boardman and colleagues who showed that human Tregs expressing a similar donor HLA class I-A2-specific CAR were able to alleviate and prevent rejection of skin transplants in a human skin xenograft transplant model [10]. This approach confers the benefit of directed non-self **major histocompatibility complex** (MHC) class I targeting with no further need for direct or indirect alloantigen presentation by **antigen-presenting cells** (APCs) via MHC I or MHC II, respectively, therefore avoiding APC stimulation dependence. Further details of these findings are provided in Box 2.

Alloreactive CAR-Tregs are primed for clinical translation, preferentially in solid organ transplantation settings where the graft only expresses the target allo-HLA molecule. However, this approach might be risky in patients receiving HSCT for residual leukemia or lymphoma because the broad expression of targeted HLA on all tissues might result in excessive immunosuppression and further increase susceptibility to infectious events. In addition to alloreactive CAR-Tregs, sev-

Box 1. Phenotypic and Immunological Characterization of Tregs

Tregs demonstrate potent immunomodulatory functions and are characterized by the high expression of CD25. A forkhead family transcription factor, FOXP3, has been shown to be a main regulatory factor and marker of Treg development [71]. Based on their origin, Tregs are stratified into two main subsets – thymus-derived (formerly nTregs) Tregs (tTregs) and peripheral-derived/peripheral-induced (formerly adaptive/induced) Tregs [72]. $CD4^+CD25^+FoxP3^+$ tTregs, which have been identified as the main Treg subset, play a crucial role in the maintenance of immunological tolerance. They modulate the activation and proliferation of Tconv, thus controlling immune reactions toward self and non-self antigens [73]. To be able to exert their immunosuppressive action, Tregs need to be stimulated by their TCR [74]. Upon activation, Tregs can suppress not only $CD4^+$ T cells but also $CD8^+$ T cells and many other immune cells when recruited to an identical APC [75,76]. The absence of a TCR signal results in fast deactivation. Tregs rely on costimulatory signals to become fully functional, and CD28 costimulation is required for T regulatory cell expansion and persistence [77]. Tregs consist of subpopulations that correspond to the phenotype of naïve (TregN), stem cell memory-like (TregSCM), central memory (TregCM), effector memory (TregEM), and terminally differentiated effector cells (TregTEMRA), according to their surface expression of CD62L/CCR7, CD45RA/RO, and CD95 [78]. More recently, human Tregs named T helper (Th)-like Tregs have been described [3]. These memory Treg cells mirror the classical $CD4^+$ Th population expressing the same chemokine receptors CXCR3, CCR6, and CCR4 that are typically expressed by T-bet^+ Th1 cells, $RORgt^+$ Th17 cells, and $GATA3^+$ Th2 cells, respectively.

Glossary

Adoptive cell transfer (ACT): the transfer of cells into an organism (e.g., a patient).
Alloreactivity: an immune reaction in response to a transplanted graft from an allogeneic donor.
Allotolerance: an induced state of non-reactivity to grafted tissue from a donor organism that would ordinarily trigger a cell-mediated or humoral immune response.
Antigen-presenting cells (APCs): cells that can process protein antigens into peptides and present them, in association with major HLA complex class II or I molecules, on the cell surface where they can interact with antigen receptors of CD4 and CD8 T cells.
Biosafety level S2 (BSL-2): a level of protection covering laboratories that work with human disease agents that pose a moderate health hazard to personnel and the environment.
Bystander suppression: the ability of Tregs not only to suppress cells carrying the target antigen but also cells in the local environment that bear other antigens.
CAR-T cells: genetically engineered T immune cells that can better recognize, for instance, cancer cells, thus allowing effective targeting and lysis.
CAR-Treg cells: regulatory T cells that have been genetically modified using CAR technology.
Chimeric antigen receptor (CAR): a receptor designed to bind to distinct proteins on the surface of target cells, such as cancer cells, following addition to immune cells (T cells) by genetic modification. The receptors are chimeric because they combine both antigen-binding and T cell-activating functions in a single receptor.
Cluster of differentiation (CD): cell membrane molecules used to classify immune cell types and related subsets as well as to establish international nomenclature standards.
Conventional T cell (Tconv): T cells that recognize processed peptide antigens that are presented within the grooves of MHC molecules on other cells based on their collectively diverse TCR repertoire.
Good manufacturing practice (GMP): the standard that medicines manufacturers must meet in their production processes; GMP requires that medicines are of consistently high quality, are appropriate for their intended

