Review

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Do Treg Speed Up with CARs? Chimeric Antigen Receptor Treg Engineered to Induce Transplant Tolerance

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Abstract. Adoptive transfer of regulatory T cells (Treg) can induce transplant tolerance in preclinical models by suppressing alloantigen-directed inflammatory responses; clinical translation was so far hampered by the low abundance of Treg with allo-specificity in the peripheral blood. In this situation, ex vivo engineering of Treg with a T-cell receptor (TCR) or chimeric antigen receptor (CAR) provides a cell population with predefined specificity that can be amplified and administered to the patient. In contrast to TCR-engineered Treg, CAR Treg can be redirected toward a broad panel of targets in an HLA-unrestricted fashion, making these cells attractive to provide antigen-specific tolerance toward the transplanted organ. In preclinical models, CAR Treg accumulate and amplify at the targeted transplant, maintain their differentiated phenotype, and execute immune repression more vigorously than polyclonal Treg. With that, CAR Treg are providing hope in establishing allospecific, localized immune tolerance in the long term, and the first clinical trials administering CAR Treg for the treatment of transplant rejection are initiated. Here, we review the current platforms for developing and manufacturing alloantigen-specific CAR Treg and discuss the therapeutic potential and current hurdles in translating CAR Treg into clinical exploration.

(Transplantation 2023;107: 74–85).

INTRODUCTION

There is a long-standing interest in organ transplantation for inducing peripheral tolerance to prevent chronic

immune-mediated rejection and to allow long-term allograft survival. Although major improvements were achieved during the last years in repressing aberrant immune reactions, general immune repression causes deleterious side effects, demanding strategies for inducing more specific and durable tolerance to the transplant. Physiologically, immune homeostasis is maintained by the concerted action of a number of immune repressive cells; regulatory T cells (Treg) are the key regulators to repress an exacerbated immune response by limiting the effector functions and the expansion of proinflammatory immune cells, ideally resulting in peripheral immune tolerance, making Treg favorite candidates for cell therapeutic intervention.[1-3]

Treg represent a separate T-cell lineage of CD4^{+} CD25^{+} cells with suppressor capacities[4,5] that generate in the thymus (tTreg) and control tolerance to self-antigen or derive from peripheral CD4^{+} T cells (pTreg) and control immune reactions toward foreign antigens.[6-8] Naive CD4^{+} T cells can be induced to become Treg by transgenic expression of the master transcription factor forkhead-box-protein P3 (FoxP3) along with stimulation through CD3/CD28, IL-2, rapamycin, and TGF-β in vitro, also named iTreg.[9,10] The tTreg development and suppressive function are determined by FoxP3;[11] FoxP3-mediated programming of Treg is crucial because loss-of-function mutation of FoxP3 abrogates Treg suppressive capacities, resulting in loss of peripheral tolerance and severe autoimmunity.[12,13] tTreg have a more stable epigenetic program[14] and are rather resistant to reversion into effector CD4^{+} T cells as they display an epigenetically stable status at the Treg-specific demethylated region (TSDR).[15] This is in contrast to pTreg and iTreg, which lack TSDR demethylation[16] with the inherent risk to convert into pathogenic CD4^{+} T-cell subsets. Treg act on a variety of proinflammatory cells, including T cells

and innate cells.[17] To maintain tolerance in the periphery in case of dysfunctional FoxP3, importantly, Treg cannot be replaced by other suppressive cells underlining their central role as key regulators in the periphery.

Because of their CD4 linage origin, antigen-driven Treg activation is physiologically restricted to HLA class II recognition through their T-cell receptor (TCR); in contrast, their suppressive activity is executed in an antigen-independent fashion through a variety of mechanisms,[18] including the release of suppressive cytokines like TGF-β, IL-10, and IL-35, and correspondingly, the inability to release proinflammatory cytokines. Treg suppressor activity is enhanced by CTLA-4-mediated CD28 blockade of antigen-presenting cells (APCs), catalysis of ATP to ADP and cAMP, LAG-3-mediated HLA class II blockade, expression of the putative checkpoint molecule TIM-3, and TIGIT-induced IL-10 release and blocking CD155–CD226 costimulation resulting in reduced TCR signaling. Treg expressing TIM-3 display a more activated phenotype and are able to supress Th1 and Th17 in a STAT3-dependent manner, in contrast to TIM-3⁻ Treg that lost the ability to supress Th17 cells in vivo.[19,20] TIGIT is able to contribute to Treg stability by maintaining FOXP3 expression and suppression of IFN-γ.[21] TIGIT has been reported to limit PI3K-AKT signaling, thereby inhibiting acquisition of a T helper type 1 (Th1) cell-like phenotype.[22] Treg expressing high levels of TIGIT are more suppressive than those expressing low levels, exhibiting superior suppression of Th1 and Th17 responses.[23]

Interestingly, antigen-specific memory Treg exhibit more potent repressor capacities than naive Treg in antigen-specific transplant models,[24,25] pointing to the impact of antigen specificity in inducing peripheral tolerance. In peripheral immunity, tolerance toward antigens of any origin is mediated by so-called type-1 regulatory T cells (Tr1 cells). Tr1 cells, first described in peripheral blood of patients who developed tolerance after HLA-mismatched fetal liver hematopoietic stem cell transplantation, modulate inflammatory and effector T cell responses in various immune-mediated diseases. These cells are self or non-self antigen-specific, express IL-10 and TGF-β once activated, and are capable to induce and mediate peripheral tolerance by limiting antigen-specific immune responses and suppressing inflammation in autoimmunity and graft-versus-host disease (GvHD).[26,27] The possibility to generate and expand Tr1 cells in vitro in an antigen-specific manner and obtaining these cells from CD4+ T cells by IL-10 gene transfer[28,29] has increased interest in their use in adoptive cell therapy.

