Title: Enhancing Human Regulatory T Cells *in vitro* for Cell Therapy Applications

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Abstract

Adoptive cellular therapies are gaining popularity as a means to treat clinical conditions, with potentially fewer risks and greater efficacy than traditional pharmacological strategies. Regulatory T cells (Tregs) are currently undergoing clinical trials in various immune-mediated pathologies, including transplant rejection and autoimmune conditions. In general, cell therapy relies upon *ex vivo* expansion of the cell product, in order to administer more cells than can be isolated from one person. *In vitro* manipulation of cell therapy products, prior to administration to patients, offers the opportunity to enhance the efficacy of the final cell therapy product in other ways. For example, cells can be exposed to reagents that enhance their longevity or functional potency after transfer into the patient. Genetic modification strategies can even permit the design of cells with bespoke functionality. Crucially, *in vitro* manipulation of therapeutic cells in isolation can exert these influences upon the biology of the therapeutic cells, without systemic exposure of the patient to the reagents being used. Quality control assessments can be integrated into the procedure prior to administration, to protect the patient from the risk of adverse events, should the procedure produce undesirable results. With a particular focus on Tregs, this review surveys the diverse strategies that are being employed to enhance the efficacy of cell therapy via *in vitro* manipulation of cells, and highlights some emerging technologies that may propel this endeavour in the future.

Keywords: regulatory T cells, Treg, cell therapy, genetic modification

1. Therapeutic Application of Regulatory T Cells

At present, immunological graft rejection, as well as autoimmune diseases, are restrained using drugs that suppress the immune system. In addition to the specific toxicities conferred by immunosuppressive drugs, global depletion or inactivation of endogenous immune cells leaves patients vulnerable to infection and malignancies[1]. Cell therapies are being heralded as the next generation of therapeutics for immune-mediated pathologies[2–6].

Regulatory T cells (Tregs) are lymphocytes whose native function is to regulate immune responses, in order to maintain immune homeostasis and prevent autoimmunity[7–10]. The most well-characterised population of Tregs are the $CD4^{+}FOXP3^{+}$ population. In humans, these cells are identified by high constitutive expression of the transcription factor FOXP3[11], associated with DNA hypomethylation at certain sites of the *FOXP3* promoter[12,13], as well as stable surface expression of the IL-2 receptor alpha chain (CD25)[14]. A deficit in FOXP3 results in IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked) Syndrome, a severe systemic autoimmunity[15], which is recapitulated in the “scurfy” phenotype of Treg-depleted mice[16]. An enrichment of Tregs observed within tumours[17,18] and at sites of infection[19] (attributed to selective recruitment, induction or expansion of Tregs)[20] has been evoked as a mechanism of immune evasion. Just as pathogens and malignant cells exploit Tregs to protect themselves from the endogenous immune response, so researchers have long recognised the therapeutic potential of manipulating Tregs as a means of protecting tissues from harmful immune responses.

The premise of Treg cellular therapy is that Tregs are isolated from the blood of the patient and cultured *in vitro*, driving the cells to increase in number through cell division. These “expanded” cell cultures are then infused back into the patient, in order to increase the proportion of the desired cell population within the immune system[3,5] (**Figure 1**).

In comparison to pharmacological agents, cell-based therapeutic agents should confer fewer side effects. Over the course of evolution, cells have undergone adaptation and selection to provide finely-tuned and “intelligent” regulation of the immune system. Cells are able to sense and respond to their environment and adapt their behaviour to provide the most appropriate activities for their context.

Moreover, adoptive transfer of *ex vivo*-expanded cells has several advantages over manipulation of a native cell population. Pharmacological agents have been developed to promote or impede the expansion or function of endogenous Tregs *in situ*[21–24]. However, the efficacy and safety of Treg-promoting drugs is dependent upon their effects being restricted to Tregs, to the exclusion of T effector cells (Teffs). The biochemical profiles of many regulatory cell types are so closely related to those of their effector cell counterparts that designing drugs to target one or other subset differentially is extremely challenging. Extracting and culturing cells *ex vivo* provides an opportunity to manipulate the desired cells in isolation, without concurrently influencing other cell populations and tissues.