Box 2. Evolving Mechanistic Insights into CAR-Tregs

After the first successful generation of CAR-Tregs targeting the HLA class I molecule A2, McDonald and colleagues further characterized these cells for their immune-suppressive capacity in alloresponses using *in vitro* and *in vivo* xenogeneic models [9]. They demonstrated that A2-CAR-Tregs have a stable phenotype and constant suppressive activity. In a murine hematopoietic stem cell transplantation model, the human A2-CAR-expressing Tregs were more efficient in the prevention/alleviation of xenogeneic HLA-$A2^+$ T cell-mediated lethal GvHD, as compared to polyclonal Tregs, without displaying cytotoxic activity [9]. In line with these outcomes, Boardman and colleagues directed human CAR-Tregs against donor MHC class I molecules by engineering two HLA-A2-specific CARs, either bearing an intracellular signaling domain (CD28 CD3ζ) or lacking this domain (ΔCAR) [10]. CAR-Tregs bearing the activated intracellular signaling domain (CD28 CD3ζ) were superior in demonstrating suppressive function/potency than cells lacking this domain (ΔCAR) or even polyclonal Tregs. At the same time, no cytotoxic activity was observed. Another interesting observation was the enhanced transmigratory potential of both CAR-Treg types toward HLA-$A2^+$ target tissues, as compared to endothelial monolayers not bearing the target alloantigen. Further verification of this suppressive ability could be obtained by transplanting human skin grafts (1.5 cm^2) onto murine recipients [BALB/c *Rag2*$^{-/-}$*γc*$^{-/-}$ mice] [10]. An alloresponse with skin graft rejection was initiated by intravenous administration of allogeneic PBMCs. Administration of CAR-Tregs attenuated alloimmune-mediated skin injury with greater efficacy than did human polyclonal Tregs, and also promote **allotolerance** induction [10].

eral autoantigen-specific CAR-Tregs were recently developed and were successfully applied in preclinical models of T1 diabetes, autoimmune hepatitis, inflammatory bowels disease, encephalitis, arthritis, and several rare diseases [11–13].

Current Status of CAR-Treg Manufacturing

CAR-Treg manufacturing adopts the initial steps of the conventional polyclonal Treg production (isolation and activation). The resulting product (referred to here as the 'Treg master product') is subsequently subjected to gene modification/CAR gene delivery (Figures 1,2). Current manufacturing processes for polyclonal Treg cells start with **peripheral blood mononuclear cells** (PBMCs) prepared from apheresis or peripheral blood as the source material (consisting of <3% Tregs) and mainly employ magnetic bead selection (magnetic-activated cell sorting, MACS), although flow cytometry-based fluorescence-activated cell sorting (FACS) devices have also been applied (Figure 1). Commonly, $CD8^+$ cells are first depleted, followed by positive selection for $CD25^{high}$ cells, resulting in the enrichment of $CD4^+CD25^{high}FoxP3^+$ Tregs (>70%). Because naïve Tregs are more stable and less contaminated with T effector (T_{eff}) cells, other approaches have relied on enriching naïve Tregs by additional depletion of $CD45RO^+$ or enrichment of $CD45RA^+$ cells [14]. After enrichment, cells are activated by anti-CD3/CD28 beads and (high) exogenous **interleukin-2** (IL-2). This procedure is repeated to expand the cells by several logs (up to >1000-fold) [15]. To prevent Treg instability through expansion of contaminating T_{eff} cells, the mTOR inhibitor rapamycin can be added for selective depletion of T_{eff} cells [16], even though high purity could also be achieved without addition of rapamycin [17]. After 2–3 weeks of expansion, the Tregs show high purity (>90% $CD4^+CD25^{high}FoxP3^+$) and high functionality upon quality control (QC) testing (Figure 1).

After isolation, activation, and expansion of the sorted Tregs, the resulting 'Treg master product' is transduced using a GMP-grade viral vector expressing the CAR construct. The generated CAR-Treg product is further expanded by continuous medium supply and restimulation with GMP-compliant anti-CD3/CD28 magnetic beads and (high) exogenous IL-2. QC assays for sterility, identity, and functionality are implemented at the end of the manufacturing workflow. The final CAR-Treg product can be subjected to further cell expansion until the desired cell count is reached, then cryopreserved or administered to the patient fresh after manufacture [18] (Figure 2).

use, and meet the requirements of marketing or clinical trial authorizations.
Human leukocyte antigens (HLAs): proteins encoded by MHC genes in humans that are present on the surface of almost all cell types.
Immunosuppression/immunotherapy: the treatment of disease by suppressing or activating the immune system.
Interleukin-2 (IL-2): a γ-chain cytokine that stimulates the growth and replication of immune cells.
Major histocompatibility complex (MHC): a group of genes in mammals coding for cell-surface polymorphic glycoprotein molecules displaying antigenic peptide fragments for T cell recognition and aid the immune system to distinguish between 'self' and 'non-self'.
Peripheral blood mononuclear cells (PBMCs): blood cells with round nuclei. PBMCs include monocytes/macrophages, lymphocytes (T cells, B cells, natural killer cells), and dendritic cells.
Regulatory T cells (Tregs): a subgroup of T lymphocytes that regulate the self-tolerance of the immune system and balance the immune response.
T cell receptor (TCR): a protein complex on the surface of T cells that is responsible for recognizing fragments of antigen that are presented as peptides bound to MHC molecules.
Treg exhaustion: a state of Treg dysfunction that controls immune tolerance and homeostasis as a result of prolonged antigen exposure (e.g., tumor-driven).