Because of their specific capacities, Treg are good candidates to induce transplant tolerance after adoptive transfer based on their capacity to dampen a proinflammatory response. In an ideal scenario, Treg are being specifically activated by alloantigens of the transplant in a localized fashion, establishing peripheral tolerance and allowing for reduction of pharmacological systemic immune suppression. The concept is underlined by a pilot safety and feasibility trial, called TASK (NCT02088931), which showed in 3 patients 6-mo surveillance of kidney transplants with subclinical inflammation after autologous Treg therapy.[30] A subset of infused Treg remained detectable in circulation for at least a month postinfusion. Graft inflammation, as assessed by the density of LCA⁺ cells, showed improvement on follow-up biopsies in the first 2 patients but not in the third patient. Although this study was not powered to detect improvement in graft inflammation, the trial demonstrates feasibility and safety outcomes, suggests that Treg infusion can reduce inflammation in the graft, and paved the way to design a full-scale protocol of Clinical Trials in Organ Transplantation-21 (CTOT-21, NCT02711826) to determine the efficacy of infused polyclonal Treg versus donor alloantigen-reactive Treg in a randomized controlled trial.[31] The ONE study revealed that Treg therapy is achievable and safe in living-donor kidney transplant recipients and is associated with fewer infectious complications but did not reduce rejection rates in the first year; however, it minimized the burden of general immunosuppression.[32]

In addition to solid organ transplantation, Treg are also explored after allogeneic stem cell transplantation aiming at preventing severe GvHD without the need for further immune-repressive drugs.[33-35] Although these Treg isolates were CD25 enriched and may be contaminated with effector T cells, Good Manufacturing Practice (GMP) production protocols were established to obtain pure Treg from cord blood or donor peripheral blood.[36] These approaches are applying polyclonal Treg with unknown specificity; here we briefly review genetically redirected Treg for inducing antigen-specific tolerance in solid organ transplantation in experimental models and early clinical trials.

Donor Antigen-specific Treg Are More Potent in Inducing Transplant Tolerance

Pre-clinical studies sustain the notion that alloantigen-specific Treg display improved capacities in repressing an immune response against the transplanted organ compared with polyclonal Treg.[37-39] Given the relevance of antigen-specific activation, current strategies are aiming at ameliorating transplant rejection upon transfer of donor-alloantigen-reactive, host Treg.[40] Alloantigen-specific Treg are selectively expanded in vitro by one round of stimulation through donor-derived APCs and consecutively expanded by a round of polyclonal stimulation to obtain clinically relevant cell numbers for adoptive transfer.[41]

Technical obstacles, however, limit so far the translation of alloantigen-specific Treg to broad clinical application. Obtaining allogenic antigen-expanded Treg requires complex in vitro procedures involving stimulation with donor antigen to activate and amplify the small number of at most 10% natural Treg that are alloantigen reactive.[42] Overall, this is an extremely laborious and inefficient process, and the success strongly depends on the number of pre-existing alloantigen-specific Treg. Repetitive TCR stimulation may also alter the Treg phenotype and functional capacities, which finally has an impact on the clinical regimens in immunosuppression applied to transplant patients. Altogether, the technical and functional hurdles are demanding for genetically redirecting Treg with predefined antigen specificity without altering their functional capacities.

CARs Provide Predefined Antigen Specificity to Treg

The overall hypothesis is that antigen-specific activation makes Treg therapy more potent than nonactivated,

poly-specific Treg. The concept is also underlined by reports on targeting Treg to the transplant upon engineering with a TCR specific for an alloantigen.[43] TCR-engineered Treg specific for allo-MHC class II favor transplantation tolerance and long-term survival of partially mismatched heart transplants in a mouse model.[44,45]

Redirected activation through a recombinant TCR recapitulates physiologic, MHC-restricted T-cell activation; however, the huge number of potential targets for TCRs in an alloantigen-specific situation makes translation to transplantation practice extremely complex. This is in contrast to autoimmunity, in which a limited number of TCR-recognized antigens are involved and a few TCRs can be applied to a relatively large number of patients.[46]

The situation is technically easier when engineering Treg with a chimeric antigen receptor (CAR) that conveys both pre defined antigen specificity and T-cell activation upon target recognition. CARs are synthetic one-polypeptide transmembrane receptor molecules with an extracellular binding domain to recognize the specific antigen and with one or more intracellular signaling domains to trigger T-cell activation upon antigen engagement (for review) (Figure 1).[47,48] The antigen binding domain is in most cases derived from an antibody and composed of the variable region of the heavy (VH) and light(VL) immunoglobulin chain for antigen binding. The intracellular domain harbors the TCR-derived CD3ζ chain for the primary activation signal (signal 1) and the intracellular domain of a costimulatory receptor (signal 2), both together providing complete and persistent T-cell activation. Such "second generation" CARs with primary and costimulatory signals were superior in the redirected activation of effector T cells and are currently in broad clinical exploration. CARs with specificity for CD19 obtained the first Food and Drug Administration and European Medicines Agency approval for redirecting effector T cells against B-cell malignancies.[47]

Because of their dual capacities, engineering with a CAR is aiming to provide Treg with predefined specificity, trigger their functional capacities upon antigen engagement, and include their regulatory capacities in inhibiting or dampening an effector T-cell inflammatory immune response.[49] In conventional T cells, CAR expression with CD28 or 4-1BB costimulatory domains is often associated with tonic signaling that may be more dramatic in Treg with respect to their activation and proliferative capacities. A CAR with the CD28 costimulatory domain is preferred to trigger Treg activation; CD28 costimulation maintained CAR Treg suppressor function, whereas 4-1BB costimulation resulted in decreased Treg lineage stability, reduced in vivo suppressive capacities, and in some cases, tonic signaling.[50-52] However, the negative effect of 4-1BB can be mitigated by transient mTOR inhibition.[52]

