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Review

Chimeric antigen receptor T_{reg} therapy in transplantation

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In the quest for more precise and effective organ transplantation therapies, chimeric antigen receptor (CAR) regulatory T cell (T_{reg}) therapies represent a potential cutting-edge advance. This review comprehensively analyses CAR T_{regs} and how they may address important drawbacks of polyclonal T_{regs} and conventional immunosuppressants. We examine a growing body of preclinical findings of CAR T_{reg} therapy in transplantation, discuss CAR T_{reg} design specifics, and explore established and attractive new targets in transplantation. In addition, we explore present impediments where future studies will be necessary to determine the efficacy of CAR T_{regs} in reshaping alloimmune responses and transplant microenvironments to reduce reliance on chemical immunosuppressants. Overall, ongoing studies and trials are crucial for understanding the full scope of CAR T_{reg} therapy in transplantation.

The advent of T_{reg} therapies

Adaptive immunity in jawed vertebrates involves a complex interplay of lymphocytes [1,2], where effector $CD4^+$ and $CD8^+$ T cells (T_{effs}) and regulatory $CD4^+$ T cells (T_{regs}) act as key calibrators of the immunogenicity of non-self antigens and tolerance to self antigens (Figure 1) [3–6]. T_{regs}, which are phenotypically characterized as $CD4^+CD25^{high}CD127^{low}$ $FOXP3^+$ T cells in humans, exhibit context-dependent suppressive activity (Box 1) and account for ~2–8% of circulating human $CD4^+$ T cells [3]. Therapeutically, $CD4^+$ T_{regs} can help to mitigate autoimmune pathology, including type 1 diabetes (T1D), alloimmune graft-versus-host disease (GvHD), and transplant rejection [7]. This review arrives at a pivotal time in transplantation, and marks the transition from chemical immunosuppressive strategies to cell-based therapies, including T_{reg} and CAR T_{reg} therapies. These advances signal a paradigm shift towards precision medicine that promises enhanced control over transplant tolerance and rejection. In this review we critically examine the outcomes of CAR T_{regs} in preclinical studies and clinical trials, describe challenges in functional CAR T_{reg} optimization, and discuss present and future domains of CAR T_{reg} research that may transform the impact of CAR T_{regs} in transplantation.

T_{reg} immunotherapy

When Joseph E. Murray led the first human kidney transplantation in 1954 between identical twins [8], the average survival of human leukocyte antigen (HLA)-unmatched allografts was <3 months. Since then chemical immunosuppressants have decidedly improved short-term allograft survival, despite older donor–recipient pairs and greater population-wide comorbidities [9]. However, the long-term prognosis of organ transplants 10 years after transplantation in the USA remains suboptimal, and >50% of allografts are lost in the first 10 years [10]. Complications from the long-term use of chemical immunosuppressants contribute to these poor outcomes, causing off-target effects, including acute nephrotoxicity and cardiovascular disease [11]. The non-specificity of these immunosuppressants can cause generalized immunosuppression, diabetes, runaway opportunistic infections, and *de novo* malignancies [12]. Accordingly, in recent

Highlights

Immune homeostasis is a dynamic balance maintained by effector and regulatory lymphocytes – $CD4^+$ T_{regs} that act as pivotal downregulators of effector responses.

CAR T_{reg} therapy can provide HLA-independent targeting of transplant antigens with improved phenotypic stability, homing, and on-target effects than polyclonal T_{regs}, and promises to improve long-term graft survival and minimize chemical immunosuppression.

Structural CAR T_{reg} optimizations can maximize the therapeutic efficacy of CAR T_{regs} in models of transplantation and graft-versus-host disease, and, by extension, possibly in the clinic.

Future studies must address key aspects of CAR T_{reg} therapy, including long-term persistence, pathological conversion, safety concerns, side effects, outcomes in sensitized patients, interactions with existing immunosuppressants, and supply chain concerns.

Significance

CAR T_{reg} therapies, extending beyond polyclonal T_{regs}, promise to improve transplantation outcomes and reduce reliance on chemical immunosuppressants. These therapies embody targeted immunomodulation to foster graft tolerance while preserving host immunity. However, their full potential, encompassing efficacy, side effects, and limitations, awaits validation through rigorous research and clinical trials that could transform transplantation immunology and address challenges in achieving operational tolerance.

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years there has been a shift towards exploring 'living' drugs such as T_{reg} therapies (Box 2), aiming to offer a more natural form of immune homeostasis [4]. To date, Phase 1 clinical trials in patients with GvHD and living-donor kidney transplantation have demonstrated the safety and efficacy of *ex vivo* expanded polyclonal T_{reg} transfer, with reduced steroid need, lower infection risks, and comparable rejection rates to standard care ([13–17]; extensively reviewed in [7]). Currently, the Phase 2b Transplantation Without Overimmunosuppression (TWO) study (ISRCTN11038572)[i] is recruiting patients to test the outcomes of polyclonal T_{regs} in living-donor kidney transplant recipients compared to standard care, focusing on the incidence of acute rejection over 18 months [18].

Despite promising results, limitations in T_{reg} therapy, such as declining T_{reg} numbers post-transfer and functional challenges, necessitate innovative approaches. In a Phase 1 trial in recent-onset T1D patients [19], pharmacokinetic studies using deuterium labeling revealed a two-phase decay of circulating T_{regs}, where half of the T_{regs} rapidly disappeared within 20 days, followed by a slower phase of decay in which 25% of the transferred T_{regs} were still present after 90 days, and the remaining T_{regs} stabilized for at least 1 year post-infusion. Notable functional limitations of human T_{regs} include loss of lineage-defining FOXP3 expression and the development of a proapoptotic phenotype on repeated antigenic stimulation via granzyme pathways [20]. T_{regs} can also convert into interleukin (IL)-17-producing effector cells when exposed to proinflammatory cytokines such as IL-1 and IL-6 [21]. Thus, key challenges in developing more effective T_{reg} therapies include increasing the survival, numbers, purity, antigen specificity, and functional stability of T_{regs}, particularly after *in vivo* transfer [22].