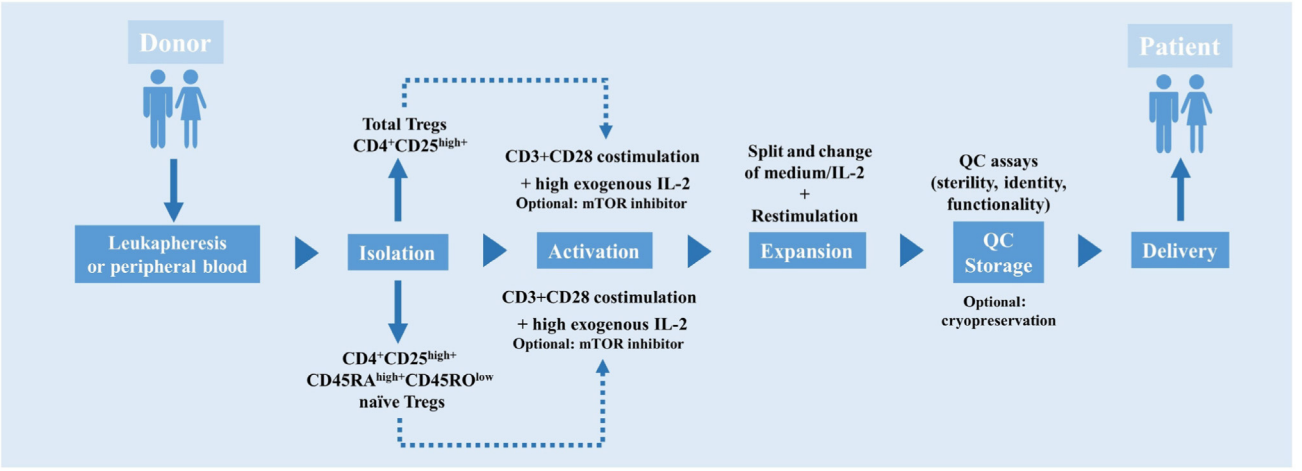

Figure 1. Current Manufacturing Workflow of Conventional Polyclonal Treg Cells. In the first manufacturing step, leukapheresis or peripheral blood is used to obtain peripheral blood mononuclear cells (PBMCs) as the starting material. Isolation of the Treg cell population from PBMCs is mainly achieved by magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) based on characteristic marker expression profiles. Activation is performed by stimulation with anti-CD3/CD28 antibody-coated magnetic beads and (high) exogenous interleukin-2 (IL-2) levels. An mTOR inhibitor can be added to prevent the expansion of contaminating effector T cells (optional). Expansion is repeated to expand the cells by several logs over 2–3 weeks via passaging and continuous medium supply using anti-CD3/CD28 antibody-coated magnetic beads plus (high) exogenous IL-2. Quality control (QC) assays for sterility, identity, and potency are performed at the end of the manufacturing workflow. The final polyclonal Treg product can be stored upon cryopreservation or freshly administered to the patient (delivery). Abbreviations: CAR, chimeric antigen receptor; CD, cluster of differentiation; CD45RA, CD45 isoform containing exon 4 sequences but lacking exons 5 and 6; CD45RO, isoform containing exons 3, 7, and 8 but lacking the RA, RB, and RC exons of CD45; GMP, good manufacturing practice; IL, interleukin; mTOR, mammalian target of rapamycin (serine/threonine-specific protein kinase); QC, quality control; Tregs, regulatory T cells.