Furthermore, the period of *in vitro* culture allows ample opportunity for manipulating the cell therapy products. This capability has been exploited in diverse and creative ways, to enhance the utility of cells for research and clinical applications. In this review, we shall explore some examples of strategies currently employed to increase the purity, yield, specificity, per-cell potency and safety of adoptive cell therapies. Some of these techniques have been applied to

Tregs, whilst others have been developed for other applications but, we speculate, may soon be adapted to address outstanding challenges in Treg therapy. Whilst this review shall focus mainly upon Tregs, the principles explored herein may be applied also to other immune-modulating cellular therapeutics.

2. Overview of Methods Employed to Modify Lymphocyte Biology *in vitro*

In common with other therapies, the major goal of cellular therapy is to achieve an effective, safe treatment that can be delivered reliably. Hence, clinical trials assess the value of a therapy with respect to three main parameters: feasibility, safety and efficacy. The principle factors that dictate these parameters in cellular therapy are yield, inherent functional potency, purity, specificity, longevity, and the means to monitor and intervene in cell function extrinsically (**Figure 2**). In the endeavour towards generating more efficacious regulatory cell therapies, approaches range from traditional chemical or biological culture supplements, through genetic engineering, to synthetic biology (**Figure 3**).

Even among lymphocyte populations, different cellular subsets respond differentially to various chemical agents, including nutrients, growth factors, cytokines and drugs. By altering the availability of these substances, it is possible to modulate the survival, proliferation and function of a target cell subset.

Gene transfer, traditionally accomplished in lymphocytes with the aid of viral vectors, is being exploited to create “designer” cell therapies (**Figure 4**). The introduction of transgenes (derived from the host species or an unrelated species) into the genome of a cell therapy product is now relatively trivial. Genetic “knockout” of endogenous genes is a little more challenging, requiring targeted mutation of the gene of interest, but silencing (“knockdown”) of gene transcription can be achieved by transducing cells with sequence-specific small

interfering ribonucleic acids (siRNAs). Genetic modifications that augment the viability, proliferation or suppressive apparatus of Tregs can be designed with the aim of generating the most potently suppressive cell therapy products. Currently, progress in the field of gene transfer technologies has mostly been directed at effector T cells for cancer therapy but several research groups and companies are developing designer Tregs for application in immune-mediated pathologies[25,26]. The advent of genome editing platforms, such as CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats) and TALENs (Transcription Activator-Like Effector Nucleases), is set to accelerate both research and application of designer cell therapies[27–29].

Beyond simply adding or removing protein-coding genomic loci, it is now becoming relatively common practice to employ synthetic biology for cell therapy applications[30–35]. Synthetic biology can be used to incorporate native signalling pathways into novel regulatory circuits, allowing the user to prescribe the manner in which a cell will respond to a particular stimulus. The stimulus itself might be endogenous or it might be synthetic, allowing those therapeutic cells to be controlled remotely via administration of the synthetic stimuli. Such synthetic circuits would offer an exclusive line of communication between a clinician and the modified cells.

3. Increasing the Yield of Tregs

3.1. Expanding Tregs with Cell- or Bead-based Stimulation

One simple-yet-effective means to increase the clinical impact of a cell therapy is to infuse a greater number of cells. From the very earliest days of Treg research, researchers have proposed varied strategies for promoting the expansion of Tregs in culture. Beyond the basic

requirements for Treg survival *in vitro* (essential nutrients and growth factors), proliferation of Tregs depends upon T cell Receptor (TCR) stimulation, co-stimulation[36] and cytokines. Interleukin-2 (IL-2) was identified as the key cytokine driving Treg proliferation[37], although there is still disagreement concerning the optimal concentration. Over time, the source of TCR stimulus and costimulatory signals has been refined. Early studies utilised combinations of allogeneic cells and antibodies to ligate the TCR, such as allogeneic feeder cells plus anti-CD3 with IL-2[38]. More recently, the development of anti-CD3- and anti-CD28-coated beads[39] and artificial antigen-presenting cells (aAPCs)[40,41] has permitted higher fold expansion of Tregs. Antibody-coated beads provide particularly robust and consistent TCR stimulation, in a polyclonal manner, irrespective of the antigen-specificity of the cell's TCR. The beads are available in a Good Medical Practice (GMP)-compatible format that has been used to expand Tregs for use in clinical trials[42–44]. In addition to increasing the number of cells, the activated state of Tregs undergoing *in vitro* stimulation is also permissive to potent suppressive activity[45].

