Review

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Targeted Therapy of Multiple Sclerosis: A Case for Antigen-Specific Tregs

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Abstract: Multiple sclerosis is an autoinflammatory condition that results in damage of myelinated neurons in affected patients. While disease-modifying treatments have been successful in slowing the progression of relapsing remittent disease, most patients still progress to secondary progressive disease that is largely unresponsive to disease-modifying treatments. Similarly, there is currently no effective treatment for patients with primary progressive MS. Innate and adaptive immune cells in the CNS play a critical role in initiating an autoimmune attack and in maintaining the chronic inflammation that drives disease progression. In this review, we will focus on recent insights into the role of T cells with regulatory function in suppressing the progression of MS, and, more importantly, in promoting the remyelination and repair of MS lesions in the CNS. We will discuss the exciting opportunity to genetically reprogram regulatory T cells to achieve immune suppression and enhance repair locally at sites of tissue damage, while retaining a fully competent immune system outside the CNS. In the future, reprogramed regulatory T cells with defined specificity and function may provide life medicines that can persist in patients and achieve lasting disease suppression after one cycle of treatment.

1. Introduction

Multiple sclerosis is a chronic neuroinflammatory autoimmune disease in which autoreactive T cells and B cells infiltrate the blood-brain barrier and wrongly attack the myelin sheath, resulting in demyelination and axonal damage in the central nervous system (CNS). The condition typically manifests as a Relapsing-Remitting MS (RRMS), although the majority of patients advance to Secondary Progressive MS (SPMS) which is characterized by disease worsening without signs of remission (Lorscheider *et al.*, 2016). About 10-15% of MS patients are initially diagnosed with Primary Progressive MS (PPMS), where the disease steadily worsens without any intervals of remission (Faissner *et al.*, 2019, Oh & Bar-Or, 2022).

Currently, the therapeutic landscape of RRMS revolves around disease-modifying treatments (DMTs) that aim to reduce pathology related to relapses to achieve a favorable clinical course (Oh & Bar-Or, 2022). However, despite a long list of approved DMTs, patients still face the progression of disease and worsening of disabilities. Unfortunately, the DMT's benefit in RRMS patients are typically not seen in patients with SPMS and PRMS (Faissner *et al.*, 2019; Oh & Bar-Or, 2022). There is increasing evidence to suggest that the accumulation of axonal loss and neurodegeneration, in association with the local inflammation, leads to irreversible neurological disabilities associated with the clinical advancement in MS. Currently, drugs that can promote neural repair are absent (Absinta, Lassmann & Trapp, 2020; Klotz, Antel & Kuhlmann, 2023). Thus, there is a need to develop novel therapies that could provide solutions to the existing unmet needs of MS patients.

2. CNS is not Immune Privileged

Although the frequency of adaptive immune cells in healthy brain tissue is low, there is a steady state of lymphocyte migration across the blood-brain border, which is often incorrectly described as blood-brain barrier (Engelhardt & Ransohoff, 2012). In addition to low level migration into brain tissue, there is also a population of CNS-resident T_{RM} cells that, in experimental models, can cause

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local tissue damage even in the absence of further T cell infiltration from the blood (Vincenti *et al*., 2022). However, the infiltration of pathogenic T cells from the blood into the CNS plays an important role in the progression of MS. Hence, reducing the migration of T cells across the blood-brain border has been a successful strategy in the management of patients with RRMS. The antibody natalizumab blocks the a4 integrin subunit of VCAM-1 and MDdCAM-1, and thus disrupts T cell adhesion to endothelial cells, resulting in the inhibition of T cell infiltration into brain and spinal cord (Engelhardt & Ransohoff, 2012). Although Natalizumab, and other disease modifying biologics that prevent T cell infiltration, have been effective in RRMS, these interventions are ineffective in SPMS and PPMS. Mechanistically, prevention of T cell infiltration can reduce inflammation and cell damage caused by autoreactive immune cells during the RRMS phase of disease, but it cannot reverse extensive demyelination and tissue damage caused by resident T_{RM} cells. Despite the success of treatment with biologics, the current approaches do not enhance tissue repair or stimulate remyelination in patients with MS.