To address some of these limitations, researchers have developed antigen-specific T_{regs}, including CAR T_{regs}, to recalibrate T_{regs} to prespecified antigens [23]. Some noteworthy therapeutic platforms beyond CARs include recombinant T cell receptors (rTCRs) [24], TCR fusion constructs (TRuCs) [25], synthetic TCR and antigen receptors (STARs) [26], HLA-independent TCRs (HIT receptors) [27], and peptide-centric CARs (PC-CARs) [28]. These major histocompatibility complex (MHC)/TCR-dependent strategies are more sensitive to lower antigen concentrations than CARs [27], which is potentially interesting for targeting uninflamed graft tissue. However, challenges such as mismatch hybridization of exogenous and endogenous TCR chains often limit the application of these strategies [29]. CARs, acting independently from MHC molecules, can target a broader range of moieties to reshape undesired immune responses and offer reduced risks of damaging normal tissues that express low amounts of antigen [27]. Moreover, CARs can enhance T_{reg} homing to local inflammation sites, which was first demonstrated in a

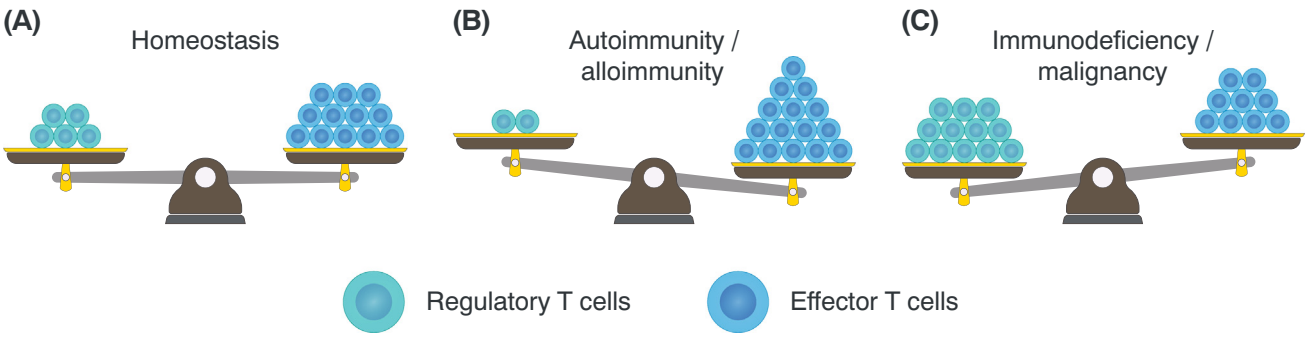

Figure 1. Regulatory and effector T cell ratios determine immune homeostasis. (A) In human peripheral blood, regulatory-to-effector $CD4^+$ T cell ratios typically range from ~1:10 to 1:50 and are crucial for maintaining a state of immune equilibrium [3]. (B) An imbalance favoring effector cells can trigger autoimmune disorders, disrupt self-tolerance, or exacerbate alloimmunity, particularly in transplantation contexts [4,5]. (C) A shift towards regulatory T cells, however, can lead to weakened immunosurveillance, potentially resulting in immunodeficiency, chronic inflammation, or oncogenesis [6].

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Box 1. Modes of T_{reg} suppression

T_{regs} are notable direct and indirect modulators of immunity at sites ranging from secondary lymphoid tissues to the peripheral circulation and inflamed tissues [97]. Mechanistically, T_{regs} can consume IL-2 as a 'cytokine sink' to suppress the growth and expansion of nearby effector cells [97,98]. T_{regs} can also diminish the costimulatory capacity of antigen-presenting cells by engaging the cytotoxic T lymphocyte-associated protein (CTLA)-4/B7 axis to negatively regulate effector T cell (T_{eff}) activation [99]. Alternatively, T_{regs} can attenuate immune responses via the secretion of immunosuppressive cytokines such as IL-10, IL-35, and transforming growth factor β (TGF-β) [97] and induce targeted apoptosis in T_{effs} via cytolytic proteases, including granzymes secreted together with perforins [100]. In addition, the CD39 and CD73 ectonucleotidase receptors at the T_{reg} surface can hydrolyze ATP to generate pericellular adenosine, thereby downregulating T_{effs} and their proliferation [101]. Recently, reduction–oxidation (redox) remodeling of T_{effs} by T_{regs} was described in mice as an extra T_{reg} suppressive mechanism that can impair T_{eff} activation and function by disrupting T cell glutathione metabolism. This process involves T_{reg}-mediated interference with T cell extracellular redox remodeling upon activation by dendritic cells, leading to downregulation of proinflammatory immune responses [102]. Finally, the shedding of T_{reg}-derived exosomes (vesicles <100 nm in diameter) containing inhibitory miRNAs has been demonstrated to be a previously unknown immunosuppressive mechanism [103]. These exosomes can suppress adaptive type 1 T helper cell (T_H1) responses by transferring specific miRNAs to T_H1 cells, thereby inhibiting their proliferation and cytokine secretion, and highlighting a cell contact-independent T_{reg}-mediated suppressive mechanism that can control systemic inflammatory responses [103].

colitis model using carcinoembryonic antigen (CEA) transgenic mice [30]. Using luciferase-expressing irrelevant CAR T_{regs} and CEA-CAR T_{regs}, CEA-CAR T_{regs} selectively trafficked to the colons of diseased mice, unlike irrelevant CAR T_{regs}, and reduced colitis severity [30].

Overall, the success of CAR therapy is evidenced by the excellent outcomes of effector CAR T therapies in patients with blood-borne cancers and many US FDA approvals of CAR T therapies for such pathologies [31,32]. In recent years, CAR T_{regs} have also shown improved treatment outcomes, with enhanced suppressive qualities over polyclonal T_{regs} in preclinical murine and humanized mouse models, including amyloidosis in mice using β-amyloid-specific murine T_{regs} (as a model of Alzheimer's disease) [33], inflammatory bowel disease in humanized NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice using flagellin-specific human T_{regs} [34], vitiligo in mice using ganglioside (G)D3-specific murine T_{regs} [35], experimental autoimmune encephalomyelitis (EAE) in mice using myelin-specific human T_{regs} (as a model of multiple sclerosis) [36], and various models of T1D in mice using various pathological MHC- and peptide–MHC-specific murine or human T_{regs} [37–39]. In addition, in murine and humanized GvHD models, CAR T_{regs} suppressed pathological antibody-producing B cells similarly to effector CAR T cells without giving rise to a cytokine storm [40–42]. Specifically, in a xenogeneic model of *HLA-A*02:01*$^+$ (HLA-A2$^+$) GvHD in HLA-A2$^-$ NSG mice, human HLA-A2-CAR T_{regs} effectively prevented GvHD more than polyclonal T_{regs} [40], and CD19-CAR T_{regs} in a xenogeneic GvHD model using NSG mice suppressed B cell differentiation and antibody production without signs of GvHD [41]. A study using murine