4. Enhancing the Potency of Treg Cell Therapy Products

Arguably, the two properties possessed by Tregs that most recommend these cells for application as an immune cell therapy are specificity and tolerogenic potential. Indeed, the principle rationale for developing cellular therapies for immune-mediated pathologies is to impose localised immune regulation, and perhaps tolerance induction, rather than global immune suppression. Rather than simply increasing the number of regulatory cells, it would be wise for cell therapy strategies to be founded upon understanding, and ultimately enhancing, these two properties in Tregs.

4.1. Enrichment of Highly Potent Treg Subsets

It may be possible to increase the potency of the cell therapy products simply by starting with the most potently suppressive cell population. Expression of molecular markers has been used to refine the population of cells that is isolated for cellular therapy. The exact combination of cell surface markers that identifies the most suppressive Treg fraction is greatly contested. However, there is some consensus that moderate expression of the IL-2 receptor α chain (CD25) and low expression of the IL-7 receptor α subunit (CD127) marks a potent population of CD4⁺FOXP3⁺ Tregs from human peripheral blood[46–49]. Other groups regard CD45RA as a useful marker to indicate potently suppressive Treg phenotype[50].

4.2. Culture Supplements to Enhance Treg Potency

The *in vitro* expansion procedure can also incorporate treatment with chemical agents to improve the purity of the final cell product or to promote the viability or suppressive function of the Tregs. Rapamycin, for example, selectively abrogates proliferation of Teffs, whilst favouring the proliferation, survival and phenotypic stability of Tregs, thus increasing the effective ratio of Tregs to Teff cells[21,51]. When administered *in vivo*, the anti-proliferative effects of Rapamycin can have adverse effects upon the parenchyma and may prove detrimental to tissue repair in the context of transplantation[52]. However, pre-treatment of Tregs during *in vitro* expansion can serve to limit outgrowth of contaminating Teffs and promote the efficacy of Tregs after infusion[51], without systemic exposure to the drug.

4.3. Engineering Designer Tregs

One of the most direct ways to enhance the suppressive potency of Tregs would be to augment the molecular apparatus that is employed by regulatory cells to exert their

immunoregulatory properties. A parallel approach has already been exploited in effector lymphocytes employed as cell therapies for the treatment of malignancies. For example, in one study, Tregs engineered to express supraphysiological levels of IL-12 exhibited enhanced anti-tumour activity[53]. In Tregs, one might drive elevated expression of anti-inflammatory cytokines (such as interleukin-10 and transforming growth factor beta) or co-inhibitory receptors (such as Cytotoxic T Lymphocyte-Associated-4). More elaborate approaches incorporate conditional induction of Treg activity, rather than simply augmenting constitutive, ubiquitous activity. Alternatively, driving expression of some Treg-associated immune-modulating molecules in Tregs has been shown to be sufficient to confer regulatory activity upon these cells, whilst retaining their native antigen-specificity [54,55].

5. Regulating the Activity of Treg Cell Therapeutics

It is believed that, rather than imposing systemic immune suppression, regulatory cells possess a degree of specificity that restricts their activity to a pathological immune response, sparing physiological immunity. Without specificity, cell therapies would be subject to the same limitations as pharmacological immunosuppression; namely increased risk of infection and malignancy. As such, ensuring the specificity of regulatory cells will be critical to realising the theoretical benefits of immune cell therapies. The nature of this specificity is not straightforward, though the term is generally used to allude to specificity for a cognate antigen, conferred by the TCR.