3. T_{reg} Biology and its Role in MS

T_{reg} cells are a subset of CD4+ T cells that are vital to prevent autoimmunity. FoxP3 is the master transcription factor for T_{reg} cells, and inherited defects in the FoxP3 gene lead to the failure of T_{reg} development which results in fatal autoimmunity in mice (Clark *et al*., 1999) and in the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome in humans (Bennett *et al*., 2001). While the majority of T_{reg} cells develop naturally in the thymus, some can be reprogrammed from conventional CD4+ T cells in peripheral tissues. The T cell receptor (TCR) of thymus-derived T_{reg} cells have been selected for high affinity to self-peptides (Jordan *et al*., 2001), and these cells play an important role in maintaining systemic tolerance. By contrast, peripherally-induced pT_{reg} are formed in response low affinity self-peptides and in response to commensal microbiota and food antigens, and these cells play a role in tissue specific hemostasis and tolerance (Raffin, Vo & Bluestone, 2019). *In vitro* T_{reg} induction from conventional CD4+ T cells has been repeatedly demonstrated by TGF-β and IL-2 to mirror the pT_{reg} lineage commitment pathway ***(Chen et al., 2003), yet these i*** T_{reg} ***cells are prone to lose FoxP3 expression and thus their*** T_{reg} ***functionality. This so-called*** T_{reg} ***plasticity is to a large extent under the epigenetic control of the*** T_{reg} ***-Specific Demethylated Region (TSDR). While the TSDR is completely demethylated in t*** T_{reg} ***cells (Toker et al., 2013), it is partially methylated in p*** T_{reg} ***and i*** T_{reg} (Dhamne *et al*., 2013), ***which may favor loss of FoxP3 expression in these cells after in vitro restimulation (Floess et al., 2007; Toker et al., 2013).*** Similarly, activated effector T cells that can temporarily upregulate ***FoxP3*** have a TSDR that is largely methylated (Baron *et al*., 2007).

T_{reg} cells are equipped with multifaceted mechanisms to achieve their two major roles - immune suppression and tissue homeostasis (Figure 1). Upon activation by cognate self-antigen and sensing a pro-inflammatory environment, T_{reg} cells are attracted to the site of inflammation, release anti-inflammatory cytokines and restrict further T cell activation by hindering antigen presentation. Additionally, local immunosuppression can be achieved by activated T_{reg} through depriving T-cell pro-survival nutrients and signals, such as IL-2, from the local environment (Duffy, Keating & Moalem-Taylor, 2019). The mechanisms by which T_{reg} can repair tissue damage are less well established. Tissue resident T_{reg} can have a direct regenerative ability, as demonstrated in skin and skeletal muscle (Li *et al*., 2018). Amphiregulin, an EGFR ligand crucial to tissue repair, is dispensable for the suppressive function of T_{reg} cells (Arpaia *et al*., 2015), while its production by T_{reg} was found to play a role during the chronic phase after stroke by accelerating neurological recovery (Ito *et al*., 2019). Moreover, the observation that T_{reg} can promote oligodendrocyte proliferation and differentiation *in vitro,* may explain how T_{reg} can enhance oligodendrocytes mediated repair of damaged CNS tissue *in vivo*, as described by Crawford and colleagues (Crawford *et al*., 2016). Mechanistic studies further revealed that CCN3, IL-10 and AIM2 expressed by T_{reg} play an important role in stimulating neuronal tissue repair (Dombrowski *et al*., 2017; Naughton *et al*., 2020; de la Vega Gallardo *et al*., 2020; Chou, 2021).

