Regulatory T cell therapy approaches

Oliver McCallion\textsuperscript{1}, Merve Bilici\textsuperscript{1}, Joanna Hester\textsuperscript{1}, Fadi Issa\textsuperscript{1§}.

\textsuperscript{1}Transplantation Research and Immunology Group, Nuffield Department of Surgical Sciences, University of Oxford.

\textsuperscript{§}Corresponding author. Postal Professor Fadi Issa, Transplantation Research and Immunology Group, Nuffield Department of Surgical Sciences, University of Oxford, John Radcliffe Hospital, Headley Way, Oxford, OX3 9DU. Email fadi.issa@nds.ox.ac.uk
ABSTRACT

Regulatory T cells (Tregs) have enormous therapeutic potential to treat a variety of immunopathologies characterised by aberrant immune activation. Adoptive transfer of ex vivo expanded autologous Tregs continues to progress through mid- to late-phase clinical trials in several disease spaces and has generated promising preliminary safety and efficacy signals to date. However, the practicalities of this strategy outside of the clinical trial setting remain challenging. Here, we review the current landscape of regulatory T cell therapy, considering emergent approaches and technologies presenting novel ways to engage Tregs, and reflect on the progress necessary to deliver their therapeutic potential to patients.

KEYWORDS

Regulatory T cell, adoptive cell therapy, gene editing, CAR-Treg, tolerance.
1. Introduction

Regulatory T cells (Tregs) play a crucial role in the maintenance of immune homeostasis and antigen tolerance. Abnormalities associated with their number and function have been implicated in the development of a wide range of diseases. Given their unique ability to powerfully control aberrant immune responses, there has been significant interest in targeting Tregs therapeutically for pathologies such as autoimmune diseases and transplant rejection. To date, Treg therapeutic approaches have predominantly comprised either traditional small-molecule or biologic administration directly to patients, or more innovatively through adoptive cellular therapy—so called ‘living drugs’—where live Tregs are infused directly into patients. Both approaches have generated exciting glimpses into potential efficacy and continue to progress through clinical evaluation. However, both approaches also have limitations that, as they stand, may limit wider adoption of the therapy. Here, we discuss current Treg cell therapy approaches, consider the barriers and limitations currently faced, and reflect on new therapeutic and technological approaches to enhance their specificity, stability, and applicability.

2. Treg biology

2.1 Classification

Tregs comprise a specific subpopulation of CD4+ T cells with immunosuppressive function, broadly characterized by their high expression of the IL-2 receptor α-chain (CD25), low expression of the IL-7 receptor (CD127), and expression of the Treg master transcription factor FOXP3. Tregs comprise 5-10% of circulating CD4+ lymphocytes and exhibit considerably heterogeneity. They may be classified as either thymus derived (tTregs, or naturally occurring nTregs) or peripherally-derived Tregs (pTregs) according to their site of origin. In the thymus the strength and duration of the T cell receptor (TCR) signal commit precursors to a regulatory lineage, whilst pTregs differentiate from naïve CD4+ T cells following TCR stimulation in the presence of TGF-β and IL-2 in the periphery. Human Tregs may also be classified as naïve (nTregs), effector memory (emTregs) or central memory (cmTregs) based on their differentiation status. Naïve Tregs have not encountered their cognate antigen and reside in secondary lymphoid organs (SLO); on antigen encounter they become activated and differentiate into cmTregs. Once activated, cmTregs differentiate into emTregs by migrating out of SLOs into the peripheral circulation or tissue. Naïve Tregs have limited immunosuppressive capacity and typically express CD45RA, whilst CD45RO is expressed by memory Treg subsets. Each subset also displays unique characteristics in terms of their metabolism and migratory capacity, which may potentially be leveraged therapeutically. For example, naïve Tregs are more dependent on IL-2 for survival compared...
with effector Tregs which rely on ICOS and IL-7 signalling\(^8\). The migratory marker L-selectin (CD62L) is expressed by cmTreg and is required for SLO retention. The chemokine receptor CCR7 is also important to distinguish Treg subsets, with naïve Tregs expressing a high level of CCR7 which is downregulated by effector and memory subsets whilst the adhesion receptor CD44 is expressed by memory and effector subsets and correlates with FOXP3 expression and suppressive ability\(^9\). As phenotype and function are intimately related, it is likely that therapies may in future capitalise on the variability in phenotype and differentiation status to, for example, localise highly suppressive subsets to specific anatomic niches.