5.1. Selecting or Imposing Antigen-Specificity

Whilst antibody-coated beads give the most powerful induction of cell cycle in Tregs, for certain applications, true antigenic TCR stimulation may be favourable. Advocates of antigen-

based TCR stimulation favour antigen-presenting cells, bearing a particular antigen or repertoire of antigens representing the target of the immune response, in the context of pertinent costimulatory molecules. In transplantation, for example, researchers have explored the benefits of selecting alloantigen-reactive Tregs, by stimulating the cells *in vitro* with donor-derived APCs (to select for cells with direct alloreactivity)[56] or autologous APCs pulsed with donor peptides (to select for Tregs with indirect allospecificity)[57,58]. By exposing Tregs to the same antigens that induce the undesired immune response, it is thought that these Tregs will preferentially regulate these pathological immune responses, thereby sparing physiological immune responses and diminishing the risk of global immunosuppression[59]. In other words, polyclonal stimulation generates a higher bulk yield of cells, maintaining the heterogeneous antigen specificities of the progenitor population; antigen-based stimulation generates a smaller pool of cells but enriches for the desired specificity within that pool.

Alternatively, gene transfer has been used to express antigen receptors of the desired specificity in Tregs. T cells, including Tregs, with defined antigen specificity have been generated by forcing expression of either transgenic TCRs[60–65] or chimaeric antigen receptors (CARs) in these cells[66–70]. Transgenic TCRs are derived from natural TCRs that have been selected for their affinity for a salient disease-associated antigen. For example, expression of MHC class I-restricted TCRs in Tregs has been explored as a means of allowing these cells to be activated by endogenous antigens presented on the more ubiquitous class I MHC, as well as MHC class II on professional APCs[71]. CARs are engineered constructs composed of variable, antigen-binding moieties of one molecule (most commonly the single chain variable fragment of a BCR) fused to the transmembrane domain of another receptor

(classically, the CD3-zeta domain)[72,73]. By splicing the antigen-specificity of one receptor onto the intracellular signalling functionality of another, one can create a T cell in which MHC-independent, high affinity recognition of a chosen antigen is coupled to activation of the cell. Several groups have generated Tregs expressing CARs with specificity for particular alloantigens, as a means to improve Treg therapy to treat allograft rejection[66–68]. Whilst there are certainly promising data emerging from these studies, some limitations remain to be resolved, such as putative toxicity and inferior potency[25,70]. For many clinical applications of cellular therapy, the challenge of identifying the pertinent antigen can be prohibitive to effective receptor design.

5.2. Engineering Bespoke *in vivo* Activity of Tregs

Aside from conferring responsiveness to a particular target antigen, genetic engineering strategies can also be employed to create “designer” cell therapy products to fit a particular specification. In nature, it is probable that Treg activity is restricted or specialised with respect to the anatomical location of their activity, the nature of the immune response that they regulate, or to a particular target cell phenotype. When developing strategies to reduce off-target suppression by therapeutic Tregs, therefore, there is scope to exploit all of these varieties of specificity, not only antigen-specificity.

Beyond transgene expression, Synthetic Biology can be applied to modulate the *in vivo* behaviour of the cells after infusion[32,74–76]. The idea of controlling cell migration is particularly appealing in light of emerging evidence of tissue-restricted activity of lymphocytes, including Tregs. Abundant evidence is emerging for specialised functions of Tregs resident within different lymphoid and non-lymphoid tissue[77–81]. For example, in

mice, exposure to the vitamin A metabolite All Trans Retinoic Acid (ATRA) induces Tregs expressing mucosal-homing receptors CCR9 and $\alpha_4\beta_7$ that tend to home to the intestine[82], where these gut-tropic Tregs promote oral tolerance[83]. Meanwhile, Tregs with a memory-type phenotype are enriched in the skin[81]. Thus, localisation of Tregs might be a mechanism by which function of adoptively-transferred Tregs can be controlled. Enforcing expression of tissue-homing molecules on the surface of Tregs might help to enrich these cells within a particular target tissue, such that their activity is, to some extent, spatially restricted.