Box 2. Array of T_{reg}-centric immunotherapies

Adoptive transfer of polyclonal and CAR T_{regs} are two extensively studied forms of T_{reg} immunotherapy. Nevertheless, other domains of T_{reg} immunotherapy are under active study to facilitate the dominant suppression of effector lymphocyte responses and the induction of self- and allotolerance in autoimmune and transplantation settings. Briefly, these include the conversion of non-T_{reg} cells into lineage-stable induced FOXP3$^+$ T_{regs} (both *in vitro* and *in vivo*) [104,105] and the expansion of T_{regs} *in vivo* using survival factors needed for T_{reg} survival such as IL-2 [106]. Bioengineering efforts have also described modified IL-2 proteins (muteins) that have near-exclusive specificity for IL-2 receptors expressed on T_{regs} versus those on proinflammatory leukocytes [107]. In addition, T_{regs} can be 'backpacked' with IL-2 payloads for local IL-2 delivery and increased T_{reg} functionality at sites of inflammation and antigen encounter [108]. A variation of the backpacking approach involves the transgenic expression of membrane-anchored IL-2, and this can stabilize CAR T_{regs} under inflammatory conditions in NSG mice treated with xenogeneic human peripheral blood mononuclear cells [109]. Notably, these approaches are only a selection of current T_{reg}-centric immunotherapies. Looking to the future, cross-field ideas from other domains of immunology may offer new perspectives on T_{reg} immunotherapy, such as *in vivo* transduction of CAR T_{regs} to circumvent the limitations of *ex vivo* CAR T_{reg} manufacturing [110] or the generation of fourth-generation CAR T_{regs} coexpressing IL-2 and/or IL-10 to support T_{regs} at sites of antigen encounter. Naturally, it is also conceivable that these and other immunoengineering strategies might be combined to create superpowered T_{reg} immunotherapies.

CAR T_{regs} specific for anti-human (h)CD19 demonstrated less acute GvHD lethality and intact graft-versus-tumor activity in hCD19 transgenic mice without eliciting GvHD compared to controls [42]. In a study using CD19-CAR T cells for systemic lupus erythematosus, effector CAR T cells also effectively depleted pathogenic B cells and induced drug-free remission with minimal cytokine release syndrome [43]; however, CAR T_{regs} may have an advantage because they potentially pose fewer cell therapy complications and risks of GvHD than CAR T cells.

CAR T_{reg} pipeline

Developing CAR T_{regs} involves three key steps. First, T_{regs} are harvested from the peripheral blood of a transplant recipient and are transduced with a CAR-expressing gene (Box 3). Next, the CAR T_{regs} are expanded *in vitro* to generate sufficient numbers for effective therapy [44]. Hypothetically, many CAR T_{regs} may be lost quickly after transfer if, similarly to polyclonal T_{regs}, half of these cells are lost within 3 weeks of transfer [19]. Unlike polyclonal T_{regs}, however, a larger proportion of CAR T_{regs} may persist due to their universal antigen specificity. Finally, the expanded CAR T_{reg} product may be used fresh for immediate infusion or can be cryopreserved for delayed therapy.

The manufacture of CAR T_{regs} takes several weeks and includes numerous quality control tests for safety, efficacy, and reproducibility. In this context, the DNA methylation status of the T_{reg}-specific demethylated region (TSDR) [45,46] can serve as a measure of T_{reg} stability and suppressiveness.

Box 3. Methods of CAR T_{reg} manufacture

There are several methods for expressing non-natural protein sequences in mammalian cells. Unguided viral gene transfer is one popular method that often involve retroviral, lentiviral, or adeno-associated viral (AAV) vectors. Retroviruses and lentiviruses, both belonging to the Retroviridae family, are commonly used as viral vectors in cell engineering. Although classical retroviruses primarily replicate their RNA via reverse transcription in the genome of dividing cells, lentiviruses, a subclass of retroviruses, have the unique ability to additionally infect quiescent cells, thus broadening the spectrum of targetable cells for therapeutic purposes [111]. It is important to note that, in cell therapy applications, self-inactivating (SIN) vectors are employed. These specialized vectors are designed to express the therapeutic gene without the capacity to replicate or infect new cells, thereby enhancing safety [112]. A known caveat of unguided gene transfer in hematopoietic stem cells, however, is the possible integration of viral DNA near a proto-oncogene. This can prompt unchecked clonal T cell proliferation and leukemia [113], although this phenomenon has not (yet) been observed in primary T cells.

In transplantation, both retroviruses and lentiviruses have been used to generate CAR T_{regs} [114,115]. Human HLA-A2-specific CAR T_{regs}, for instance, have been generated using lentiviruses with stable expression of key T_{reg} markers, including high CD25 and FOXP3 and low CD127 [40]. These CAR T_{regs} proved to be efficacious *in vitro*, and suppressed HLA-$A2^+$ peripheral blood mononuclear cells, and *in vivo* outperformed control T_{regs} in a xenogeneic GvHD mouse model by enhancing mouse survival and delaying GvHD – underlining their suppressive abilities and potential as a therapeutic tool [40]. Preliminary comparative analyses in our center have demonstrated that, although murine cells showed improved transduction rates with retroviral CAR vectors {such as the murine stem cell virus (MSCV) pMIG backbone [116]}, human cells responded better to lentiviral CAR vectors (such as the pWPXL backbone: http://n2t.net/addgene:12257), as evidenced by flow cytometric analysis of surface-expressed tags (c-MYC and FLAG; unpublished data), although this remains to be validated.

AAVs, derived from the Parvoviridae family, are small, non-enveloped vectors that are capable of infecting both dividing and non-dividing cells. Unlike their wild-type counterparts, these vectors do not integrate their genetic material into the host genome [111]. Acting as episomal DNAs, AAV vectors minimize the risk of insertional mutagenesis and exhibit low immunogenicity, thus enabling repeated administration [92]. However, because of several limitations, including small payload capacity and episomal dilution during expansion, AAVs have not been the first choice for CAR T_{reg} engineering, and there are presently no AAV-based CAR T_{regs}.

Recently, non-viral techniques have become available, and CRISPR-Cas is the most extensively studied. This approach allows guided insertion of CAR constructs to avoid insertional mutagenesis and permits the ablation of endogenous TCR sequences [117]. AAVs have been used in this context to deliver homology-directed repair (HDR) templates for CRISPR-mediated knock-in [117]. Importantly, combining CRISPR-Cas with endogenous TCR gene ablation minimizes competition of CARs and TCRs for target antigen binding [118], resulting in CAR T cells with exclusive CAR target antigen specificity and enhanced survival over CAR T cells transduced via unguided viral vectors that are prone to tonic activation [119].