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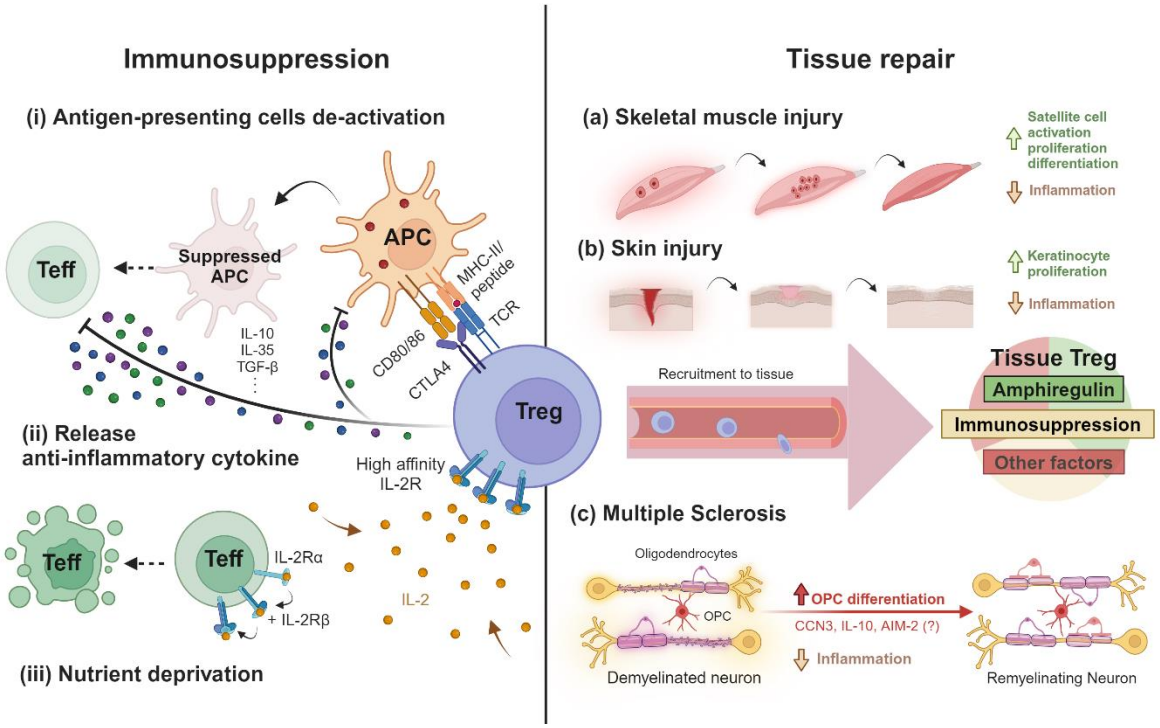

Figure 1. The dual functionalities of T_{reg} in immunosuppression and tissue repair. In lymphoid organs, resting T_{reg} cells will be activated by the cognate antigen presented by antigen-presenting cells (APCs) and elicit immunosuppressive functions, including APCs suppression through (i) dampening the costimulatory molecules CD80/86 by CTLA4 binding and (ii) the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, as well as (iii) scavenging signals and metabolites essential to the survival and functionality of pro-inflammatory cells. On the other hand, circulating T_{reg} cells that are recruited to the tissues by their respective chemokines resolve local inflammation and release amphiregulin and other cellular factors with regenerative functions. Amphiregulin, an EGFR ligand, can promote the activation and proliferation of (**a**) muscle stem cells (satellite cells) and (**b**) keratinocytes to form new muscle fibres and skin layers, respectively. For demyelinating neurons in multiple sclerosis, CCN3, IL-10 and AIM2 produced by T_{reg} cells can promote the differentiation of oligodendrocyte progenitor cells (OPC) achieving remyelination, although the suggested role of CCN3 has been described in vitro and not in vivo. The figure is produced by Biorender.com.

Recent studies have uncovered some abnormalities of T_{reg} in MS patients, yet it has been difficult to establish whether this is a cause or consequence of MS pathogenesis. Direct comparisons of the frequency of peripheral T_{reg} and infiltrated T_{reg} from cerebrospinal fluid between MS patients and healthy individuals had generated unconclusive results (Duffy *et al.*, 2018). However, e*x vivo* phenotyping of peripheral T_{reg} of MS patients revealed reduced FoxP3 expression and impaired suppressive activity (Viglietta *et al.*, 2004; Haas *et al.*, 2007). A study of untreated RRMS patients showed an increased number of Th1-like, IFN-γ-producing FoxP3+ T cells compared to healthy controls (Dominguez-Villar, Baecher-Allan, & Hafler, 2011). This functional plasticity could be due to the highly pro-inflammatory environment of CNS lesions, characterized by IFN-γ, TNF-α, IL-1β, IL-6 and IL-17 (Duffy *et al.*, 2018). The use of animal models has provided more mechanistic understanding of the role of T_{reg} cells in MS disease. Using the most widely used MS mouse model, Experimental Autoimmune Encephalomyelitis (EAE), it has been shown that T_{reg} depletion or dysfunction can promote disease progression which can then be rescued by the adoptive transferr of functional T_{reg} (Duffy *et* al., 2017; Stephens *et al.*, 2009; Kim *et al.*, 2018; Duffy, Keating & Moalem-Taylor, 2019). Considering the dual actions of T_{reg} cells, it is likely that the beneficial effect of adoptive T_{reg} therapy is the concerted result of immune suppressive activities and their ability to stimulate tissue repair.