2.2 Suppressive mechanisms
Tregs both directly and indirectly suppress CD4\(^+\) and CD8\(^+\) T cell, B cell, dendritic cell (DC), macrophage, and natural killer (NK) cell proliferation and function\(^10\). Contact dependent mechanisms include expression of granzymes and perforin\(^11\), which directly disrupt target effector cell membranes resulting in apoptosis, and through expression of inhibitory molecules, for example CTLA-4 and PD-1\(^{12,13}\). CTLA-4 and PD-1 interact with CD80/86 and PD-L1 receptors on antigen presenting cells (APCs) respectively, resulting in APC suppression and indirect inhibition of T conventional cells\(^14\). Contact independent mechanisms include secretion of immunomodulatory cytokines such as IL-10, TGFβ\(^13\) and IL-35\(^15\) that suppress conventional T cells and NK cells. In addition, Tregs modulate the extracellular environment to be resistant to effector activation. For example, Tregs express the ectoenzymes CD39 and CD73 which dephosphorylate extracellular ATP to adenosine resulting in effector T cell suppression\(^16\). In addition, Tregs express the trimeric IL-2 receptor comprising IL-2Rβ, IL-2Rγ, and the high-affinity IL-2Rα chain (CD25), in contrast to effectors which express a lower-affinity dimeric IL-2 receptor. As Tregs do not produce IL-2 they may act as an IL-2 sink depriving IL-2 from effector populations thereby inhibiting their function\(^17\).

3. Treg cell therapy: current approaches.
Strategies to enhance Treg number and function provide a more targeted alternative to current therapeutics which rely on generalised non-specific immunosuppression. Numerous preclinical models have demonstrated significant potential to treat multiple pathologies including autoimmune-mediated diseases\(^18-22\), graft versus host disease (GvHD)\(^23\), and to induce tolerance following solid organ transplantation\(^24-27\). At present, administration of \textit{ex vivo} expanded Tregs to patients is the most commonly adopted approach\(^28\).
3.1 Adoptive Treg transfer approaches

Adoptive Treg transfer, or more broadly adoptive cellular therapy (ACT), involves a bespoke infusion of Tregs that have been isolated, expanded, and sometimes genetically manipulated, ex vivo (Figure 1). There are several manufacturing protocols, all of which involve collecting starting material from the donor—commonly autologous peripheral blood or an autologous leukapheresis product—from which Tregs are isolated and expanded in culture over multiple rounds of stimulation. Our manufacturing process starts with peripheral blood, from which CD4⁺CD25⁺ lymphocytes are magnetically enriched from PBMCs, and then expanded by TCR stimulation in the presence of IL-2 and rapamycin; the resulting polyclonal product is cryopreserved until infusion. Alternatively, antigen specific Tregs may be generated by expansion in the presence of professional APCs, most commonly B cells or DCs. However, whilst adoptive transfer of antigen specific Tregs may be more potent, their precursor frequency is typically low. As such, expanding sufficient cells for therapeutic administration can be challenging particularly as intensive expansion may impair Treg suppressive capacity.

3.1.1 Treg ACT: clinical experience

Early studies into the safety and feasibility of Treg ACT were predominantly conducted in the setting of haematopoietic stem cell transplantation to prevent or treat GvHD, paving the way for trials in autoimmunity and transplantation tolerance to be initiated. In the setting of type 1 diabetes (T1DM), administration of autologous Treg ACT to children recently diagnosed with T1DM up to 30 x 10⁶/kg was well tolerated and resulted in clinical remission in two thirds of patients at 12 months. Similarly, treatment of 14 adult patients with recent onset T1DM with autologous Treg ACT up to 26 x 10⁶ per dose in a phase 1 clinical trial was also well tolerated. However, the recently completed phase IIa Sanford Project T-Rex Study (NCT02691247) was reported by the manufacturer Caladrius Biosciences to have missed its primary outcome of improving C-peptide levels at 12 months. Nevertheless, the complete trial outcome is awaited and conceivably may identify subgroups demonstrating an effect given the heterogeneity of the trial population. In the setting of solid organ transplantation, Treg ACT has been administered to both kidney transplant and liver transplant recipients with excellent effect. For example, administration of a Treg enriched product to 10 liver transplant recipients resulted in successful immunosuppression withdrawal in 70% with stable graft function between 5.2 and 6.8 years following cessation. More recently, the ONE study, a global consortium initiating harmonised phase I trials of several cellular therapies, demonstrated the safety of administering polyclonal (up to 10 x
$10^6$/kg) Tregs following living donor kidney transplantation, with promising preliminary efficacy signals$^{39,40}$. Following on from these early data, we have recently initiated the TWO study, a currently-recruiting phase 2b clinical trial to examine the ability of autologous Treg ACT to facilitate immunosuppression minimisation$^{41}$.