Although TSDR methylation status does not dictate FOXP3 amounts, it does stabilize T_{reg}-specific FOXP3 expression [47–49]. TSDR methylation can differentiate between transient activation-induced FOXP3 expression in T cells (methylated TSDRs) and lineage-stable T_{regs} (demethylated TSDRs) [45]. Moreover, current magnet and flow cytometry-based T_{reg} sorting relies on phenotypic surface markers that provide inhomogeneous T_{reg} products in which contaminating effector T cells can be transduced along with T_{regs} [7], potentially risking allograft health and treatment outcomes. Ongoing research seeks to refine cell culture and isolation techniques, such as incorporating mechanistic target of rapamycin (mTOR) inhibitors in culture that are known for their T_{reg}-supportive properties and role in stabilizing T_{reg} homeostasis [50,51].

Presently, concerted efforts are underway to standardize polyclonal T_{reg} and CAR T_{reg} manufacture at a clinical scale using fully closed systems such as CliniMACS® Plus and CliniMACS Prodigy® [52]. These approaches can yield up to 2 billion T_{regs} with >90% $CD4^+CD25^{high}CD127^{low}FOXP3^+$ expression in 2 weeks [52]. The automated process integrates anti-CD3/CD28 beads,IL-2, and rapamycin, concluding with bead removal and a ready-to-use product; this provides equal suppression of effector T cells as manually cultured T_{regs}, and promises upscaling of clinical CAR T_{reg} manufacturing.

Heterogeneity of CAR T_{reg} sources and subtypes

Natural human T_{regs} isolated from blood based on the $CD4^+CD25^{high}CD127^{low}$ phenotype encompass diverse T_{regs} with different origins and functions [53]. These include thymus-generated T_{regs} (tT_{regs}) that canonically regulate tolerance to self antigens, and peripheral blood T_{regs} (pT_{regs}) that specialize in controlling immune responses against foreign antigens [54,55]. Alternatively, TGF-β-rich *in vitro* cultures can generate induced T_{regs} (iT_{regs}) from conventional T cells (T_{cons}) [56]. Despite their diversity, no distinct phenotypic markers can presently differentiate these T_{reg} subsets, although TSDR methylation status can inform the expressional stability of FOXP3 [45,46]. Specifically, tT_{regs} have the highest level of TSDR demethylation, whereas pT_{regs}, iT_{regs}, and conventional T cells have more methylated TSDRs [57]. In addition, although tT_{regs} are epigenetically stable [58], they may still alter their phenotype upon *in vitro* and *in vivo* activation, and can differentiate into memory T_{regs} with T helper cell (T_H)-like phenotypes, such as T_H17-like T_{regs} [59]. Overall, the heterogeneity of T_{regs} highlights the challenges in identifying the best-suited subtype and source of T_{regs} for engineered T_{reg} therapies [60].

Beyond the use of natural T_{regs}, the transgenic expression of FOXP3 in T_{regs} or conventional T cells may also yield effective immunosuppressive agents [61]. For example, in a murine EAE model, myelin-targeted $CD4^+$ CAR T cells coexpressing FOXP3 reduced central nervous system inflammation and improved EAE outcomes compared to conventional $CD4^+$ T cells [62]. Similarly, in a GvHD mouse model, HLA-A2-CAR and FOXP3-transduced human $CD4^+$ T cells outperformed polyclonal T_{regs} by reducing liver and lung inflammation, and by limiting the expansion of grafted $CD3^+$ T cells [63]. Finally, in a humanized mouse model of GvHD, HLA-A2-CAR T_{regs} with transgenic FOXP3 expression maintained their phenotype and function, and achieved allograft-specific immunosuppression on a par with conventional CAR T_{regs} [64].

Design considerations for CAR T_{regs}

Although it is beyond the scope of this article to extensively review CAR design [65] (Figure 2A–F, Key figure; and Box 4), some aspects are worth discussing. In transplantation models, for instance, CAR T_{regs} with CD28 costimulation have proved to be superior to CAR T_{regs} with other costimulatory domains [66,67]. In xenogeneic human skin transplantation using NSG mice, only CD28-costimulated CAR T_{regs} effectively prevented graft rejection [66], while a study using human HLA-A2-CAR T_{regs} with 10 different costimulatory domains in a humanized GvHD

Key figure

Structure and function of chimeric antigen receptors (CARs) in regulatory T cells (T_{regs})