Forced expression of native chemokine receptors has been used to direct the homing properties of effector T cells, resulting in improved anti-tumour efficacy[84,85]. Synthetic biology can go further, by facilitating extra regulation. Transgenic receptors, coupled to endodomains of endogenous chemokine receptors that modulate the migration of cells, have been used to exert user control over the recruitment of engineered lymphocytes to a chosen anatomical site. By co-opting endogenous chemokine receptor signalling pathways, synthetic regulatory circuits have been engineered to allow lymphocyte migration to be induced by a synthetic stimulus, such as light[33] or synthetic chemical ligands[34].

Similarly, different Treg suppressive mechanisms, of which there are a multitude, are adapted to suit different requirements. For example, IL-10 appears to be the predominant mediator exploited by Tregs in the maintenance of tolerance to environmental antigens in the mucosae[86]. In mice, Treg-derived IL-10 was shown to be required for Treg-mediated suppression of skin graft rejection[87], and protection from experimental colitis[88], and for tolerance to leishmania[89]. Yet IL-10 is dispensable for *in vitro* suppression of T cell proliferation[90]. The particular complement of suppressive mechanisms employed by a

given Treg is most likely determined by the life history and prevailing microenvironment of that cell. Thus, modulating the predominance of different suppressive mechanisms might be another means to direct the activity of Tregs.

5.3. Improving the Safety Profile of Treg Therapy

In order to reduce off-target effects, methods of enhancing the specificity of regulatory cells would be desirable. Aside from antigen-specificity, activation requirements and tissue-homing have also been the subject of genetic manipulation to restrict off-target activity of putative cellular therapeutics, as discussed above. Additionally, cells can be engineered with genetic “safety switches” that can be induced by a clinician to enable rapid reversal of the treatment if adverse effects are detected[91,92]

Some designs incorporate inhibitory receptors to transiently inactivate engineered T cells as a means of reducing on-target off-tumour activity[93]. More sophisticated genetic engineering strategies incorporate regulatory circuits, such that the expression or activity of the transgene is inducible[94]. Transgenic receptors for artificial ligands can be coupled to the intracellular apparatus for lymphocyte activation, in order to place the activity of cell therapy products under the control of the user who administers the stimulus. Synthetic signalling circuits designed to act as Boolean logic gates, whereby the activity of an engineered T cells is dictated by multiple molecular parameters, are being explored for refining the safety and efficacy of engineered cell therapy products[95–98]. One ingenious example of this type of genetic switch was recently described in a study by Wu et al in Tregs for cancer therapy. In this study, a chimeric antigen receptor was engineered such that its intracellular signalling capacity was dependent upon a synthetic small molecule drug, as well as upon ligation of its

cognate antigen[99]. This genetic circuit constituted a Boolean “AND” gate, which ultimately placed the activity of the CAR-expressing T cells under the control of a clinician administering the small molecule. No doubt such synthetic signalling circuits will soon be adapted and applied to immune regulatory cell therapy products.

5.4. Promoting Longevity of Cellular Therapeutics *in vivo*

One of the major limitations of immune cell therapy is that immunogenicity of allogeneic cells restricts clinicians to using autologous cells. In certain scenarios, it would be beneficial to administer generic pre-prepared cell products from a biobank. For example, patients suffering from certain autoimmune pathologies have inherent deficits in their regulatory immune cell populations that make retrieval of a sufficient quantity and quality of cells very challenging[100,101]. Meanwhile, in order to infuse cells during a “window of opportunity” in a patient’s treatment regimen, whilst accommodating the process of *ex vivo* expansion, the progenitor cells must be retrieved several weeks in advance of the treatment starting. Yet, in cadaveric donor transplant procedures, for example, the date of transplantation cannot be predicted in advance. Cells sourced from a biobank would necessarily be derived from allogeneic or xenogeneic sources.