3.2 IL-2 therapy approaches

IL-2 was first identified as indispensable for effector T cell survival, proliferation, and function, before its importance in Treg homeostasis was recognised$^{42}$. However, the potential for IL-2 to stimulate both effector and regulatory T cells, in addition to its short half-life (under 15 minutes) necessitating high-dose administration and frequent, occasionally fatal, dose-limiting toxicities curbed early clinical adoption as a therapeutic to stimulate antitumour effector populations$^{43,44}$. However, preferential expansion of Tregs through exploiting IL-2 signalling differences, namely the high affinity ($K_d \sim 10^{-11}$ M) of the trimeric IL-2 receptor expressed by Tregs, is under active investigation through administering ultra-low dose IL-2, carefully optimised to promote Treg without effector expansion$^{45-47}$. This approach has been trialled in multiple disease spaces including type 1 diabetes$^{48}$, vasculitis$^{49}$, GvHD$^{50}$, and solid organ transplantation. Ultra-low dosing regimens appear to have a tolerable safety profile, in particular with respect to the incidence of venous thromboembolism which are especially problematic at higher doses$^{51}$. Nevertheless, the therapeutic window of this approach is very narrow, and the potential to inadvertently expand effector populations, and thereby worsen the immunopathology, remains significant. This is well illustrated by recent attempts to use low-dose IL-2 to facilitate immunosuppression withdrawal in liver transplant recipients, where subcutaneous injection of $1 \times 10^6$ IU of IL-2 daily resulted in 100% rejection associated with expanded CD25$^+$ T effector and NK cell populations$^{52}$. Even when combined with Treg ACT, recombinant low dose IL-2 to treat T1DM results in the expansion of NK, MAIT, and effector CD8$^+$ T cell expansion without improvement in metabolic function$^{53}$.

4. Barriers to routine autologous Treg therapy

Given the safety and early clinical efficacy signals outlined above, consideration of how potential therapeutic benefit may be delivered to larger patient populations outside of the clinical trial environment merits attention. However, scaling up autologous Treg therapy raises several biological, technical, and economic problems requiring innovative solutions. From a biological perspective, the pathology for which Treg ACT is indicated may be associated with intrinsic Treg dysfunction$^{54-57}$;
expanding and adoptively transferring dysfunctional Treg in these settings is likely to be of limited benefit. Similarly, not all patients are able to donate a sufficient volume of blood or undergo leukapheresis from which a product may be generated—particularly children and patients with significant co-morbidities. On the technical side, expansion of a sufficient number of Tregs requires several weeks in culture which precludes their acute administration, for example to recipients of deceased donor organs. Equally, the manufactured product may fail to meet the required number, function, purity, and sterility criteria necessary for release and clinical administration. Finally, bespoke cell therapies do not fit well within current small molecule and biologic manufacturing processes. Instead, ACTs require specific facilities and expertise to produce, which are less amenable to economies of scale, a fact that is reflected in significant per-product cost. It is not clear whether these facilities should be centralised or established within academic centres.

5. Treg cell therapy: next-generation approaches

Multiple strategies to mitigate current limitations are under active evaluation, leveraging the natural opportunity to apply genome editing and bioengineering technologies to enhance Treg ACT specificity, stability, potency, and source (Figure 2). Major advances in this domain have recently been achieved, paving the way for translation of these technologies into the clinic.

5.1.1 Improving Treg specificity with Chimeric Antigen Receptors (CAR)

CAR technology has produced several landmark highly-effective novel therapeutics for B-cell precursor acute lymphoblastic leukaemia and diffuse large B cell lymphoma; the same technology demonstrates significant promise to direct Treg cell therapies specifically and potently towards a known antigenic target. Broadly, CARs comprise an extracellular signalling domain, most commonly a single chain variable fragment (scFv), and an intracellular signalling domain, most commonly the CD3ζ activating domain of the T cell receptor that may be potentiated by a co-stimulatory molecule such as CD28. These are linked by an extracellular hinge region and transmembrane domain. Expression of the wild-type CD28 co-stimulatory domain appears to be particularly important for CAR-Treg generation given that expression of other co-stimulatory domains destabilises their suppressive function. On the manufacturing side, autologous CAR T cell therapies are produced by either lentiviral or gamma-retroviral transduction of activated autologous T cells.
with a CAR-encoding vector. Transduced cells are expanded and either used directly or cryopreserved before near-patient thawing and administration.\textsuperscript{65} CAR-Tregs present a route to alloantigen specific tolerance with therapeutic relevance towards numerous pathologies including type 1 diabetes\textsuperscript{66}, inflammatory bowel disease\textsuperscript{67}, multiple sclerosis\textsuperscript{68}, haemophilia\textsuperscript{69}, vitiligo\textsuperscript{70}, transplant rejection, and GvHD\textsuperscript{71,72}. In the transplantation space, our predominant strategy has been to exploit frequent donor-recipient human leucocyte antigen (HLA) mismatches by transducing Treg with a CAR specifically targeting donor HLA\textsuperscript{73}. In this way, CAR-Treg encounter their cognate antigen only within the allograft resulting in targeted local immunosuppression. This approach has mainly been validated with Tregs expressing CARs targeting HLA-A2, a commonly mismatched antigen between transplant donors and recipients, although only a small number of CARs would be required to cover most potential mismatches. In humanised skin allograft models of transplant rejection, HLA-A2\textsuperscript{+} CAR Tregs infiltrate the graft and prolong its survival in comparison to polyclonal Tregs\textsuperscript{74-76}. Equally, HLA-A2\textsuperscript{+} CAR Tregs prevent the development of GvHD following reconstitution with HLA-A2\textsuperscript{+} human PBMCs\textsuperscript{77,78}. However, the ability of CAR-Tregs to induce tolerance appears to be lost when animals are pre-sensitised to donor antigen, suggesting that their ability to control memory responses are limited\textsuperscript{76}, an important consideration for translational studies. Excitingly, autologous HLA-A2\textsuperscript{+} CAR-Tregs have recently progressed to phase I/IIa clinical trial (STeadfast, NCT04817774). The study team aim to recruit twenty-one unsensitised living donor kidney transplant recipients to receive autologous naïve Treg (CD4\textsuperscript{+}CD25\textsuperscript{−}CD127\textsuperscript{lo/-}CD45RA\textsuperscript{+}) transduced with a lentiviral vector encoding an anti-HLA-A*02 CAR in the post-transplant period.