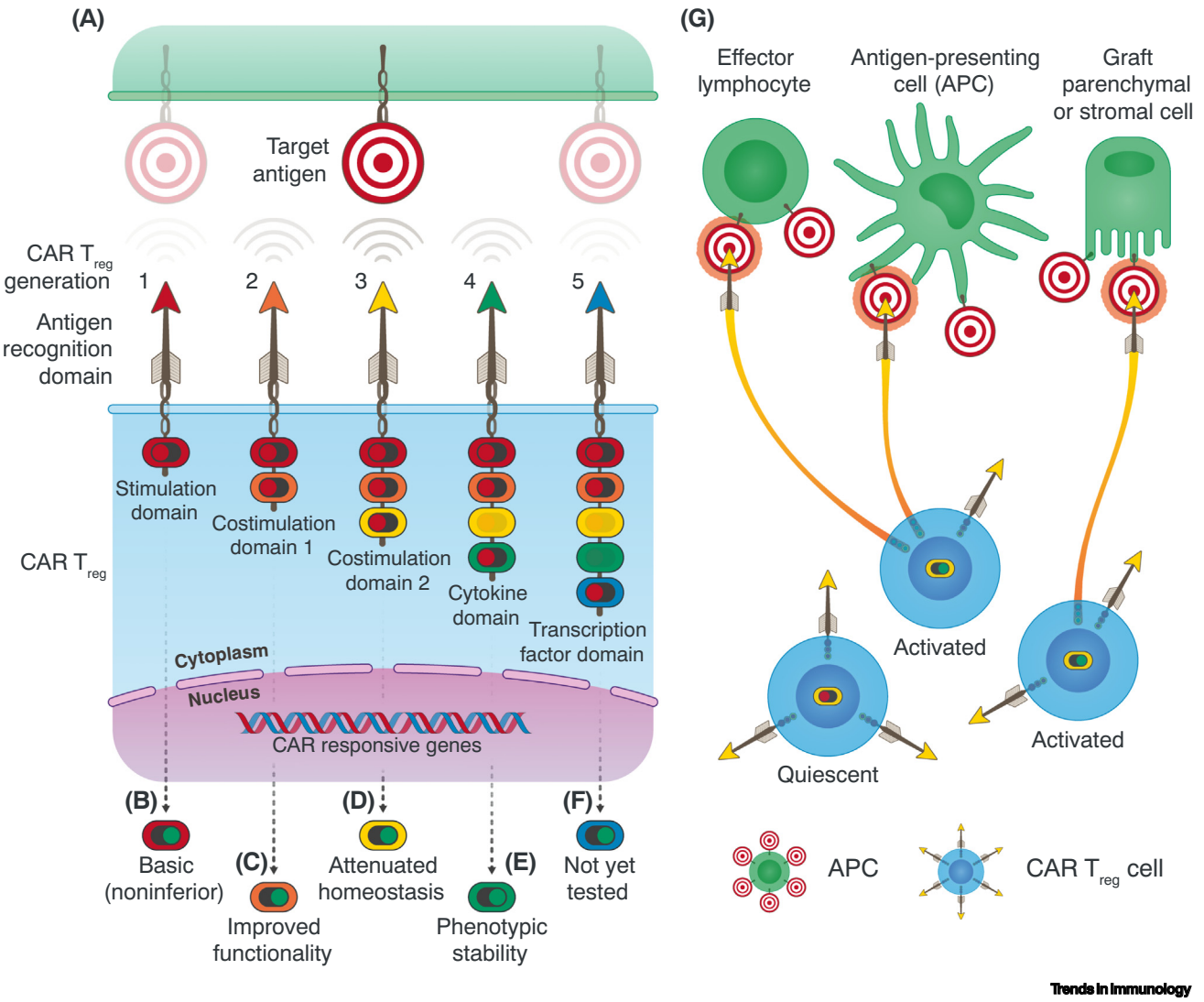

Figure 2. (A) CARs enable non-T cell receptor (TCR)-mediated interactions between T_{regs} and target cells through an antigen-recognition domain and target antigen. All CARs comprise three critical components: an extracellular antigen binder, a hinge connecting extra- and intracellular domains, and an intracellular signaling domain with phosphorylation-sensitive moieties. (B) First-generation CARs feature a single core stimulatory signaling domain, such as the CD3ζ ITAM-rich tail [136]. Although first-generation CAR T_{regs} were not tested in a clinical setting, in immunocompetent murine receiving HLA-A2-mismatched skin grafts, first-generation HLA-A2-CAR T_{regs} proved non-inferior to second-generation CAR T_{regs} with CD28 costimulation [68]. (C) Second-generation CARs include a costimulatory domain, such as CD28 or 4-1BB [137,138]. In CAR T_{regs}, CD28 costimulation, particularly in low-costimulation environments, enhances CAR T_{reg} functionality [66,67]. (D) Third-generation CARs incorporate dual costimulatory domains, but their hypothesized benefits for T cell homeostasis compared to second-generation CARs remain unfounded [140]. (E) Fourth-generation CARs integrate transgenic cytokine expression or transcription factors [62–64,69,141,142], potentially stabilizing the CAR T_{reg} phenotype and boosting T_{reg} potency in immunocompetitive environments. (F) Fifth-generation CARs contain intracellular cytokine receptor signaling domains, such as JAK–STAT kinases, for a comprehensive immune response [143], although such CAR T_{regs} remain to be explored. (G) *In vivo*, CAR T_{regs} can target various antigens [30,77], including cells bearing target antigens such as effector lymphocytes, dendritic cells, and allograft cells, to spatially restrict the immunosuppressive effects.

Box 4. CAR generation and structures

Each CAR domain has tailored functions. The primary goal of the target recognition domain is to direct CAR-expressing T_{regs} to the antigenic target (see Figure 2A in main text). Accordingly, various antigen-binders have been used, including scFvs, natural receptor ligands, and switchable, universal peptide-based or molecular binders [120,121]. Among these, scFvs are most commonly used. Notably, scFv-based CAR T_{regs} maintain the affinity and specificity of the original antibody, thus allowing MHC-independent recognition of a wide range of antigens with near-infinite target customizability.

The target recognition domain determines most of the CAR therapeutic efficacy by affecting its target and trafficking [30]. Engineering of this extracellular domain can include not only the derivation of downsized antibody-based binders, including nanobodies (from camelids, llamas, and shark Fv portions), diabodies, and bi-specific scFvs [122–124] but also non-antibody-based binders such as affimers, aptamers, and designed ankyrin repeat proteins (DARPins) [125–128].

The hinge (or spacer) domain bridges the extra- and intracellular CAR domains, and impacts on both the expression stability and functionality of the CAR [129]. Optimized hinge design has been shown to enhance effector CAR T cell persistence and antitumor activity, leading to improved disease outcomes [130]. Adjusting the length and sequence of this domain directly affects CAR binding to its target cell, and thus impacts host cell activation and therapeutic outcomes [131,132]. Sequence alterations in the spacer using flexible versus rigid amino acids [133] can determine the orientation and flexibility of the CAR. Furthermore, the sequence origin of the CAR hinge domain, typically CD8 or CD28, can influence CAR di- and multimerization with native CD8 and CD28 receptors, thus affecting CAR surface expression and, by proxy, CAR T cell activation and effector functions [134]. Unsurprisingly, optimal CAR T_{reg} design requires hinge domain optimization to favor immunological synapse formation and enhance downstream CAR T_{reg} signal transduction.

The intracellular signaling domains of a CAR must then relay essential T cell signals to ensure robust activation, including antigenic activation (signal 1), costimulation (signal 2), and cytokine signaling (signal 3) (Figure 2B–F) [135]. First-generation CARs consist only of an scFv fused to a T cell-activating domain [136] (Figure 2B). Notably, these constructs were never clinically tested in T_{regs} [68]. The addition of a costimulatory domain marks second-generation CARs (Figure 2C), and has improved homeostasis and reduced T cell 'exhaustion' in effector CAR T cells [137,138]. Interestingly, recent high-throughput screening of 700 000 CARs with novel intracellular domain combinations in NSG mice inoculated with NALM6 leukemia cells revealed new CD40-CD3ε-DAP12 CAR T cells with improved antitumor outcomes compared to 4-1BB-CD3ζ CAR T cells [139]. This approach in CAR T_{regs} might help to identify enhanced signaling domain combinations for transplant alloimmunity. Next, two separate costimulatory domains mark third-generation CARs (Figure 2D), although the advantages of third-generation CARs over second-generation CARs are dubious [140]. In immunodeficient *Rag*$^{-/-}$ mice grafted with CEA^{+} tumors, second-generation CEA-specific CAR^{+} cells with a CD28 costimulatory domain delayed tumor progression more effectively than those with CD28 and OX40 costimulatory domains [140], and there was greater activation-induced cell death in third-generation CARs. Fourth-generation CARs, such as T cells redirected for antigen-unrestricted cytokine-initiated killing (TRUCKs), integrate pro-homeostatic cytokine expression or inducible transcription factors as an add-on to second or third-generation CARs [141,142] (Figure 2E). Finally, fifth-generation CARs incorporate intracellular cytokine receptor domains, such as the intracellular tails of the IL-2 receptor that can engage the JAK–STAT pathway [143] (Figure 2F).