Early studies provided experimental evidence for the concept; adoptive transfer of CAR-engineered mouse Treg with specificity for carcinoembryonic antigen dampened experimentally induced colitis.[53] CAR Treg with specificity for myelin oligodendrocyte glycoprotein-controlled experimental autoimmune encephalomyelitis.[54] Engineering human Treg with a CAR specific for HLA-A2 is rendering the cells highly suppressive and capable of preventing lethal GvHD in an artificial xenogenic model of HLA-A2-positive effector T cells in immune-deficient mice.[55] Treg with a CAR of irrelevant specificity did not prevent lethal GvHD.

More recently, the expression cassette for the anti-HLA A2 CAR was inserted specifically into the TCR α-chain locus, thus replacing the endogenous TCR and expressing the CAR under the control of the TCR regulatory elements.[56] Such HLA A*02-specific CAR+ TCR− human Treg maintained both Treg phenotype and function in vitro, selectively accumulated at transplanted HLA-A*02+ islet cells without impairing their function in a mouse model, and exhibited antigen-dependent in vivo suppression, independently of TCR expression.[56] A most recent report demonstrates in a clinically relevant model the capability of CAR Treg with specificity for HLA-A*02 to prolong the survival of vascularized heart allografts in the fully immunocompetent mouse, however, with some signs of chronic rejection.[57]
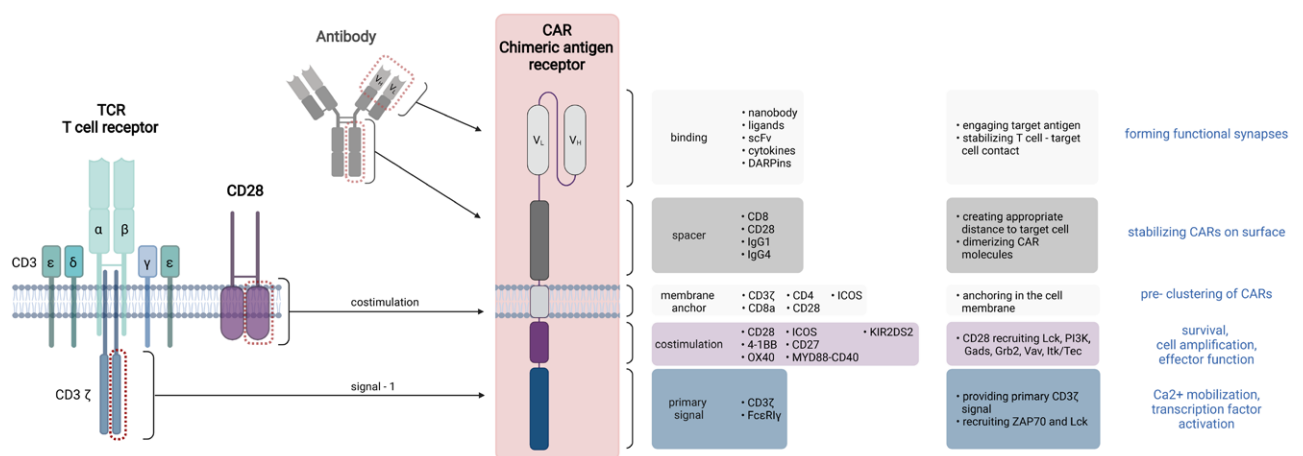
FIGURE 1. The modular composition of a chimeric antigen receptor (CAR). The CAR is a recombinant composite trans-membrane receptor composed in the extracellular part of an antibody-derived scFv (single-chain fragment of the variable regions of immunoglobulin light [VL] and heavy [VH] chains) that is linked by a "spacer" to a membrane anchor and the intracellular signaling domains derived from the T-cell receptor (TCR) complex and costimulatory molecules. Although the first-generation CARs harbor only the CD3ζ chain for providing the primary signal, second-generation CARs deliver additional costimulation through an integrated costimulatory signaling domain. The modular composition allows combining multiple variants of each module to set up a CAR with optimized functional properties for the respective host cells and functional needs.

Semi-allogeneic donor cells mismatched at MHC class I and indirectly activated can enhance tolerance to subsequent skin allografts in mouse models.[58] Human alloantigen-specific CAR Treg suppress skin rejection in xenogenic mouse models;[59] HLA-A*02-specific CAR Treg are more potent in protecting humanized skin transplants than polyclonal, nonengineered Treg.[59,60] In skin transplantation models, it became also obvious that, although limiting allograft rejection in naive mice, CAR Treg do not prevent rejection in sensitized mice.[61]

The choice of CAR-targeted antigen is difficult when aiming at specifically targeting the transplanted organ. For instance, using HLA-specific CARs raises the difficulty that many HLA allele proteins belong to an evolutionary closely related family of proteins that differ by only a few amino acids; anti-HLA CARs harbor the risk of systemic Treg activation by cross-reactive HLA alleles expressed by the recipient. In this situation, the well-annotated database of eplets expressed by different HLA alleles will be helpful.[62-64] Eplets are small configurations of polymorphic amino acid residues on HLA molecules and are considered essential components of HLA epitopes recognized by antibodies. These antibodies can then serve as targeting domains for CARs redirecting Treg in a well-defined, specific fashion. In this situation, Dawson et al[65] provided a platform to map the alloantigen specificity of humanized HLA-A*02:01-specific CARs, which allows to isolate and test specificity and cross-reactivity of humanized alloantigen-specific CARs for use in adoptive cell therapy.