In the pre-clinical setting, efforts to potentiate CAR-Treg stability and function through modulating the construct-encoding sequence also continue. One such approach is to include the sequence encoding IL-10, a key immunosuppressive cytokine, within the HLA-A2\textsuperscript{+} CAR vector, resulting in constitutive or induced IL-10 expression\textsuperscript{79}. HLA-A2\textsuperscript{+} CAR-Tregs constitutively expressing IL-10 have an enhanced ability to suppress effector T cell proliferation \textit{in vitro}, although \textit{in vivo} constitutive IL-10 expression is insufficient to maintain a suppressive phenotype\textsuperscript{80}. This points towards a significant and more general concern regarding CAR-Tregs, namely \textit{in vivo} stability given chronic antigen exposure—particularly considering the known propensity for Tregs to skew from a regulatory to effector phenotype based on microenvironmental cues\textsuperscript{81,82}. This is illustrated in the case of a high-affinity CAR-Treg with a first-generation scFv recognising soluble factor 8 (FVIII) in a haemophilia A
model which adopted a pro-inflammatory phenotype akin to CAR-Tconv, producing high levels of IL-10, IL-4, and IFN-γ, and ultimately increasing the formation of αFVIII IgG. Interestingly, neither targeted mutation of the CD28 intracellular co-stimulatory domain, or constitutive expression of IL-10 in this case is sufficient to restore suppressive phenotype, although reducing the scFv surface expression by transducing a construct where the scFv is complexed to CD3ɛ (a TCR fusion construct, or TRuC) does⁸⁰. Hitting the ‘goldilocks zone’ when it comes to TCR signal strength and duration is of critical importance in realising the benefit of antigen specific Treg suppression. Incorporating safety features such as suicide switches or molecules that can be targeted for depletion may ultimately be required to mitigate the plasticity risk.

5.1.2 Genome editing Treg ACTs
Capacity to edit the genome of primary human cells has historically been limited, with the major available technologies—zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)—being expensive, difficult to synthesise, and having limited delivery vectors⁸³. The discovery of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9) system, a component of the bacterial adaptive immune system, has however massively increased the ease and availability by which sequence-specific double stranded DNA (dsDNA) breaks can be introduced⁸⁴. In the CRISPR/Cas9 system, a guide RNA (gRNA) sequence directs the Cas9 protein to its complementary genomic sequence. HNH and RuvC-like nuclease domains then introduce precise dsDNA breaks in the complementary and non-complementary strands respectively which are repaired by one of two endogenous repair mechanisms: non-homologous end joining (NHEJ), an error-prone but rapid process that often introduces insertions or deletions (INDEL) into the target sequence resulting in inactivating knock-out (KO) mutations; or homology directed repair, a precise but slower process, where a DNA template is utilised to de novo synthesise the complementary sequence which may be used to knock-in (KI) genes of interest⁸⁵. A key strength of the CRISPR/Cas9 system is its versatility and ease of delivery as gRNA can target any sequence with an upstream Cas9-species specific protospacer adjacent motif (PAM) resulting in a highly customisable system. Since the first descriptions of utilising CRISPR/Cas9 to generate precise KOs and KIs in primary human T cells⁸⁶,⁸⁷, there has been an explosion of interest in editing Tregs to enhance their survival, durability, stability, and function.
5.1.2.1 Gene engineering to potentiate Treg ACT survival