model in NSG hosts confirmed that CAR T_{regs} with wild-type CD28 costimulation were the most immunosuppressive [67]. However, a recent study describing HLA-A2-mismatched skin transplantation in immunocompetent mice showed that CAR T_{regs} with different costimulatory domains, including ICOS, PD-1, or GITR, offered similar protection against skin rejection as CD28 [68]. Further comparisons between first-generation CAR T_{regs} (lacking a costimulatory domain) and CD28-encoding second-generation CAR T_{regs} showed similar rates of skin rejection and suppression of anti-HLA-A2 antibodies, suggesting that costimulation through endogenous antigen-presenting cells *in vivo* nullified CAR-mediated costimulation [68]. Although the current literature emphasizes the advantages of CD28-encoding CAR T_{regs}, especially in environments with limited costimulatory signaling, the results highlight the growing interest in expanding CAR designs.

The advent of fourth-generation cytokine-expressing CAR T_{regs}, such as those expressing IL-10, offers promising avenues for achieving a more stable immunosuppressive phenotype and enhanced functionality. Recent evidence suggests that human IL-10-expressing HLA-A2-CAR T_{regs} not only maintain a stable phenotype post-transduction but also demonstrate enhanced *in vitro* suppression of effector T cells compared to polyclonal T_{regs}, especially when activated by HLA-A2^{+} B-lymphoblastoid cells [69]. The *in vivo* effects of these IL-10-coexpressing CAR T_{regs}, however, remain to be explored [69].

Concurrently, the challenge of ensuring the *in vivo* persistence of CAR T_{regs} requires that immunogenicity concerns are addressed, especially those related to foreign CAR sequences. In clinical trials, CARs with mouse-derived single-chain variable fragments (scFvs) triggered immune responses that led to their premature elimination, shifting the focus to human(ized) CAR sequences and simpler structure target-binding domains to improve CAR T outcomes [70]. A Phase 1 clinical trial in B cell non-Hodgkin lymphoma patients using a fully human CD19-CAR showed reduced CAR-specific immune responses and lower toxicity than mouse-derived scFv CARs [71], highlighting the likely benefits of humanizing CAR T_{reg} designs for improved persistence and efficacy.

Transplant antigens for CAR T_{reg} targeting

Clinical applications of CAR T_{regs} are diverse, although GvHD and the prevention of allograft rejection are two evident uses. In alloimmunity, donor tissues and organs feature distinct HLA molecules, making them ideal therapy targets [72]. Among the various HLA classes and subtypes, *HLA-A*02:01* (HLA-A2) is highly prevalent across North America, Europe, North Africa, and Western Asia, and is expressed in ~20% of North Americans (Table 1) [73]. Consequently, HLA-A2-CAR T_{regs} have been developed, and these demonstrated attenuated effector T cell proliferation and reduced histological signs of graft rejection compared to polyclonal T_{regs} in preclinical models of GvHD and skin allotransplantation in humanized NSG mice [74,75]. Moreover, in a murine heterotopic heart transplantation model using species-hybrid C57BL/6 mice expressing human HLA-A2 (B6.A2) and wild-type B6 mice, HLA-A2-CAR T_{reg} therapy extended allograft survival from 35 to 99 days compared to polyclonal T_{regs} [76], indicating that HLA-A2-CAR therapy can have allograft prolonging outcomes. However, using B6.A2×BALB/c F1 hybrids as donors, HLA-A2-CAR T_{regs} only modestly increased median graft survival from 11 to 14 days compared to no therapy, which could be extended beyond 100 days in combination with a 9 day course of rapamycin [76]. These findings collectively underscore the potential of HLA-targeted CAR T_{reg} therapy in transplantation and the potential merits of synergizing CAR T_{regs} with existing immunosuppressants. The encouraging outcomes of these preclinical findings have paved the way for the multicenter Phase 1/2 'STeadfast' trial (NCT04817774)[ii] that is recruiting living-donor kidney transplant recipients of HLA-$A2^+$ grafts, and will compare allograft rejection in transplant recipients treated with HLA-A2-CAR T_{regs} (TX200-TR101 [77]) to those receiving standard care.

Table 1. Most common HLA alleles across the globe at the *HLA-A*, *-B*, *-C*, and *-DRB1* loci

Region	Most common allele per HLA locus, median frequency (% as whole integer)							
	HLA-A		*HLA-B*		*HLA-C*		*HLA-DRB1*	
North America	*02:01	22%	*35:01	7%	*04:01	15%	*07:01	9%
South and Central America	*02:12	31%	*35:43	14%	*07:02	14%	*14:02	10%
Europe	*02:01	26%	*07:02	8%	*07:01	14%	*07:01	13%
North Africa	*02:01	13%	*50:01	10%	*06:02	21%	*07:01	17%
Sub-Saharan Africa	*23:01	12%	*07:02	6%	*06:02	15%	*15:03	12%
Western Asia	*02:01	15%	*35:08	7%	*04:01	18%	*03:04	27%
North-East Asia	*24:02	23%	*51:01	8%	*01:02	17%	*09:01	11%
South-East Asia	*11:01	21%	*40:01	10%	*07:02	15%	*09:01	14%
South Asia	*11:01	13%	*40:06	12%	*06:02	12%	*07:01	18%
Australia	*34:01	38%	*13:01	24%	*04:01	25%	*14:01	13%
Oceania	*24:02	31%	*15:02	12%	*01:02	21%	*12:02	19%

Although the STeadfast trial focuses on HLA-A2-CAR T_{regs}, there is a growing need to develop CAR T_{regs} targeting other HLA variants, reflecting the diverse HLA landscape across different populations. For instance, targeting *HLA-A*24:02* in North-East Asia and *HLA-C*06:02* in Africa might markedly diversify the eligible patient population of HLA-CAR T_{regs} (Table 1), and creating CARs for other common HLA-A subtypes such as *HLA-A*24:02*, *HLA-A*03:01*, *HLA-A*01:01*, and *HLA*11:01*, particularly in North America, might further expand the reach of the therapy (Table 2).