Taken together, the experimental models used to demonstrate CAR Treg activities are confirmatory; however, they mostly do not represent the human situation in a sufficient manner in that human Treg survive only for a couple of days in the mouse and therefore repress the initiation phase but not the ongoing chronic phase of allograft rejection.

How to Manufacture CAR Treg

How to Isolate Treg

Major hurdles for translating the CAR Treg concept have been, so far, the low abundance of natural Treg in the peripheral blood, the isolation with minimized risk of contamination with effector T cells, the effective engineering with a CAR, and the ex vivo expansion to clinically relevant numbers. During the last years, several groups successfully developed procedures to isolate, amplify, and genetically engineer human Treg ex vivo under GMP conditions (**Figure 2**).[66] Because of the small proportion in peripheral blood, Treg are mostly isolated based on CD4+CD25+ expression, with or without CD8+ T-cell depletion. CD4+CD127lowCD45RA+CD25high Treg as starting population for clinical scale manufacturing seems to be safe[65] because this population is depleted of effector T cells and suitable for ex vivo amplification.[67,68] CD127 is a suitable marker in this respect because CD127 inversely correlates with FoxP3 and suppressive function.[69,70] CD4+CD25high Treg, sorted based on low CD127 expression, provide a more potent therapeutic capacity than conventional Treg.[71] nTreg isolated and amplified in this manner consistently maintain FoxP3 expression in comparison to CD45RA− memory Treg[72,73]; these cells also show a stable epigenetic phenotype.[74] Stability of repressor phenotype is crucial because a reverted effector T cell in the engineered T-cell population would, upon antigen engagement, drive a proinflammatory and cytotoxic response and finally cause severe side effects.

CD45RA+ Treg are isolated from autologous or donor peripheral blood as starting material[55] or further purified by serial enrichment procedures[75] to reduce the risk of contaminating T effector cells. Although the risk of contaminating effector T cells remains with these procedures, high-speed GMP-compliant sorting of CD45RA+ Treg entered clinical exploration for acute GvHD (EudraCT 2012-002685-12).[76,77] Taken together, purification procedures under GMP conditions are established to provide a Treg population that showed a stable phenotype during ex vivo amplification.[68,72]

Treg can also be induced in vitro to obtain iTreg that can be used for manufacturing starting from conventional CD4+ T cells by culture in the presence of TGF-β or retinoid acid[78,79] to induce FoxP3 expression; alternatively, FoxP3 expression can be enforced by viral transduction or gene editing.[10,80] Full conversion to Treg requires activation-independent, high FoxP3 levels; ectopic FoxP3 expression alone however, is not sufficient to induce the transcriptomic landscape of isolated Treg questioning the suitability of ectopic FoxP3 expression in the long term.[81,82]

How to Stimulate and Transduce Treg

Isolated Treg are activated by CD3 plus CD28 stimulation in the presence of IL-2, with or without rapamycin or TFG-β,[49] and transduced by a retro- or lentivirus encoding the CAR.[83,84] Engineered cells are expanded afterward to cell numbers suitable for clinical application by TCR/CD3 plus CD28 stimulation in the presence of IL-2. For instance, umbilical cord blood-derived Treg can be expanded using anti-CD3/CD28 monoclonal antibody-coated beads to provide TCR and costimulatory signals.[36] Artificial antigen-presenting cells (aAPCs), like KT64/86 cells preloaded with anti-CD3/CD28 antibodies, achieve similar expansion properties while increasing TGF-β secretion and Treg suppressor function.

How to Engineer a CAR for Redirecting Treg

Engineering a CAR for redirecting Treg basically follows the rules of CAR design previously developed for effector T cells (for review).[85] However, not all rules in the composition of a successful CAR seem to be transferable from the experience on effector T cells demanding systematic testing of each CAR domain for Treg, which is still a trial-and-error process.[49]