There are several putative targets that may potentiate Treg survival. c-Jun NH\textsubscript{2}-terminal kinase (JNK) signalling is found in multiple cell types and is implicated in diverse and often contrasting cellular functions including proliferation, cytokine production, and apoptosis via, amongst other mechanisms, activation of the AP-1 transcription factor\textsuperscript{98-90}. In T cells, JNK signalling has been implicated in co-stimulatory signal integration, increased IL-2 signalling, potentiated effector function, and ordered cell death\textsuperscript{91-94}. Interestingly, Tregs with selective siRNA-mediated knockdown of JNK-1 demonstrate superior survival, secondary to enhanced expression of antiapoptotic proteins, in addition to superior suppressive capacity resulting from enhanced production of immunosuppressive cytokines IL-10 and TGF-β and surface expression of LAG-3\textsuperscript{95}. This manifests as an enhanced ability to prevent rejection of allogenic islet cell transplants in mice\textsuperscript{95}. Similarly, overexpression of c-Jun (a downstream JNK target) in CAR-T cells renders them resistant to exhaustion, likely mediated through disruption of AP-1 driven expression of exhaustion-associated gene networks\textsuperscript{96}. Instead of targeting pathways driving initiation of exhaustional programmes, the same technique has instead been directed towards retaining the stemness of transduced cells. For example, deletion of the DNA methyltransferase 3 alpha (DNMT3A) from T cells prior to retroviral CAR delivery enhances their antigen-specific proliferative capacity and function\textsuperscript{97}. This is associated with retained expression of transcription factors that normally become repressed on repeated antigen exposure, and retention of a stem-like transcriptional state. However, mutations in DNMT3A are also associated with numerous haematological malignancies\textsuperscript{98}, which is of particular concern when considering CAR Tregs given that the point is to preserve their cognate antigen, unlike in the case of malignancy where antigenic clearance is desired. The potential for similar edits to result in leukaemic conversion in this setting may therefore be more significant.

Targeted gene edits may be introduced to provide a competitive survival advantage to Treg ACT in specific disease settings. For example, following transplantation, patients require lifelong non-specific pharmacological immunosuppression (IS) to prevent graft rejection. However, ACT administered to immunosuppressed individuals are also therefore susceptible to IS-mediated dysfunction. A mainstay of IS following transplantation is the calcineurin inhibitor tacrolimus, which exerts its immunosuppressive effect through the cytoplasmic protein FKBP12, preventing calcineurin-mediated NFAT (nuclear factor of activated T cells) translocation and transcriptional activation. FKBP12 KO renders T cells resistant to tacrolimus-induced immunosuppression. GMP-compliant CRISPR/Cas9 mediated KO of
fkbp12 in CMV-specific effector T cells results in superior virus-specific effector function and stimulation in the presence of tacrolimus\textsuperscript{99}. A similar technique applied to Treg ACTs has significant potential to reduce the effective ACT dose for transplant recipients.

Innovatively, genome editing approaches may also be combined with small molecule therapeutics to modulate signalling responses and promote specific survival of adoptively transferred cells. One strategy to increase the specificity of IL-2 treatment is to \textit{ex vivo} retrovirally transduce Treg with an orthogonal IL-2 receptor, which retains native IL-2 signalling through STAT5 but responds selectively to an engineered orthogonal IL-2. Importantly, orthogonal IL-2 binds only to the transduced orthogonal receptor and therefore does not stimulate endogenous populations with either the dimeric (βγ) or trimeric (αβγ) IL2R. Following transduction Tregs remain functional as reflected by their ability to promote donor haematopoietic stem cell engraftment and prolong heart allograft survival in a mixed chimerism model\textsuperscript{100}. Orthogonal IL-2/IL-2R systems have successfully been applied to human CAR-T cells\textsuperscript{101}. Whilst their relative scarcity may hinder similar attempts to transduce primary human Treg, the potential to modulate Treg number \textit{in vivo} in real time through altering IL-2 dosing is intriguing, offering the prospect of ‘fine tuning’ ACT function in response to clinical changes.