Overall, because the application of HLA-CAR T_{regs} may increase, HLA typing is set to play a pivotal role in expanding the applicability of these therapies. Presently, HLA-CAR T_{regs} are mostly focused on living donor–recipient pairs owing to the constraints of HLA-typing of deceased-donor grafts. However, HLA-typing advances such as Nanopore sequencing promise to revolutionize speed and accuracy [78–80] such that graft typing at presentation may soon become available. Alternatively, HLA genotyping may become an integrated part of health records or be included in 'emergency' citizen identification with other key medical details, although these remain prospective developments rather than a current reality.

Beyond targeting major histocompatibility complexes [74–77,81], minor histocompatibility antigens, such as Y-chromosome-specific H-Y antigens, offer another promising avenue for CAR T_{reg} therapy. A study involving 118 kidney transplant recipients revealed that antibodies against H-Y antigens developed most frequently in gender-mismatched male-to-female kidney transplant pairs and correlated strongly with acute rejection [82]. In addition, in female mice receiving a male bone-marrow transplant, intranasal H-Y peptide infusion could induce tolerance to male grafts [83], and, in female TCR transgenic mice specific for H-Y peptides [84], daily H-Y infusions elicited the conversion of naïve T cells to $FOXP3^+$ T_{regs} that tolerized female hosts to male skin grafts [85]. These findings suggest that H-Y-specific CAR T_{regs} might be a suitable therapy in gender-mismatched male-to-female transplantation.

Overall, in CAR T_{reg} targeting (Figure 2G), host T_{regs} must be directed towards both ubiquitously and exclusively allograft-expressed antigens to promote tolerogenic graft-specific responses with minimal off-target effects. Mechanistic studies are underway to identify novel candidate target antigens [86] to enhance the versatility of CAR T_{regs} in transplantation. This might not only improve the therapeutic window of CAR T_{reg} therapy but also extend its eligibility criteria.

Future perspectives of CAR T_{reg} therapy

Antigen-specific immunoregulatory therapies may offer targeted immunosuppression with fewer side effects than polyclonal alternatives. However, although CAR T_{regs} have evident clinical potential, several challenges stand in the way of their translation.

For instance, CAR T_{reg} therapy requires the harvesting of (large numbers of) host immune cells. The manufacture of universal, allogeneic CAR T_{regs}, instead, might allow large-scale production, streamlined pre-transfer evaluation, and cryopreservation of 'off-the-shelf' CAR T_{regs} – either third-party or pluripotent stem cell-derived [87]. In addition, future trials must demonstrate whether CAR T_{regs} maintain their lineage stability *in vivo*, especially within inflammatory

Table 2. Most common HLA alleles in North America at the *HLA-A* locus

Region	*HLA-A* allele frequency (% as whole integer)				
	*02:01	*24:02	*03:01	*01:01	*11:01
North America	22%	13%	7%	6%	5%

environments that might affect T_{reg} plasticity and drive their pathological conversion to proinflammatory cells [21,88]. Finally, although CAR T therapies can cause severe toxicities, including cytokine storm [89], the full spectrum of side effects associated with CAR T_{reg} therapy remains to be established. Because T_{regs} do not produce many, if any, proinflammatory cytokines, CAR T_{regs} are not expected to cause cytokine storm or neurotoxicity. Of note, a recent study found that post-infusion CAR T_{regs} were a key marker of resistance to effector CD19-CAR T therapy in patients with large B cell lymphoma [90], and the rate of neurotoxicity correlated inversely with CAR T_{reg} prevalence. Importantly, these findings allay neurotoxicity risks but also hint at the immunosuppressive potency of CAR T_{regs}.

Expected side effects of CAR T_{regs} are likely to mirror those of chemical immunosuppressants, including generalized immunosuppression and an increased risk of immunodeficiency, opportunistic infections, and impaired antitumor immunity (Figure 3A–C) [12,91]. Of note, in NSG mice receiving human skin transplants and HLA-A2-CAR T_{regs}, no cytotoxicity towards HLA-$A2^+$ epithelial cells was reported, implying that CAR T_{regs} may spare bystander tissues following target engagement [75] – a phenomenon worth substantiating further in clinical studies. In addition, we posit that insertional mutagenesis after viral transduction of CAR T_{regs} (that is possible with any unguided gene