The function, potency, and persistence of conventional T cells redirected by a CAR is substantially tailored by choice of the costimulatory domain.[86] The Treg requirements for CAR-mediated stimulation are different to conventional T cells; however, Treg consistently require CD28-mediated stimulation along with the primary CD3ζ signal,[87-89] which no other member of the CD28 superfamily can substitute.[50] Systematic evaluation of costimulatory CARs, primarily developed for effector T cells, recently confirmed that Treg benefit most from CD28-ζ costimulation with respect to phenotypic stability, cytokine production, survival, and CAR-induced gene expression profile with respect to the enrichment of genes of the NK-κB pathway and their target genes.[50]
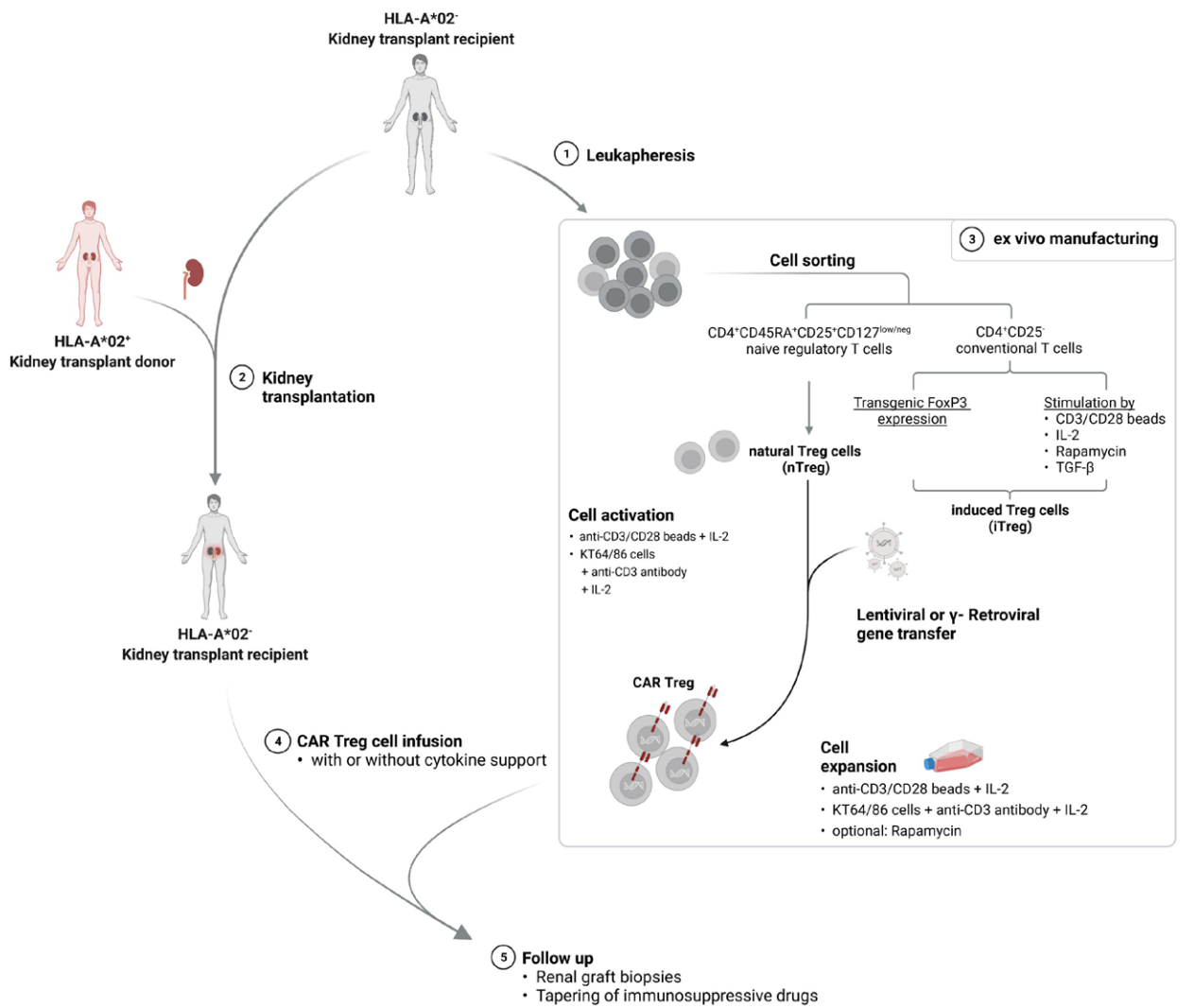
FIGURE 2. Outline of the CAR Treg therapy and options in ex vivo manufacturing. Treg are isolated from patient's leukapheresis product by cell sorting procedures to obtain naive Treg (nTreg); alternatively, conventional CD4^{+} T cells can be induced to convert to induced Treg (iTreg) by transgenic FoxP3 expression or by stimulation through their TCR/CD28 plus IL-2, TGF-β, and rapamycin. Treg are activated and engineered by retro- or lentiviral transfer to express the CAR and subsequently amplified to clinically relevant numbers under various stimulatory conditions. Finally, after passing a panel of quality control procedures, the CAR Treg product is applied to the patient.

While optimizing the procedure to engineer CAR Treg, some basic considerations need to be taken into account:

(1) CAR targeted antigen needs to be selectively expressed by the transplant or in the vicinity of the transplant to locally restrict CAR redirected Treg activation;
(2) the optimal level in binding affinity to antigen and in CAR expression to induce ideal suppressor capacities needs to be determined for each individual CAR;
(3) Treg require a strong CD28 activation signal along with CD3ζ signaling upon antigen engagement;
(4) Treg require continuous "feeding" with IL-2 along with CAR signaling to survive, amplify and execute their suppressor functions[90]; and
(5) Engineered Treg need to display a stable phenotype to maintain immune tolerance over time. In case of insufficient persistence, Treg need to be repetitively administered, which requires manufacturing and expansion of higher cell numbers than for therapy by one course of Treg administration.

Early Clinical Exploration of Treg Therapy

Clinical Treg trials were initiated in transplantation medicine more than a decade ago (summarized in Ref. [91]), mostly using nonengineered, ex vivo amplified Treg. The transferred Treg populations are polyclonal, although with unknown antigen specificities. The central role of Treg is clinically demonstrated by protecting from autoimmune kidney disease, maintaining tolerant kidney grafts, and preventing kidney allograft rejection.[92-94] More recent trials demonstrated the safety and persistence of adoptively transferred Treg in transplant patients.[95]

Amplified natural Treg with polyclonal specificities administered to kidney transplant patients proved safe in the multicenter ONE study[32] as well as in a liver transplant trial (NCT 02145325);[31] both trials did not reveal significant adverse events such as rejection. In 2 kidney transplant patients, transferred Treg amplified in vivo and maintained their tolerogenic gene expression profile under immune

TABLE 1.