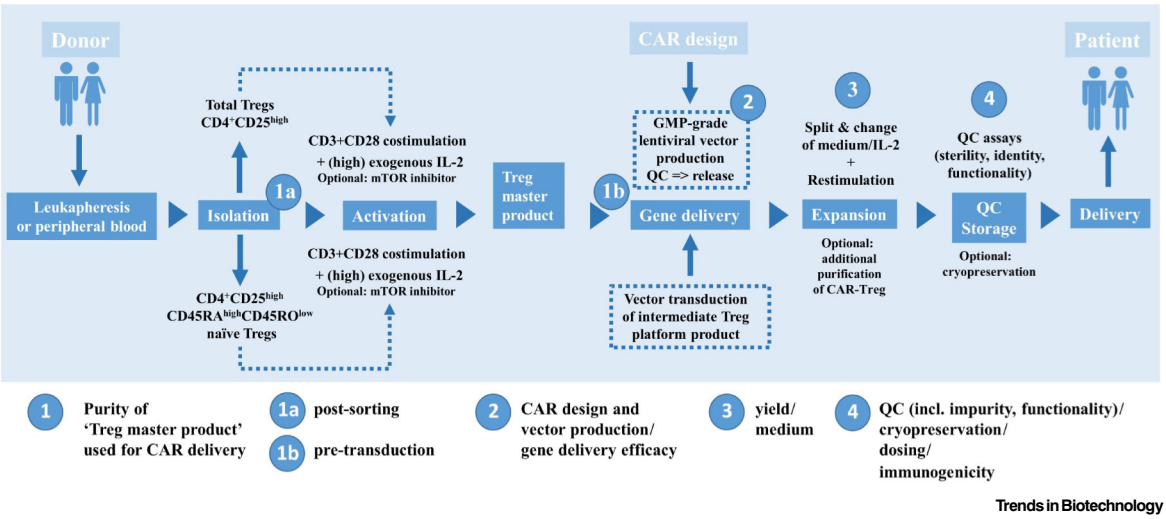

Figure 2. Current Manufacturing Workflow of CAR-Treg Products and Associated Challenges. In the first manufacturing step, leukapheresis or peripheral blood is used to obtain peripheral blood mononuclear cells (PBMCs) as the starting material. Isolation of the Treg cell population from PBMCs is mainly achieved by magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) based on characteristic marker expression profiles. Activation is performed by stimulation with anti-CD3/CD28 antibody-coated magnetic beads and (high) exogenous interleukin-2 (IL-2) levels. mTOR inhibitor can be added to prevent the expansion of contaminating effector T cells (optional). The obtained Treg master product further undergoes genetic modification with a CAR construct using a GMP-grade lentiviral vector. For expansion, CAR-Treg cells are subjected to passaging and are supplied continuously with medium containing anti-CD3/CD28 beads plus (high) exogenous IL-2 levels. Increase purity of the CAR-Treg product upon gene delivery, enrichment processes (e.g., using a CAR transduction marker and/or FOXP3) may be carried out during expansion (optional). Quality control (QC) assays for sterility, identity, and potency are performed at the end of the manufacturing workflow. The final polyclonal Treg product can be stored upon cryopreservation or freshly administered to the patient (delivery). Challenges related to respective processing steps are indicated as 1, 1a, 1b, 2, 3, and 4. Abbreviations: CAR, chimeric antigen receptor; CD, cluster of differentiation; FOXP3, forkhead box protein P3; GMP, good manufacturing practice; IL, interleukin; mTOR, mammalian target of rapamycin (serine/threonine-specific protein kinase); QC, quality control; Tregs, regulatory T cells.

Challenges and Opportunities in Manufacturing CAR-Treg Cells

The manufacturing process of cell and gene therapies is known to be complex and costly [19,20], which subsequently influences the translational success of these products [21,22]. The current manufacturing process for CAR-Tregs demonstrates several challenges in cell purification, yield, expansion, vector production/transduction, cryopreservation, and product release testing (Figure 2).

The Purity of the 'Treg Master Product' Used for CAR Delivery

Tregs represent a tiny population among PBMCs (<3%). One of the main obstacles is to obtain sufficient yield and purity under GMP-compliant sorting conditions. Effective preclinical sorting strategies based on FACS that achieve high purity (>98%) are not yet GMP-compliant. The majority of current GMP protocols are based on semi-closed MACS devices. Although the relatively low post-sort purity (60–80%) using MACS can be overcome by selective outgrowth of Tregs during expansion (discussed in 'Expansion and Yield'), expanded Tregs display highly variable purities resulting from **conventional T cell** (Tconv) contamination or potential Treg instability in terms of maintaining stable FOXP3 expression, as discussed later (Figure 2).

Thus, a particular challenge in manufacturing CAR-Tregs is to prevent contaminating Tconv cells from being redirected by CAR transfer to the specific target, given that gene delivery of the CAR construct occurs at an intermediate stage of Treg expansion when purity is still limited (Figure 2). Redirecting the antigen specificity of Tconv cells by CAR expression raises safety issues, due to their undesired, increased antigen targeting. In particular, for Tregs equipped with disease-relevant CARs (e.g., allo/autoantigens), the risk of generating unpredictable numbers of pathogenic Tconv cells with disease-amplifying potential must be tightly controlled. Despite encouraging safety data from recent clinical trials using first-generation Tregs [23,24] (NCT02371434), in which the few contaminating Tconv cells with polyclonal **T cell receptor** (TCR) repertoire in the final product were kept under control, the issue requires further attention for CAR-Tregs, and the strategies discussed below could be employed.

Using Naïve Tregs

There is consistent evidence that Tregs themselves demonstrate high heterogeneity. Because the functional stability of induced Tregs *in vivo* is poorly understood so far, thymus-derived natural Tregs, particularly $CD3^+CD4^+CD25^{high}FoxP3^+CD45RA^+CD62L^+$ cells, are the source of choice for manufacturing CAR-Tregs (Box 1). Naïve Tregs are functionally more stable during expansion [14]. Therefore, depletion of $CD45RO^+$ or enrichment of $CD45RA^+$ Tregs increases safety. However, these strategies are associated with lower yields from the source used, since the majority of Tregs in adults express a memory phenotype [78].

Refined Sorting Strategies

Compared to MACS, enrichment of Tregs by FACS using multicolor strategies enhances post-sort purity by applying additional selective markers, such as $CD127^{low}$, and employing density-defined cut-offs for sorting [14,17,25]. The downside is that currently available FACS equipment is not accepted as GMP-grade by most European regulatory authorities and is not suitable for scaling up in late-stage clinical trials and marketing authorization [25] (Box 3). The newest generation of such droplet-based cell sorters attempts to fulfill GMP requirements [26], and these are being used in two clinical trials for engineered bone marrow grafting (NCT03802695, NCT04013685). Another potential alternative could be the use of microfluidic switch technologies, such as the MACSQuant® Tyto® cell sorting platform, and fluid-channel sorters.