5.1.2.2 Gene engineering to potentiate Treg ACT number and stability

A second potential application of genome editing is to create lineage-stabilising KI or KOs to strengthen the durability of the Treg phenotype on encountering a destabilising inflammatory milieux. The importance of FOXP3, the Treg master transcription factor, is well illustrated by the clinical syndrome IPEX (immunopathology, polyendocrinopathy, enteropathy, X-linked) resulting from a loss-of-function FOXP3 mutation and causing often-fatal autoimmunity from an absent Treg compartment\textsuperscript{55,102-104}. The prospect of inducing expression of FOXP3 and therefore a regulatory phenotype in bulk T cells is an appealing route to overcome the challenges of low precursor frequency. Successful HDR-mediated integration of a promoter into the FOXP3 locus of human CD4\textsuperscript{+} cells has indeed been achieved, which results in the acquisition of an immunosuppressive phenotype. However, these cells are less potently suppressive, requiring a higher edited Treg:effector ratio, and demonstrate key transcriptomic differences from tTreg, notably in the expression of IKZF2\textsuperscript{105}. This is congruent with previous observations that FOXP3 expression is necessary but not sufficient for Treg function\textsuperscript{106-108}. Alternative approaches to induce FOXP3 expression include HDR-
mediated KI of FOXP3 or forced demethylation of the Treg-specific demethylation region (TSDR) within the FOXP3 promotor. The former has been investigated as a therapeutic strategy for IPEX, through delivering a functional FOXP3 template alongside CRISPR/Cas9 targeting the mutated version, although this approach is likely of limited applicability for developing Treg cellular therapies. The latter strategy of forcing TSDR demethylation, which conceivably is of greater applicability to the Treg ACT field, has unfortunately had limited success to date with successful TSDR demethylation—achieved by localising the demethylating enzyme TET1 specifically to TSDR by fusion to cas9—not translating into a regulatory phenotype. It is likely that FOXP3 may need to form part of a broader combination of edits to produce functional Tregs, with HELIOS being a potential second candidate. HELIOS is a member of the Ikaros transcription factor family, which exhibit diverse functions within lymphoid cells regulating both lineage development and mature effector functions. Whilst HELIOS function remains incompletely defined within Tregs, its expression has nevertheless been implicated in Treg differentiation, stability, and suppressive capacity. As such, inducing durable FOXP3 and HELIOS co-expression may provide a more stable edited Treg product. CD4+ and CD8+ lymphocytes retrovirally transduced with full-length HELIOS and FOXP3 do indeed acquire a regulatory phenotype, although unfortunately they also exhibit destabilised proliferative potential.

Others have approached maintaining Treg phenotypic stability by targeting signalling pathways responsible for promulgating phenotypic skewing. For example, PKC-θ acts as a negative feedback loop to restrain Treg suppressive function, although its important role in immune-synapse formation, differential physiological effects between T cell subsets, and structurally homologous related proteins renders it a challenging target at a systemic level. Specific inhibition of PKC-θ via engineered antibodies tagged with a cell-penetrating peptide mimic, however, results in increased Treg suppressive capacity in vitro and potentiates the therapeutic efficacy of adoptively-transferred Treg preventing GvHD development in a humanised xenograft model. Co-opting a similar approach with an editing strategy may sidestep the necessity to systemically administer these therapies and potentiate Treg ACT.

Alternatively, targeting microenvironmental cues at the initiation of their respective signalling cascades may represent a precise method to maintain Treg ACT stability. This technology heralds from the immuno-oncology field, where it has been applied to potentiate effector function within harsh tumour microenvironments. For instance, NK cells can successfully overcome immunosuppressive TGF-β signalling by fusing the extracellular TGF-β receptor
with the stimulatory intracellular domain NKG2D, the NK-stimulatory DNAX-activation protein 12 (DAP12), or a synthetic Notch-like receptor\textsuperscript{120,121}. Whilst examples of a similar approach in Tregs are scarce, the principle of converting a pathogenic Treg destabilising cue into a lineage-stabilising intracellular cascade is appealing. However, given that Notch1 signalling appears to skew Treg towards a Th1 phenotype, a distinct approach may be required for Treg ACT\textsuperscript{122}.

5.1.2.3 Towards allogeneic Treg ACT

Multiple limitations of autologous Treg cell therapy could be overcome by designing and banking an allogeneic ‘third-party’ Treg product available for off-the-shelf administration. However, unmodified alloTreg products, much like unmatched solid organ transplants, are likely to be recognised by the replete recipient immune system. Here, the best-case scenario would be an inefficacious product whilst in the worst-case allogeneic therapy could conceivably result in exacerbation of underlying immunopathology, acute rejection, and sensitisation. Genome editing technologies may potentially be leveraged to abrogate allogeneic cell therapy immunogenicity. In the induced pluripotent stem cell (iPSC) field, there has been significant progress towards this aim. Initial approaches created hypoimmunogenic lines by inhibiting MHC class I and II expression, most commonly through disruption of \textit{B2M} encoding the \(\beta_2\)-microglobulin required for mature MHC I expression, or class II transactivator (CIITA) encoding a key component of the transcription factor complex required for MHC II transcription. Unfortunately, in concordance with the ‘missing self’ hypothesis\textsuperscript{123}, such approaches render iPSCs susceptible to NK-mediated depletion\textsuperscript{124}. Strategies to mitigate this include inducing expression of inhibitory proteins such as non-classical HLA\textsuperscript{125-127} or CD47\textsuperscript{128} (a transmembrane glycoprotein that inhibits macrophage-mediated phagocytosis), or through selective retention of HLA-C\textsuperscript{129}. However, as most protocols to generate Tregs from iPSCs presently involve retroviral transduction of \textit{FOXP3}\textsuperscript{130,131}, the ability to generate a Treg therapy from hypoimmune iPSCs remains to be established.