Immunogenicity is a major obstacle to infusion of non-autologous cell therapies[102], as the therapeutic cells may be depleted by the recipient’s immune system before they can exert their effects and this response may exacerbate existing inflammation. For this reason, some researchers are focussing on methods to reduce the immunogenicity of cells prior to infusion. Scientists have genetically-engineered porcine donors to prevent them from expressing highly immunogenic molecules[103]. In an analogous manner, genome editing can be utilised

to delete HLA (Human Leukocyte Antigen) genes from the genome of allogeneic lymphocytes for cell therapy[62,104,105].

The durability of the therapeutic effect conferred by Tregs is likely to be a function not only of cellular longevity, but also the ability of Tregs to propagate their suppressive activity to other cells. One of the major mechanisms by which Tregs deter the activation of T cells is by impeding the maturation of DCs. Whilst mature DCs prime T effs and promote immunity, immature DCs exhibit a more tolerogenic phenotype, producing higher levels of anti-inflammatory cytokines and co-inhibitory molecules[106]. Via modulation of DCs and establishment of an anti-inflammatory milieu (including IL-10, TGF β [107] and adenosine[108]), Tregs can promote expansion of other Tregs and induction of Tregs from T eff precursors in the periphery[109], thereby propagating the tolerogenic microenvironment[110]. By enhancing the ability of Tregs to establish this self-propagating feed-forward mechanism, the effects of a small number of precursor Tregs could be amplified[111].

6. Conclusions and Future Perspectives

It must be acknowledged that there are certain drawbacks to the application of genetic engineering to cell therapy products, compared with pharmacological manipulation. Genetic engineering involves a more cumbersome procedure than simply adding a compound to the culture, as generation of the viral vector takes several days, additional reagents and a host cell line. More importantly, the risks (though often perceived rather than actual) associated with genetic modification *per se*, and with introduction of a viral vehicle[112–114], are not inconsequential. Off-target effects upon the genome are a concern with any genetic engineering strategy. Viral vectors that integrate into the genome do so in a non-site-specific

manner. In fact, strong evidence has been reported that viral integration occurs preferentially within permissive open chromatin conformations that are enriched at actively-transcribed genomic sites[115,116]. In theory, the random integration of a virus into a locus that encodes or regulates an oncoprotein or tumour suppressor gene could result in cancerous transformation of the cell culture. Infusion of a transformed cell therapy product would be of particular danger to an immunosuppressed patient. Furthermore, viral antigens and the transgene product may provoke an immune response[117].

Nonetheless, there are indisputable benefits to the genetic engineering approach to enhance cell therapy products, compared with pharmacological methods. Unless designed otherwise, the biological effects of the genetic modification are stable, not dependent upon continued exposure to a compound. Thus, modification of the therapeutic cells, in isolation, can be conducted entirely *in vitro*, without collateral effects upon other cells within the body that might result from attempts to sustain the treatment of therapeutic cells *in vivo* after infusion. From an economic perspective, it would also be cheaper to apply a single treatment of cells *in vitro* than to administer repeated doses of drugs. Furthermore, genetic engineering can be more specific with respect to its molecular target than pharmacological interventions. Of course, the specificity of the outcome still depends upon intelligent target selection and transgene design.

With the advance of site-specific genome editing platforms, such as zinc-finger nucleases, Transcription Activator-Like Effector Nucleases (TALENs) and Cas9-CRISPR[118], the requirement for virus-mediated transduction to achieve stable genetic modification may become obsolete. Only transient transfection is required to introduce the enzymes and templates required for genome editing. Once the genome editing has occurred, the

modification is permanent. Genome editing technologies theoretically overcome the risk of insertional mutagenesis by virtue of their sequence-specificity. The Cas9-CRISPR system, for example, utilises oligonucleotides to direct enzymatic DNA repair to a unique DNA sequence, which could be a target locus, or to a safe harbour. For lymphocytes, transient transfection remains technically challenging[119] but the development of new vehicles for both oligonucleotide and protein transfection promises to ameliorate these restrictions in the near future[120]. In other respects, genome editing methodologies can outperform traditional genetic engineering techniques by permitting modification of regulatory modules, as well as protein-coding loci, to achieve more subtle or controlled outcomes. Further advances in the design of synthetic regulatory elements promise to add a further layer of control of cellular activity. For example, the convergence of genome editing and optogenetics in the form of photoactivatable CRISPR-Cas9 constructs[121] might permit induction of genome edits simply by exposing engineered cells to light. Additionally, genome editing can be used to inactivate endogenous genes, which would be particularly useful for eliminating the native TCR when re-directing lymphocyte specificity. Inspiration can also be drawn from fields as apparently disparate as materials engineering, which is providing nanomaterials to modulate the function of immune cells with great precision[122,123]. Naturally, these new technologies are also subject to limitations and concerns[124].