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4. Novel T_{reg} Based Therapies for MS

To ultimately achieve an increased number of functional T_{reg} in MS patient, a variety of non-cell-based and cell-based therapies are under development. Whilst the former aims at rescuing the patient's own T_{reg} repertoire, cell-based therapies replenish the patient with *ex vivo* expanded and potentially engineered T_{reg}. The toolbox of non-cell based therapies comprises primarily three synergistic approaches, which are peptides for antigen-specific expansion, variants of IL-2 for preferential T_{reg} survival, and small molecules for reinforcing functionality (Eggenhuizen, Ng & Ooi, 2020). Although these strategies have not yet entered the clinic, novel biologic designs aiming at combining all three keys of the toolbox are emerging. Particularly, a recent proof-of-concept study demonstrated the feasibility and efficacy of microparticles decorated with MHC-II molecules containing peptides of myelin oligodendrocyte glycoprotein (MOG) used as a T_{reg} vaccine, which led to targeted expansion of MOG-specific T_{reg} cells via the incorporation of a modified IL-2 molecule stimulating the IL-2 receptor of T_{reg}, as well as simultaneous inhibition of effector T cell proliferation via the release of rapamycin (Rhodes *et al.*, 2023). However, these types of strategies rely on the presence T_{reg} with the vaccine-targeted specificity in MS patients, and they carry the risk of inadvertently expanding pathogenic effector T cells that have the same specificity. Thus, adoptive transfer of antigen-specific T_{reg} represents an alternative therapeutic approach.

5. Antigen-Specific T_{reg} Cell Therapy for MS and its Benefits

Therapeutic T_{reg} can be generated in three ways (Figure 2), which include (i) polyclonal expansion or (ii) antigen-specific expansion of autologous T_{reg} obtained from patients, and (iii) genetic engineering of T_{reg} specificity (Raffin, Vo & Bluestone, 2019).