5.1.3 Non-genomic Treg ACT bioengineering

In addition to the genetic-engineering approaches described above, the generation of Treg cell therapies offers an opportunity to apply other bioengineering approaches to potentiate survival and function. A recent innovative approach to equip Tregs with a ‘nanogel backpack’ has been achieved by impregnating a nanogel with engineered IL-2/Fc conjugated to an
anti-CD45 antibody and then incubating this construct with Tregs ex vivo. Elegantly, nanogel backpacks only release their payload on Treg activation by exploiting the up-regulation of reducing agents expressed on the cell surface following TCR ligation, and the high-affinity Treg IL2Rα rapidly consumes locally released IL-2 minimising off-population stimulation. Tregs equipped with nanogel backpacks prolong skin graft survival in a humanised mouse model of transplantation more effectively than standard Tregs associated with antigen-specific Treg expansion and an enhanced Treg:Teff ratio within the graft. This approach is particularly appealing given the ease with which it could be integrated into existing workflows for generating Treg ACT.

5.2 Targeting Treg in vivo
An alternative attractive approach to harnessing Treg therapeutic potential is to directly and selectively target endogenous Tregs, thereby circumnavigating the technical and costly process of generating a Treg cell therapy product. Such approaches include developing cytokine therapeutics to selectively expand native Treg populations, or by redirecting Tregs towards specific microanatomical niches to specifically localise their immunosuppressive effects.

5.2.1 In vivo approaches with cytokine-targeting therapeutics
Several approaches to increase the specificity of IL-2 therapy for Treg populations are under preclinical evaluation (comprehensively reviewed in). IL-2 muteins are generated by targeted mutation of specific amino acid residues which disrupt binding of the dimeric IL-2 receptor whilst preserving binding of the trimeric IL-2 receptor. Muteins may be fused to either whole antibodies or antibody fragments to stabilise their pharmacodynamic properties. Alternatively, human recombinant IL-2 may be complexed with a monoclonal antibody or PEGylated to improve its in vivo stability. Interestingly, these approaches may be used to bias preferential trimeric IL2R binding, and thereby preferential Treg specificity. Proposed mechanisms for this phenomenon include steric hinderance, where IL-2 is physically blocked from binding to the dimeric receptor, and triggered exchange, where IL-2 is preferentially liberated from the immune complex in the presence of CD25. Conversely, other IL-2 binding antibody clones may conformationally stabilise IL-2 to promote binding of IL2Rβ. Interestingly, a manufactured IL-2 and CD25 fusion protein unexpectedly expands Tregs selectively through periodically dissociating trans dimerization of the trimeric IL2R on Tregs. More recently agonism of the dimeric IL-2 receptor has been achieved.
directly with a bispecific antibody and conceivably a similar approach could be taken with a trispecific antibody\textsuperscript{139}. However, IL-2 targeting approaches need careful \textit{in vivo} evaluation considering the potential for effector T cells to also express CD25 on encountering their cognate antigen, meaning that even trimeric IL2R specific therapeutics cannot currently exclusively stimulate the Treg compartment.

The tumour necrosis factor receptor superfamily member 25 (TNFRSF25, DR3) has also demonstrated promise as a pre-clinical target to stimulate \textit{in vivo} Treg expansion. Administration of an anti-TNFRSF25 4C12 antibody in mice results in pronounced Treg expansion up to 30-35\% of total CD4\(^+\) T cells in an IL-2 and MHC-class 2 dependent manner\textsuperscript{140}. Administration of anti-TNFRSF25 in mouse models of allogeneic heart, skin, and islet cell transplantation also causes massive \textit{in vivo} Treg expansion and prolongs graft survival\textsuperscript{141-143}. Similarly, preferential Treg enrichment in HSCT products, achieved by administering anti-TNFRSF25 to donor animals, facilitates recipient engraftment and reduces early post-transplant sequelae\textsuperscript{144-146}. Antibody stimulation of a related TNF receptor superfamily member, TNFR2, also results in dose dependent proliferation of Treg from human peripheral blood\textsuperscript{147}, whilst combination of a TNFR2-specific mutein with IL-2 is also able to expand Tregs from mice\textsuperscript{148}. However, agonist antibodies remain complex to develop, illustrated by the limited successes targeting other TNRRSF members, and the distribution of both TNFRSF25 and TNFR2 are not restricted to Tregs, presenting potential challenges in therapeutically translating this approach\textsuperscript{149}.