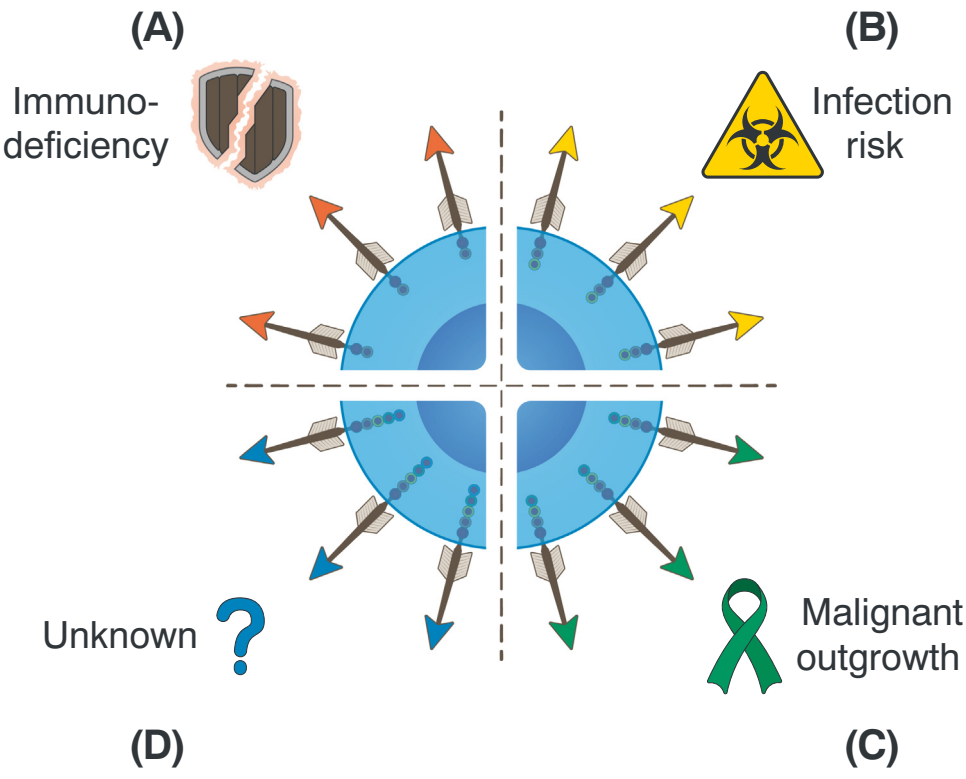

Figure 3. Potential side effects and toxicities of chimeric antigen receptor (CAR) regulatory T cell (T_{reg}) therapy. The only CAR T_{reg} trial in transplantation, the 'STeadfast' trial (NCT04817774)[ii], is currently ongoing in living-donor kidney transplant recipients. Evidence of CAR T_{reg}-mediated toxicities is therefore limited, but it is hypothesized the possible side effects will be similar to those of chemical immunosuppressants and polyclonal T_{regs}. (A–D) Potential toxicities include (A) generalized immunosuppression leading to immunodeficiency, (B) heightened risk of opportunistic infections, and (C) compromised antitumor immunity, potentially accelerating tumor progression [12,91]. (D) Uncharted side effects could include insertional mutagenesis [92] with subsequent complications and unintended transformation of T_{regs} into proinflammatory cells, undermining therapeutic goals [21,88].

transfer [92]) and the potential pathological conversion of CAR T_{regs} to proinflammatory cells warrant further study (Figure 3D) [21,88]. Importantly, the STeadfast trial (NCT04817774)[ii] in kidney transplantation should provide essential insights into the clinical safety profile of (HLA-A2-)CAR T_{regs}.

Future research should also focus on whether CAR T_{regs} can suppress alloimmunity in both naïve and sensitized recipients because HLA-A2-CAR T_{regs} recently showed no benefits in HLA-A2-sensitized recipients [93]. In a murine HLA-A2-mismatched skin transplant model, HLA-A2-specific CAR T_{regs} significantly delayed skin graft rejection and diminished donor-specific antibodies (DSAs) in naïve mice compared to irrelevant CAR T_{regs}. In HLA-A2-sensitized mice with preformed memory alloreactivity, however, neither HLA-A2-CAR T_{regs} nor irrelevant CAR T_{regs} could delay rejection or attenuate DSA formation [93]. If CAR T_{regs} prove to be effective only in unsensitized recipients, their application would be restricted to ~60–70% of the kidney transplant recipient pool – specifically those with <1% calculated panel-reactive antibodies [94]. This limitation poses implications for all sensitized recipients, including patients with previous transplants, blood transfusions, or pregnancies.

Finally, an intriguing area of future exploration is the combination of CAR T_{regs} with chemical immunosuppressants. Although calcineurin inhibitors, belatacept, and basiliximab agnostically suppress T_{cons} and T_{regs} [95], mTOR inhibitors such as rapamycin and sirolimus spare T_{regs} over T_{cons} and support T_{reg} expansion [50,96]. The latter might permit rapamycin to act synergistically with CAR T_{reg} therapy to extend allograft outcomes [76]. Aligning CAR T_{reg} therapy with selective immunosuppressants that support T_{reg} homeostasis might help to reduce long-term systemic immunosuppression and improve transplantation outcomes.

Overall, future CAR T_{reg} studies must answer diverse questions, including the optimal number of CAR T_{regs} for clinically relevant immunosuppression, the merits of repeated CAR T_{reg} transfer, the ideal treatment time, the clinical potential of multivalent CAR T_{regs}, the efficacy of CAR T_{regs} in sensitized recipients, and the optimal combination of CAR T_{reg} therapy and chemical immunosuppressants.

Concluding remarks

Recent advances in T_{reg} therapies, particularly antigen-specific CAR T_{regs}, demonstrate that they have a clear potential for managing autoimmune and transplant pathology. Notably, CAR T_{regs} can offer improved phenotypic stability, homing, and therapeutic outcomes over polyclonal T_{regs} [77]. Although preclinical results in transplantation models are promising, future research will be necessary to formalize the merits of CAR T_{regs} in the clinic (see Outstanding questions). Moreover, research investigations into enhanced targets of alloimmunity that can distinguish transplanted tissues and organs from host tissues will be invaluable in guiding future therapeutic interventions. The advent and availability of omics in research can expedite the search for novel transplant-related antigens and help to consolidate the promises of CAR T_{reg} and related immunosuppressive cell therapies. Eventually, we posit that the evolution and development of effective CAR T_{reg} therapies in transplantation may minimize or even obsolesce chemical immunosuppression and its associated side effects.

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Declaration of interests

The authors declare no conflicts of interests.

Resources

[i]https://www.isrctn.com/ISRCTN11038572

[ii]https://clinicaltrials.gov/study/NCT04817774

Outstanding questions

Although CAR T_{reg} therapies have shown promise in preclinical murine transplantation models, what are their safety and efficacy outcomes in the human setting?

How can the functional instability of (CAR) T_{regs} in humans be addressed and obviated, such as the loss of lineage-defining FOXP3 expression and the induction of T_{reg} apoptosis through granzyme pathways upon repetitive antigenic stimulation?

How can human CAR T_{regs} be protected from pathological conversion in proinflammatory environments? For instance, given that T_{regs} can convert into IL-17-producing effector cells upon IL-1 and IL-6 signaling, how can CAR T_{regs} be armored against this harmful transformation?

What are the possible side effects of human CAR T_{reg} therapy, and how can they be mitigated? Do CAR T_{regs}, for instance, precipitate generalized immunosuppression and increase the risk of acquired immunodeficiency, opportunistic infections, and *de novo* malignancies?

How can the manufacturing process of human CAR T_{regs} be streamlined and automated to allow large-scale production, including rigorous pre-transfer evaluation and perhaps even cryopreservation of 'off-the-shelf' CAR T_{regs}?

What antigens universally expressed at sites of human alloimmunity can CAR T_{regs} be targeted against to generate universal CAR T_{regs} with fewer restrictions and extended eligibility criteria?

Will fourth- or fifth-generation human CAR T_{regs} with transgenic coexpression of stabilizing cytokines and/or transcription factors improve the therapeutic outcomes of CAR T_{reg} therapy, including in sensitized transplant recipients? Alternatively, what add-on therapies to second-generation CAR T_{regs} will be necessary to overcome the underwhelming preclinical outcomes of CAR T_{regs} in sensitized recipients?

Which chemical immunosuppressant drug groups (e.g., mTOR inhibitors) and in which combinations and

dosages can most effectively synergize with human CAR T_{reg} therapy to improve transplantation outcomes and minimize immunosuppressant side effects?

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