Early phase trials using CAR-engineered Treg to prevent solid organ transplant rejection

Sponsor	Acronym	Status	ClinicalTrials.gov Identifier	Clinical centers	Patient cells	Cell product	Disease	Target antigen	Reference
SangamoTherapeutics	STeadfast	Phase 1/2	NCT04817774	University Hospitals Leuven, University Medical Center Groningen, Erasmus MC, University Medical Center Rotterdam, Oxford University Hospitals NHS Foundation Trust	nTreg	Autologous CAR Treg	Renal transplant rejection	HLA-A2	https://www.sangamo.com/
Quell Therapeutics Limited	LIBERATE	Phase 1/2	NCT05234190	Cambridge University Hospitals NHS Foundation Trust, Royal Free London NHS Foundation Trust, King's College Hospital NHS Foundation Trust	nTreg	Autologous CAR Treg	Liver transplant rejection	HLA-A2	https://www.quell-tx.com/

suppressive treatment.[96] A caveat is that adoptive transfer of donor-alloantigen-reactive Treg after immune suppressive preconditioning in living-donor kidney transplantation did not prevent transplant rejection that resulted from weaning of immunosuppression.[97] Complete cessation of pharmacological immune suppression was not achieved despite demonstrating functionally suppressive Treg in vitro. The reasons may be multiple, including insufficient Treg dose, loss of repressive capacities, or vast depletion of administered Treg. In a Phase I trial, kidney transplant recipients received Treg at day +5 posttransplantation in lieu of induction immunosuppression (EuDra CT No 2011-004301-2). Autologous Treg therapy showed feasible and safe results in a long-lasting dose-dependent increase in peripheral blood Treg and potentially associated with a lower rejection rate than standard immunosuppression.[98]

A pilot Treg therapy study (UMIN-000015789) using an ex vivo–generated Treg-enriched cell product showed to be safe and effective for drug minimization and operational tolerance induction in living-donor liver recipients with nonimmunological liver diseases.[99] In a subsequent trial, patients enrolled while awaiting liver transplantation or 6–12 mo posttransplant adoptively transferred Treg transiently increased the pool of circulating Treg and reduced anti-donor T-cell responses (NCT02166177), moreover implying that Treg therapy may facilitate the reduction or complete discontinuation of immunosuppression following liver transplantation.[100]

The ONEnTreg13 trial confirmed that the application of autologous nTreg was safe and feasible even in patients who had a kidney transplant and were immunosuppressed.[101] The CELLIMIN trial revealed that upon Treg transfer, the activation of conventional T cells was reduced and nTreg shifted in vivo from a polyclonal to an oligoclonal TCR repertoire.[102]

Within kidney transplant patients receiving ex vivo expanded autologous Treg, circulating Treg levels amplified in a sustained manner. Clinically, all Treg doses tested were safe with no adverse infusion-related side effects, infections, or rejection events up to 2 y posttransplant.[31] Also, the administration of donor-alloantigen-reactive Treg (darTreg) in liver transplantation (deLTa trial, NCT02188719) showed transient increases in Treg in recipients. The adoptively transferred cells retained a broad FoxP3$^+$ CD25^{high} CD127^{low} Treg phenotype in the long term.[103] Taken together, trials applying Treg in kidney or liver transplant patients showed safe and provided some evidence of persistence and reduced activation of conventional effector T cells.

Exploring CAR Treg Therapy in Transplantation

CAR or TCR-engineered Treg with redirected specificity are currently evaluated in trials or are in an advanced preclinical stage (**Table 1**). "STEADFAST" is a multicenter trial (NCT04817774, EudraCT 2019-001730-34) in which a CAR specific for HLA-A*02 is engineered into autologous naive Treg from an HLA-A*02-negative patient awaiting an HLA-A*02-positive donor kidney.[104] The CAR recognizes HLA-A*02 expressed by the donated kidney with the aim to specifically accumulate at the transplant and induce lasting immune tolerance. The control participants will undergo kidney transplant as per the planned standard of care with no CAR Treg administered.

Recently started, the trial is aiming at evaluating safety and the therapeutic efficacy of HLA-A*02-specific CAR Treg on the donated kidney in living-donor kidney transplant recipients infused several weeks after kidney transplantation. Secondary objectives include the evaluation of the effect of CAR Treg on acute graft-related outcomes and on long-term safety, chronic graft function, the ability to reduce immune suppression, and the accumulation of CAR Treg in kidney transplant. The first patient was treated with HLA-A*02-specific CAR Treg in March 2022.[105]

In the "LIBERATE" trial (Quell Therapeutics), CAR Treg are aimed to be applied to patients with liver transplants to reduce or eliminate systemic immune suppression.[106] Treg are engineered with an HLA-A2-specific CAR to redirect Treg of HLA-A2 negative recipients to the HLA-A2–positive donor liver. CAR T cells are also engineered with a FoxP3 phenotype lock module and a safety switch for specific Treg elimination in case of adverse events. The study is designed to induce durable operational tolerance by preventing a host-vesus-graft immune response resulting in organ rejection.

Given together, these first trials will provide the opportunity to monitor the localization, phenotype, persistence, and function of the CAR Treg in the targeted transplant tissue and in the periphery and to assess the induced modulation of the alloimmune response in transplant patients.

Conclusions and Future Perspectives

Adoptive therapy with regulatory T cells, with or without engineered targeting specificity, may become a realistic option in the treatment or prevention of transplant rejection. The protocols of GMP-conform isolating, amplifying, and genetically engineering Treg are developed for being translated to clinical applications. However, a number of issues need to be considered in developing the strategy for broad clinical application (**Table 2**).