Box 3. Translational Challenges Facing CAR-Treg Development

Repeated Application and Immunogenicity

The potential risk of initiating an immune response against CAR epitopes following patient administration represents a significant challenge. Preclinical studies have demonstrated that antibodies against a CAR construct can be generated. Repeated injection of antigen-specific CAR-Tregs into the same mice producing CAR antibodies led to even shorter cell survival [79]. Similarly, the immunogenicity of CAR-Tconv cells in clinical settings has been observed [80]. The risk of immunogenicity is associated with the presence of non-human sequences in the CAR construct, suicide domains, or the presence of residual viral or other non-human proteins used in the editing process. Both humoral and cellular responses have been described, with varying degrees of influence on CAR-T expansion and persistence, and on subsequent overall safety and clinically meaningful treatment response [80].

Treg Exhaustion and Persistence Optimization

Treg exhaustion phenomena limit their clinical efficacy following patient administration. Although some studies indicate that CD137 (4-1BB) costimulatory signals may alleviate exhaustion in CAR-Tconv, thus favoring persistence, the majority of CAR-Treg products incorporate CD28 costimulatory signals because of their importance in Treg development, expansion, and function [81–83,31,101]. A better understanding of how Tregs coordinate immune tolerance and their homeostasis with surrounding signals will be necessary to address this issue. For instance, a recent finding demonstrated beneficial effects of the tumor-suppressor protein LKB1 (liver kinase B1) which combats Treg functional exhaustion by coordinating metabolic and immunological homeostasis [84].

Regulatory Requirements

Differences in GMP requirements among countries may hamper the establishment of consistent and standardized GMP-compliant manufacturing processes. For instance, although it is possible to implement standard non-GMP-compliant cell sorters for Treg isolation and purification from peripheral blood sources in the USA, the European regulatory framework does not allow this. According to EU regulations (Directive 2003/94/EC and Annex 2), flow cytometry-based sorting for manufacturing involving cellular products requires GMP compliance. This regulation is particularly challenging for widely applied droplet-sorters that make use of a freely accessible fluid stream. This represents an open system that may not be GMP compliant because of the potential risk of contamination and aerosol formation.

CAR Design and Vector Production

There are two major challenges in the design of the CAR constructs for Treg manufacturing.

Selection of the Targeted Antigen

Compared to anticancer strategies that must mediate the killing of specific cancer target cells, one advantage of Tregs is their efficacy in the surrounding tissue, called **bystander suppression** [27,28]. The first clinical trials are likely to start with alloreactive CAR-Tregs which target HLA-A2 in HLA-A2-negative solid organ transplant recipients receiving an $A2^+$ graft. In this case, the target antigen is only expressed in the graft and eventually on some circulating donor-derived cells. Bystander suppression overcomes not only the HLA-A2 mismatch but also other MHC mismatches as presented on the same target cells. As mentioned earlier, autoreactive CAR-Tregs have also been tested in preclinical models; however, direct targeting of allo/autoantigen on parenchymal cells by CAR-Tregs might have some disadvantages regarding safety and long-lasting efficacy.

Functional Signaling via CAR

The activation step that is essential to successfully manufacture CAR-T cells derived from Tconv cells is usually accomplished by stimulation of CD3 and CD28 using expansion beads. Whereas first-generation CAR constructs consisted only of the extracellular CAR antigen-binding domain, a transmembrane domain, and an intracellular CD3 signaling chain, signaling domains from CD28 and/or 4-1BB (CD137) have been introduced into next-generation CAR constructs [4]. In addition, γ-chain cytokines (IL-2 and others) are added as a medium supplement [29]. According to Vormittag and colleagues, who analyzed the manufacturing process of CAR-T cells in clinical trials between 2002 and 2017, the most

commonly applied process for T cell activation is the anti-CD3/CD28 antibody-coated magnetic bead approach (65.8% of all analyzed trials). The second most commonly applied method is the combinatorial anti-CD3/CD28 monoclonal antibody plus IL-2 approach (29.3% of trials) [30].

However, CAR constructs in Tregs may need to fulfill different requirements to achieve optimal signaling for activation and expansion while preserving functionality. There is no clear consensus regarding the necessity of CD137- versus CD28-mediated intracellular signaling. Nevertheless, most research support the use of CD28 in allograft settings [10,31,101]. Other challenges relate to the *in vivo* survival of antigen-specific CAR-Tregs upon patient administration, the homing abilities of Tregs to the site where the target antigen is expressed, and **Treg exhaustion** and persistence (the latter is discussed in more detail in Box 3). Although the antigen specificity of CAR-Tregs should confer better homing capabilities, initial nonclinical studies showed only limited long-term survival at target sites. *In vivo* imaging upon adoptive CAR-Treg application in a murine colitis model revealed that these cells indeed migrated with high priority to the target tissues, but they only persisted for 7–9 days after adoptive transfer and were not detectable after day 9 [33,34]. Short-term survival could be a significant issue for manufactured CAR-Tregs, either due to disappearance of the antigen from the target tissue, cell death via continuous CAR-mediated stimulation ('overstimulation'), potential lack of exogenous IL-2 sources, or even immune responses initiated against distinct epitopes of the CAR itself. The optimal design of CAR signaling for long-term *in vivo* efficacy is an ongoing focus of research.

