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Review

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.

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] (Fig. 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

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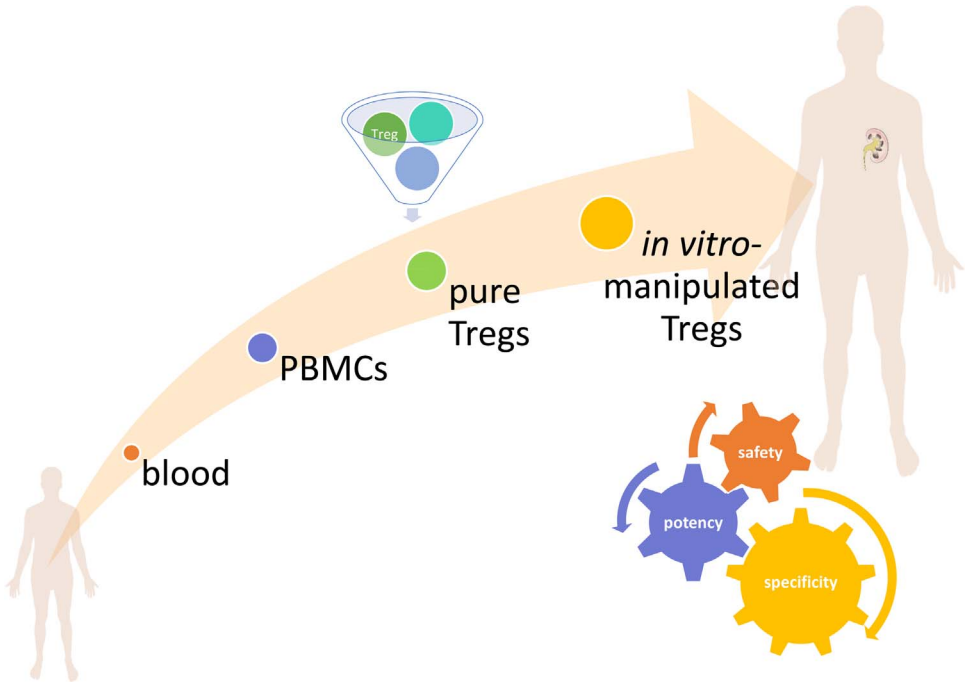

Fig 1. Cell therapy using Tregs that have been manipulated *in vitro* prior to infusion.
Therapeutic cells extracted from a patient (or a donor) can undergo various processes prior to administration as a cellular therapy. Firstly, the cells of interest must be extracted from the appropriate tissue and 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.

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 (Fig. 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 (Fig. 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 (Fig. 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 T-ALENs (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