5.2.2 \textit{In vivo} approaches with biologics and nanoparticles

Specific Treg targeting with more conventional small molecule and protein therapeutics would naturally present fewer translational challenges. An interesting approach inducing antigen-specific expansion of Tregs has been demonstrated in animal models using antigen-conjugated tolerogenic poly(lactide-co-glycolide) nanoparticles, which significantly prolong islet graft survival and impair effector T cell responses\textsuperscript{150}. \textit{In vivo} generation of antigen specific Tregs is an enticing prospect, although the approach is also contingent on the accurate identification of the pathogenic antigen repertoire; in settings where the antigenic repertoire is likely to comprise multiple peptides that are highly variable between patients, such as following transplantation, this approach may have limited applicability. Finally, novel Treg differentiators have more recently been investigated, for example the small polypeptide miPEP31, which transcriptionally represses microRNA-31 a negative regulator of Treg
differentiation\textsuperscript{151,152}. However, whilst directly targeting Tregs with small molecules remains an appealing prospect, the translational potential of new approaches requires careful evaluation.

6. Summary

Remarkable progress towards harnessing Treg therapeutic potential has been made since their initial description, and innovative new technologies continue to equip scientists with the tools to produce the next generation of Treg cell therapies. There are several directions of travel, and which is most successful remains to be established. However, it is an exciting prospect to envision multiple broadly applicable Treg cell therapy approaches that will facilitate delivery of their therapeutic potential to patients.
REFERENCES


57 Beers, D. R. et al. ALS patients’ regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. *JCI Insight* 2, doi:10.1172/jci.insight.89530 (2017).


Wang, D., Quan, Y., Yan, Q., Morales, J. E. & Wetsel, R. A. Targeted Disruption of the β2-Microglobulin Gene Minimizes the Immunogenicity of Human Embryonic Stem


FIGURE 1: Current approaches to Treg therapy.

A schematic overview of the general Treg therapy approaches that have progressed into clinical trials, created with biorender.com. The left-hand panel illustrates adoptive cellular therapy (ACT) approaches starting with donation of whole blood or a leukapheresis product from the patient from which the cell therapy is generated. Broadly, Treg ACTs may be classified as: polyclonal, indicating a broad TCR repertoire within the product; antigen specific, indicating a restricted TCR repertoire; or as Tregs transduced with a CAR comprising an ScFv towards a single antigen. The manufactured product is then infused back to the patient donor. The right-hand panel illustrates the approach taken to preferentially expand endogenous Treg in vivo through low dose IL-2 therapy. Here, the exogenously administered IL-2 is expected to preferentially stimulate and expand Treg expressing the high affinity IL2Rα chain CD25 compared with the lower affinity dimeric IL2R expressed by effector cells.

FIGURE 2: Future approaches to Treg therapy.

A schematic overview of selected published experimental approaches adopted to potentiate Treg cell therapy in the pre-clinical setting, created with biorender.com. Broadly, genetic and bioengineering strategies have been investigated to induce antigen specificity in polyclonal Treg products, enhance Treg in vivo survival and function through modulating availability and responses to essential Treg homeostatic signals, and to explore use of third party Treg products through inhibiting cell surface expression of HLA, with or without induced expression of additional pro-survival proteins.
DECLARATIONS

- ARRIVE guidelines
  No animal work was undertaken for this review and therefore ARRIVE guidelines are not applicable.

- Data Availability statement
  No novel data were generated for this review and therefore this statement is not applicable.

- Competing interests
  The authors have declared that no conflict of interest exists.

- Funding statement
  OM is a Medical Research Council Clinical Research Training Fellow (MR/V000942/1). MB receives funding from Turkish Ministry of Education. FI is a Wellcome Trust CRCD Fellow (211122/Z/18/Z). The work on advanced cellular therapies in the authors’ group is supported by the UK Medical Research Council (MR/N027930/1) and the European Union’s Horizon 2020 research and innovation program under grant agreement No 825392 (ReSHAPE).

- Acknowledgements
  This statement is not applicable for this work.

- Author contributions
  OM: investigation, writing – original draft, writing – review & editing, visualisation. MB: investigation, writing – original draft. JH: conceptualisation, supervision, writing – review & editing. FI: conceptualisation, supervision, writing – review & editing.

- Permission to reproduce (for relevant content):
  The article does not contain content requiring permission to reproduce.
Figure 2

Next-generation Treg ACTs may be engineered to facilitate

- Antigen specificity
- Enhanced functionality
- Off the shelf therapy
- Enhanced survival

Regulatory T cell

Genetic engineering approaches

- CRISPR/Cas
- TALENs
- transduction
  - dsDNA break
  - NHEJ
  - HDR
  - integration

Targeted KD
Targeted Ki

Orthogonal IL-2 receptor
HLA KD
nonclassical HLA KI
IL-2 backpacks
TNFR signaling