With so many emerging technologies at our disposal, there is great scope for creative minds to optimise medical interventions for immune-mediated pathologies. Future strategies may well integrate cell therapy with genome editing, synthetic biology and pharmacological treatment or bioengineered materials. Cells could be programmed to respond to instructions encoded in chemical or physical cues that are under the control of a clinician, whilst still

obeying their inherent biological rule book. Such a strategy could provide the optimal trade-off between efficacy, safety and economy.

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Figure Legends:

Figure 1: Cell therapy using Tregs that have been manipulated *in vitro* prior to infusion

Therapeutic cells extracted from a patient (or a donor) can undergo various processes prior to administration as a cellular therapy. Firstly, the cells of interest must be extracted from the appropriate tissue and fractionated so that the cells of interest are enriched. In the case of lymphocytes therapies, such as Treg therapy, blood is typically fractionated to retrieve total peripheral blood mononuclear cells. From this pool of enriched blood cells, the specific cell population that exhibits the desired characteristics can be isolated with high purity, using technologies such as Fluorescence-Activated Cell Sorting (FACS). The purer the resulting population, the more effective the therapy is likely to be, on a per-cell basis. These purified cell can be subject to various procedures with the aim of achieving the greatest feasibility, safety and efficacy.

Figure 2: Goals of *in vitro* manipulation of therapeutic cells

Ultimately, improvements to cell therapies are directed towards optimising safety, feasibility and efficacy of the resulting therapy. Various beneficial properties promote the fulfilment of one or more of these goals. Feasibility is largely a function of cell yield and longevity of function (which might determine whether repeated dosing is required). The efficacy of the cell therapy product is determined by factors including purity, intrinsic functional potency of the cells, the ability of cells to sustain their function *in vivo*, and the specificity of cells (for either their target or their anatomical site of action, corresponding to the site of the pathology). Safety is dependent not only upon specificity and purity of the cell product but also upon the ability to monitor such parameters *in vivo*, and to intervene, should a high-risk scenario become apparent. All of these properties can be the subject of manipulation *in vitro*, prior to administration of the cell therapy, in order to target the therapeutic cells in isolation.

Figure 3. *in vitro* culture supplements to promote optimal efficacy of cell therapy products

Various classes of culture supplements have been exploited to condition or modify therapeutic cells *in vitro* before administration, with the aim of promoting optimal function of these cells *in vivo*. These supplements include chemical reagents (such as drugs that modify intracellular signalling pathways) and biological agents (such as viral vectors for delivering genetic material to the cell nucleus for genetic modification).

Figure 4. Applications of genetic modification to enhance cellular therapy

Genetic modification is one of the most powerful and versatile tools via which cells can be manipulated *in vitro*. By promoting the expression of effector molecules, one might enhance

the intrinsic potency of each cell. New functionalities could also be introduced via transgenic or synthetic constructs. Cellular specificity can be modified by modulating expression of homing molecules that prompt migration to the desired site of action. For lymphocyte therapies, specificity can be imposed by the introduction of transgenic, synthetic or modular antigen receptors. Safety features, such as inducible genetic switches and “suicide genes” (that cause cells to activate their therapeutic functionality, or to undergo cell death, respectively) can be introduced to give a clinician control over the cells after infusion into the patient.

Genetic Modification for Cellular Therapeutics

safety

- Suicide genes
- Safety switches

specificity

- Transgenic TCRs
- Chimaeric antigen receptors
- Homing molecules

potency

- High/constitutive expression of key proteins
- new proteins/
signalling circuits