The Efficiency of Gene Delivery

Currently, CAR transduction commonly involves efficient but costly lentiviral-based gene delivery. This requires a separate manufacturing process, including QC assays, but can be purchased off-the-shelf from independent contract manufacturing organizations. The process is not trivial and requires experience in utilizing standard packaging cell line strategies for viral vector production (e.g., HEK 293T cell lines), in a **biosafety level S2** (BSL-2) environment, to enable reliable transient transfection and delivery of the packaged vector particles. Although no safety issues have been raised so far in numerous clinical trials using lentiviral-based CAR-Tconv cells, non-viral approaches have been gathering considerable interest as a potentially safe and cost-effective option [35]. A more recent approach relied on plasmid-based delivery of CAR transgenes via transposon/transposase systems. Briefly, CAR transgenes are inserted within a transposon sequence, and the corresponding plasmid is introduced into T cells via electroporation before activation. The transposase excises the transposon sequence, including the CAR transgene, from the donor plasmid and subsequently inserts it into the T cell genome. For manufacturing CAR-T cells based on this approach, the sleeping beauty (SB) system is preferably applied [36–39]. Initial approaches relying on ‘naked DNA’ transfection showed low integration efficiencies compared to established viral delivery systems, whereas novel transposon approaches have yielded integration efficiencies closer to those of viral delivery systems [38,102].

A drawback for both virus- and plasmid-based delivery systems is the potential risk of causing gene disruption or oncogenic events due to semi-random DNA integration. According to recent data, the 'Sleeping Beauty' transposon system may be safest in this respect because it may target 'safe' harbor loci within the human genome [40]. Minimizing vector copy numbers per cell also reduces the risk of insertional oncogenesis,

for example by gradually diminishing plasmid DNA levels after transfection as a result of DNA degradation or dilution by cell division. However, viral transduction still represents the predominant method for CAR transgene delivery, where 94% of clinical trials used this technique. Lentiviral systems (54%) seem to be preferred over retroviral methods (41%) for viral gene delivery [30].

Expansion and Yield

In addition to conventional cell culture flasks (T-flasks) and G-Rex® semi-closed cell culture platform with gas-permeable basement membranes, which enable large media volumes without impairing gas exchange, many CAR-T cell manufacturing processes also employ static culture bags and rocking-motion bioreactors [30]. The trend is moving toward (semi)automatic systems that allow almost closed processing, leading to reduced costs and batch-to-batch variability. Given the increasing success of anticancer Tconv therapy, massive investments are being made into technology developments which CAR-Treg processing can benefit from. Particularly for academic sites, closed automated devices to expand autologous cells even outside A/B clean rooms are of interest [41]. For instance, 'functionally closed system' manufacturing platforms, such as the CliniMACS® Prodigy, which is currently being tested at a multitude of centers for different indications, could be implemented in CAR-Treg manufacturing processes.

Another issue concerns the medium applied during the CAR-Treg expansion step. Almost all protocols use medium containing either fetal calf serum or human serum. Both have similar limitations regarding biosafety and sufficient supply for scaling up. Although there are ongoing efforts to develop serum-free medium, with some success for Tconv cells [42], Tregs show different growth properties than Tconv cells, probably because of their specific metabolism [43]. Further efforts will be necessary to optimize serum-free, xeno-free medium for CAR-Treg expansion.

A further challenge relates to stabilizing FOXP3 expression in expanded Tregs. FOXP3 expression is essential for maintaining the Treg phenotype and lineage identity, thus promoting Treg persistence by ensuring that they do not acquire T_{eff} cell activities during prolonged expansion or, later on, upon administration to patients [44–48]. Initial scientific findings suggest that the addition of the mTOR inhibitor rapamycin and/or restimulation events during expansion can provide benefits in terms of both the function and stability of the expanded Tregs [49,50]. However, there is still a gap in our knowledge regarding the detailed molecular mechanisms controlling FOXP3 protein stability in homeostatic and pathologic conditions. CRISPR/Cas9-based epigenetic editing might be a novel approach to stabilize Tregs [51].

QC, Cryopreservation, and Dosing

QC assays for release of Treg/CAR-Treg products are not yet standardized. Purity is commonly validated by flow cytometric analysis (minimally $CD4^+CD25^{high}FoxP3^+$), sometimes complemented by epigenetic demethylation analysis of the *FOXP3* promoter region (the Treg-specific demethylated region, TSDR). Unfortunately, no predictive functional assays are available. The commonly used suppression assay where Tregs inhibit the proliferation of Tconv cells is time-consuming, shows high interassay variance, and has poor predictive value for the *in vivo* function of Tregs [52]. Moreover, a recently developed rapid assay evaluating Treg functionality [53] was shown to yield inaccurate results in estimating their suppressive capacity [54]. It is crucial, particularly for CAR-Tregs, to consider developing and applying functional impurity markers to test the product for potential contamination

with putative T_{eff} cells which may constitute a safety concern. The absence of CD40L expression and release of inflammatory cytokines (interferon-γ, IL-2) following *ex vivo* stimulation has been introduced as a useful functional impurity marker which can be combined with both polyclonal and target antigen-specific stimulation [15,55].