Source of Treg

Most approaches use natural Treg isolated from the peripheral blood for engineering; other sources may also be considered. For instance, thymus-derived Treg would have the advantage of higher and more stable suppressive capacities in the inflammatory environment. Alternatively, tissue-resident Treg display a specific gene signature to persist and execute their function in the specific organ. Finally, Treg may be derived in vitro by specifically induced differentiation from pluripotent stem cells. The specific advantages of the different Treg sources need to be compared side-by-side with respect to feasibility in manufacturing, stability in function, and finally, clinical efficacy. On the other hand, alternative immune cell species may be explored in the context of a redirected control of a solid organ transplant; in particular, an immunotherapeutic strategy using CAR-modified natural killer (NK) cells in transplantation treatment was recently reported.[107]

Optimizing the CAR Design

The overall aim in optimizing a given CAR for clinical application is to combine optimized domains within a CAR that shows preserved expression, clearly defined antigen specificity, and less tonic signaling. A CAR redirecting Treg requires CD28 costimulation along with the CD3z signal; other costimulatory domains seem to be less effective.[50] T cells engineered with human or humanized CARs show improved efficacy and less severe side effects compared with CARs with murine elements.[108-110] The antibody-derived CAR binding domain is a dominant immunogenic epitope within the CAR molecule, although not the only one. Procedures to generate humanized CARs with a panel of modifications including the framework region of the binding antibody[65] are being established by empirical testing.

There is a positive correlation between antigen binding and T-cell activation; however, the optimal CAR affinity for redirecting Treg activation toward its cognate antigen is unknown. The kinetics and structure of the immune

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TABLE 2.

Options to improve CAR Treg therapy

Source of Treg	• Peripheral blood-derived natural Treg
	• umbilical cord blood-derived naive Treg with high intrinsic proliferative potential
	• thymus-derived Treg with high suppressiveand stable functional capacities
	• tissue-resident Treg with defined homing capacities
	• induced pluripotent stem cells (iPSCs)-derived Treg
Optimizing CAR design and expression	• optimize CAR binding affinity
	• adapt CAR spacer with respect to the CAR target
	• combine primary CAR signaling with appropriate costimulation
	• optimize ex vivo CAR Treg manufacturing
	• precision engineering of the CAR into the TCRα-chain locus
Improving Treg homing and persistence	• support Treg survival and persistence by cytokine support
	• strengthen signaling pathways by engineering
	• improve tissue homing by transgenic expression of chemokines and/or homing receptors
	• attract and activate other suppressor cells at the transplanted organ
Managing Treg toxicities	• engineer Treg with a suicide gene
	• engineer Treg with an inducible apoptosis switch like iCasp9
	• engineer Treg with a targetable CAR or co-expressed protein for antibody-mediated depletion
Editing Treg function	• express transgenic FoxP3
	• edit specific genetic loci involved in Treg function
	• add functional capacities by induced release of transgenic proteins

synapses formed upon contact with target cells is crucial; however, the CAR-induced synapses upon antigen binding are fundamentally different from TCR–HLA/peptide synapses with respect to size, number, structure, and kinetics[111] making predictions of CAR modifications on T-cell activation even more difficult.

Improving CAR Treg Engraftment, Homing, and Persistence

The engraftment and homing of engineered CAR Treg to the transplant is essential to establish tolerance. It is so far unclear whether or not preconditioning lymphodepletion improves Treg engraftment after administration, which requires thorough evaluation in clinical trials. A2-specific CAR Treg accumulate preferentially at the HLA-A*02+ allograft and stay there for at least 3 wk, whereas polyclonal Treg do less in a short-term transplantation model.[61,65] A similar observation was made in CAR Treg treatment of islet transplants.[112] On the other hand, alloreactive Treg may lose their functional capacities upon extensive ex vivo amplification, in particular with respect to downregulation of their CAR and/or their immunoregulatory capacities and persistence.[113]

It is so far also unresolved whether CAR Treg require additional homing receptors to improve their side-directed accumulation to the transplant[114] and long-term survival. Although polyclonal Treg are also found at the affected transplant after infusion, engineering Treg with specific chemokine receptors like CXCR3[115] or IL12 induction of CXCR3[116] are aimed at favoring more actively their accumulation at sites of the produced ligands CXCL9 and CXCL10.

Migration to the lymph node may also be crucial during the process of establishing true tolerance.[117] HLA class II-positive APCs may prime alloreactive Treg in the lymph node; CAR-redirected activation at the side of inflamed organs may then booster and prolong their activation. The ideal target for redirecting Treg may potentially be combining lymph nodes and transplant organ recognition. Once activated, Treg require cytokine support to persist for a sufficient period of time and to prevent exhaustion and apoptosis, eg, by systemic IL-2 application to support Treg survival. Finally, Treg activation in the targeted organ may co-activate bystander cells that also help control effector T-cell activation against solid organ transplants.

There is generally a paucity of models for testing human Treg migration and function in vivo. For instance, the model of xeno-GvHD clearly demonstrates the power of the CD28-ζ CAR to stimulate suppression by TGF-β and CLTA-4 signaling in Treg.[50] The model, like other transplantation models, however, does not fully recapitulate the human situation, as there is no network of lymph nodes, poor engraftment of human APCs, and poor cross-reactivity with mouse/human homing receptors, among others. To address the requirements and physiology of CAR-redirected Treg activation, it will be necessary to use alloantigen-driven syngeneic models to avoid some limitations of cross-species models.[61]