The final formulation and packaging of the cell therapy product should be swift to avoid loss of cell viability. Most Treg products are delivered to patients as 'fresh' products because the functionality and yield of cryopreserved Treg cells are not satisfactory so far [56]. Nevertheless, cryopreservation would enable a sustainable and more convenient supply chain [57]. The final product should also be tested for cell numbers (dose) before release. The optimal cell dosing for clinical testing of Treg products remains uncertain. Clinical trials with polyclonal Tregs have used 0.1–3 × 10^6 cells/kg body weight with no apparent effects of dosage and dosing frequency on efficacy and safety [8,24]. Preclinical studies suggest that roughly 100-fold more polyclonal Tregs must be manufactured and administered to obtain an effect, as compared to allospecific/antigen-specific Tregs [58–64]. However, the need to administer large cell numbers is accompanied by an elevated risk of non-specific immunosuppressive events after infusion into patients. A transient elevation of virus reactivation parameters was seen in patients who received cord blood-derived polyclonal Tregs [65]. Moreover, there is high batch-to-batch variation for each Treg product resulting from the variability of the starting material (Treg subset composition, TCR repertoire) as a function of age, preexisting disease, and prior treatments. Given these current limitations and potential safety concerns associated with administering CAR-Tregs, a comprehensive benefit–risk assessment should be performed when designing the dose for clinical testing [24].

A Proposed Framework for Manufacturing GMP-Compliant CAR-Tregs

The encouraging results achieved with CAR-T cell therapies in cancer represent an exciting opportunity to expand the CAR technology to Tregs for therapeutic purposes. After highlighting key challenges that are unique to the manufacture of polyclonal Tregs and CAR-Tregs, in addition to other translational hurdles (Box 3), we propose a framework that may pave the way to achieve standardized and robust GMP-compliant manufacture of CAR-Tregs (Table 1, Key Table).

The current state of knowledge implies that the $CD4^+CD25^{high}FOXP3^+CD45RA^{high}CD45RO^{low}$ naïve Treg cell subpopulation is the best phenotype for isolation: it has the most homogenous expression of FOXP3, the lowest risk of introducing contaminating Tconv/Teff cells, and the highest potential for *ex vivo* expansion [66]. The isolation step would benefit from currently available functionally closed and automated GMP-grade sorting systems, as well as standardized QC tests for impurity testing. Activation and stimulation of Tregs under *ex vivo* conditions should rely on a combinatorial approach applying GMP-grade magnetic bead systems (continuous stimulation, easy removal, reduced loss of Tregs and antibodies) that have already been established for CAR-T cell manufacturing, and (high) exogenous IL-2 stimulation [67]. CAR transgene delivery into Tregs should be accomplished by applying GMP-grade lentiviral vectors that provide the highest efficacy rates and are safer than retroviral alternatives. Genes integrated via lentiviral vectors are less affected by gene silencing, and their high carrying capacity allows the integration of larger gene sequences [68,69]. Robust assays for vector release validation and testing of transduction efficacy need to be implemented. Expansion processes should be conducted in full GMP-compliant rocking-motion bioreactors with GMP-grade culture bags. These systems enable continuous nutrient supply and waste removal; they are also more efficient, less

Key Table

Table 1. An Optimized Manufacturing Framework for the Generation of GMP-Compliant CAR-Treg Cells

Processing step	CAR-Treg manufacturing framework (GMP compliant)	Future perspectives	Refs
Isolation	$CD4^+CD25^{high}FOXP3^+$ $CD45RA^{high}CD45RO^{low}$ naïve Treg cells Functionally closed and automated GMP-grade sorting systems (e.g., CliniMACS® Prodigy; CliniMACS® Plus system) Standardized QC tests for purity/impurity	Fully closed systems GMP-compliant cell sorter allowing multiparameter sorting (e.g., MACSQuant® Tyto®; BD FACSAria™ III)	[66,96]
Activation	Anti-CD3/CD28 antibody-coated magnetic beads plus (high) exogenous IL-2 stimulation GMP-compliant magnetic microbeads (e.g., Gibco™ CTS™ (Cell Therapy Systems); Dynabeads™ CD3/CD28) GMP-accredited IL-2 products (e.g., Proleukin®; CellGenix® recombinant human IL-2)	Self-dissolving or easily removable beads Soluble nano-sized matrices conjugated to humanized CD3/CD28 agonists (e.g., MACS® cGMP-compliant T cell TransAct™ reagent)	[97]
CAR design and gene delivery	Off-the-shelf lentiviral transduction Packaging cell lines for viral vector generation within a separate manufacturing process Implement assays for: (i) vector release validation (ii) transduction efficacy	UniCAR platform Non-viral approaches: (i) Sleeping Beauty transposon system (ii) CRISPR/Cas9	[86,98]
Expansion	Rocking-motion bioreactor plus culture bags; e.g., BIOSTAT® RM (TX) full GMP-compliant wave-mixed bioreactor with Flexsafe® RM (TX) Bags Continuous medium supply with GMP-compliant anti-CD3/CD28 magnetic beads designed for Treg expansion plus (high) exogenous IL-2	Semi- or fully automated, closed bioreactors with full automated process controls TCR-independent expansion Serum-free, xeno-free medium Purification by unique Treg marker signatures	[55,99]
QC assays formulation and filling	Consistent in-process and release testing Standardized purity/impurity assays Clinical grade GMP-compliant medium composition and filling devices for batch production	Improved potency assays Semi- or fully automated batch production with automated in-process controls	[100]