Managing Potential CAR Treg Elimination

CAR redirected Treg therapy may potentially cause a more generalized immune repression, which affects immune regulation in healthy tissues. A major trigger may be activation of CAR Treg in healthy tissues in addition to activation in the transplanted organ with the risk of a reduced immune response against opportunistic infections. This potential situation may require the elimination of CAR Treg, which can basically be addressed by applying depleting antibodies that recognize an epitope on the extracellular CAR domain, for instance, an antibody recognizing RQR8[118]; coexpressed transgenic EGFR may also be targeted by a depleting antibody.[119] However, successful and complete CAR Treg elimination requires sufficient high antibody concentrations in organs where the engineered Treg accumulate. Alternatively, CAR Treg can be engineered with so-called "suicide" gene(s) to allow specific elimination by administration of an activating drug to the patient.[118] For instance, CAR Treg equipped with herpes simplex virus thymidine kinase (HSV tk) convert pyrimidine and acycloguanosine nucleoside analogs like ganciclovir into phosphorylated, toxic metabolites that terminate DNA chain elongation and specifically kill engineered cells.[120,121] Also, T cells engineered with inducible caspase-9 (iCasp9), that is based on the fusion of caspase-9 to a modified FK-binding protein, enter apoptosis after dimerization and iCasp9 activation upon adding a small molecule drug.[122] The clinical feasibility and efficacy in eliminating iCasp9-modified T cells was demonstrated in particular after haplo-identical hematopoietic stem cell transplantation; one dose of the dimerizer drug (AP1903) eliminated >90% of the modified T cells, resolving the clinical symptoms.[122]

Engineering With Additional Functional Capacities

Recent developments in CAR technology allow to reprogram engineered immune cells for additional functional capacities. T cells can be engrafted with an additional, CAR-inducible expression cassette to produce and release a transgenic protein, for example, a repressive cytokine, triggered by CAR engagement of cognate antigen in the transplanted tissue (**Figure 3**). Such "TRUCKs" are triggered by drug factories, that is, CAR T cells, that produce and release the transgenic protein "on demand" when and where the CAR T cell encounters a cognate target.[123] A broad variety of repressive or regulatory proteins for the transgenic release can be envisaged in this context.

Advances in genome editing technologies will engineer Treg more precisely with a CAR or TRUCK, for instance by using clustered regularly interspaced short palindromic repeat-associated protein-9 (CRISPR/Cas9). Accordingly, specific genes providing additional capacities were knocked out in Treg without affecting the overall gene expression landscape.[124] Treg-associated GARP gene expression can be specifically induced by using nuclease-deficient Cas9.[124] Likely, gene editing technologies may be used in the near future to promote the expression of other key genes in regulating the repressive capacities or the release of repressive cytokines shaping engineered Treg toward more specific cell therapeutics.

Taken together, there is growing evidence that the hurdles in site-specific genetic engineering, manufacturing clinically relevant CAR Treg numbers, stabilizing their repressive phenotype, and minimizing the risk of conversion can be addressed in the near future to allow safe
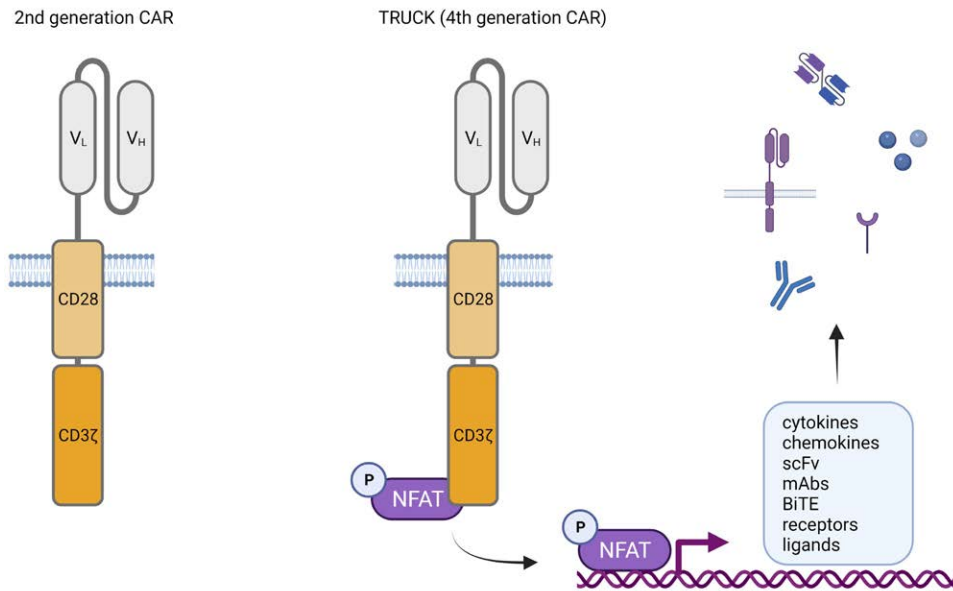
FIGURE 3. The second and fourth generation of CARs for use in redirecting Treg. CARs with the intracellular moiety of CD3ζ for primary signaling and additionally a costimulatory signaling domain represent a CAR of the second generation. A TRUCK ("T cells redirected for unrestricted cytokine initiated killing") represents the fourth generation of CARs that consist of a second-generation CAR that upon signaling induces the expression and release of a transgenic protein for therapeutic purposes; a variety of transgenic proteins can be envisaged in this context. BiTE, bispecific T cell engager; NFAT, nuclear factor of activated T cells; mAbs, monoclonal antibodies; scFv, single-chain fragment of variable region antibody.

therapy with CAR Treg in clinical practice.[80] A side-by-side comparison of different protocols needs to identify the most effective and robust procedure for manufacturing, engineering Treg with CAR-mediated specificity, transduced functional capacities, and prolonged persistence to improve their therapeutic efficacy. However, establishing true tolerance by redirected Treg transfer still needs to be shown in clinical trials; some trials are initiated or in advanced stages of planning. Early markers for tolerance or rejection will be helpful to plan and dose Treg therapy over time.[125] On the other hand, genetic engineering predefined specificity to human Treg also opens applications beyond transplantation medicine, such as in the therapy of deregulated immune responses and autoimmune diseases.

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