prone to contamination, and probably introduce less batch-to-batch variability [70]. Media perfusion systems are also compatible with magnetic bead activation. This approach reduces the loss of Tregs and antibodies because the application of a magnetic field can retain both. Finally, the formulation and filling processes should comprise GMP-compliant medium compositions and filling devices for batch production, and consistent in-process and release testing, as well as standardized assays for impurity testing (Table 1).

In addition to this proposed framework for improving CAR-Treg manufacturing process, we suggest several technology developments which may further improve the quality and reliability of the CAR-Treg production for clinical applications (Box 4 and Table 1).

Box 4. Future Considerations for Improving CAR-Treg Manufacturing

Isolation and Sorting

Supporting the development of a multi-parameter flow cytometry panel with consistent marker profiles may allow selection of the most efficient Treg subpopulation. For instance, the recent availability of GMP-compliant cell sorters (e.g., MACSQuant® Tyto® Cell Sorter) will allow for multi-parameter sorting with higher initial post-sorting purity of defined (subsets of) Tregs.

Activation

Streamlining the manufacturing process could be achieved by implementing self-dissolving/easily removable beads. Polymer-based nano-sized matrices conjugated to humanized CD3/CD28 agonists (e.g., MACS® GMP T Cell TransAct™, Miltenyi Biotech) may provide an alternative to current standards (GMP-grade microbeads). A key benefit relies on their solubility, thus avoiding 'de-beading' steps during the process, further reducing the complexity of the manufacturing process. However, there is still a clear need for identifying potential risks associated with the use of nanomatrices for manufacturing Tregs.

CAR Design and Delivery

Batch production using flexible module methods, such as UniCAR, can improve both safety and cost–benefit ratios [11,32]. Optimizing non-viral platforms (transposon or CRISPR/Cas technologies) may improve safety and efficacy (e.g., enhancing cellular functionality) [85]. In particular, Sleeping Beauty transposon systems hold great promise in terms of cost reduction by eliminating the need for clinical-grade viral vector manufacturing [36,86,87] and lowering the risk of insertional oncogenesis [40]. Using CRISPR/Cas9 with non-viral delivery of template DNA to eliminate large genes and for orthotopic placement of transgenic constructs opens new opportunities for non-viral delivery [89,89]. However, safety aspects such as off-target effects have yet to be adequately addressed [90–94].

Expansion

Semi- or fully automated closed-system bioreactors with integrated gas transfer and fully automated process control could pave the way toward an automated and standardized *ex vivo* expansion procedure and also improve safety and decrease the costs of the manufacturing process. The implementation of hollow-fiber bioreactors may also be a promising approach that deserves further attention [95]. Enabling additional purification during expansion by using unique Treg marker signatures [55] may enhance the consistency of the final product.

Off-the-Shelf Products

The implementation of allogeneic off-the-shelf products, genome-editing technologies to eliminate endogenous TCRs and MHC molecules, and express NK cell inhibitory molecules, as well as iPSC-derived Treg technology, will allow third-party modularly generated CAR-Treg cell banks with production-scale batches and improved safety [103,104].

Outstanding Questions

What is the best CAR construct for optimal Treg functionality and sustainability?

Do we need to adhere to the current 'gold standard' for CAR transgene integration/gene delivery in Tregs (lentiviral transduction), or is there a clear need to accelerate the development of virus-free gene modification systems?

What is the ideal potency test that can be predictive of the biological activity of the CAR-Treg product *in vivo*?

Is there a Treg subset that shows particularly long-lasting *in vivo* efficacy, and which source of cells is most suitable as the starting material for production (PBMCs vs tissue-derived cells vs inducible pluripotent stem cells, iPSCs)?

How can the current processing steps for CAR-Treg manufacturing be improved in terms of automation?

How can we increase the level of transparency and sharing of technical information on established manufacturing procedures among developers in the field?

Concluding Remarks

Increasing knowledge about Treg subset specificity and functionality, the existing successes of CAR-T cell manufacturing, and the still early phase of CAR-Treg development create a unique opportunity to advance the CAR-Treg research field. To enable the transfer of the CAR-Treg technology from the research stage into safe and effective clinical-grade cellular products, there is a need for further optimization of manufacturing methods, associated equipment, and product specification and characterization procedures. Further improvement will include optimization of CAR constructs, delivery techniques (viral or non-viral systems), potency testing, and manufacturing automation platforms (see Outstanding Questions). Once the highlighted challenges and outstanding issues are adequately addressed, the therapeutic opportunities for using CAR-Tregs to alleviate or even cure several debilitating diseases become immense.

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