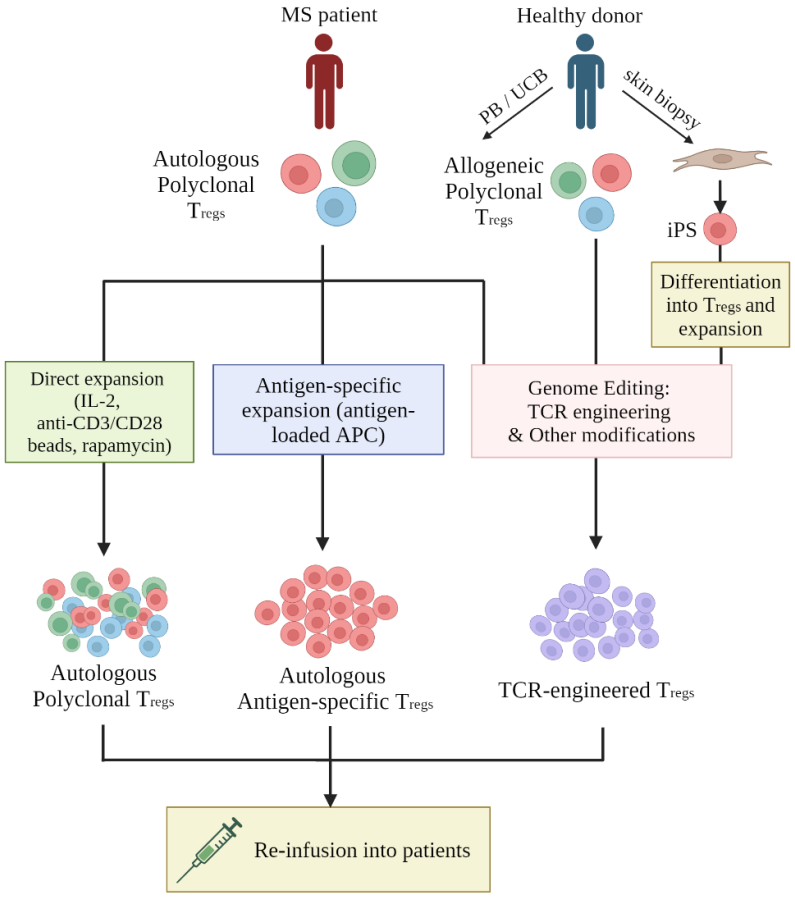

Figure 2. The summary of T_{reg}-based therapy. Several promising sources, including the T_{reg} cells extracted from the peripheral blood (PB) or umbilical cord blood (UCB) or developed from the

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fibroblast-to-induced pluripotent stem (iPS) cell developmental pathway. The figure is produced by Biorender.com.

The observation that multiple autoantigens have been identified as the cause of MS, and more than one clone of autoreactive T cells can be found in patients (Bronge *et al*., 2022), raised the question whether adoptive transfer of polyclonal T_{reg} is preferable over T_{reg} cells with a single specificity. However, T_{reg} cells with specificity for one specific antigen, for example myelin basic protein (MBP), can achieve local suppression of effector T cells present in the same microenvironment, even if these effector cells are specific for several other proteins of the neuronal myelin sheet. For example, experiments in the murine EAE model showed that T_{reg} specific for MBP were able to suppress pathology caused by effector T cells specific for MOG (McGovern *et al*., 2022). The murine EAE model was also used to demonstrated that T_{reg} with defined specificity are more potent than polyclonal T_{reg} in controlling MS-like symptoms (Stephens *et al*., 2009; Kim *et al*., 2018), which is consistent with better disease control by antigen-specific T_{reg} cells observed in other autoimmune animal models (Tang *et al*., 2004; Tarbell *et al*., 2004; Zhou *et al*., 2004; Fujio *et a*l., 2006; Tsang *et al*., 2008). The improved efficacy of antigen-specific over polyclonal T_{reg} is related to the enhanced antigen-driven migration of T_{reg} into target tissues, and the continued TCR-stimulation at the site of pathology which is required for T_{reg} to exert their optimal suppressive function (Eggenhuizen, Ng & Ooi, 2020).

6. TCR-Engineered T_{reg} Versus CAR-Engineered T_{reg}

A population of antigen-specific T_{reg} can be produced by *ex vivo* stimulation with antigen, or by genetic T_{reg} engineering. The frequency of naive T_{reg} that are specific for a desired antigen is very low, and the expansion of these cells by in vitro stimulation is technically difficult and unreliable, compared to the robust process of genetic engineering of T_{reg} specificity (Serra & Santamaria, 2019). Specificity engineering can be achieved by TCR and CAR (Chimeric Antigen Receptor) gene transfer. Both methods involve designing and transducing a transgenic receptor, yet each has its strengths and limitations. Whilst antigen recognition by transgenic TCR is restricted by the HLA genotype of patients, the antigen-binding domain of CAR is a synthetic single-chain variable antibody construct that allows antigen recognition irrespective of HLA. However, in contrast to TCRs, CARs can only recognize proteins present on the cell surface but not proteins in the cytosol or nucleolus, whereas HLA-presented peptides can be derived from any cellular protein for recognition by TCRs. Hence, the number of cellular proteins that can be targeted by TCRs is vastly greater than the proteins targetable by CARs. Currently, the preclinical development of CAR-T_{reg} for MS is lagging behind the use of TCR-engineered T_{reg} (Depil *et al*., 2020).

7. Source of T_{reg}

As shown in Figure 2, human T_{reg} cells can be isolated from Peripheral Blood (PB), Umbilical Cord Blood (UCB) or *in vitro* differentiated from Induced Pluripotent Stem Cells (iPSC). Autologous T_{reg} from PB remain the most common source of T_{reg} used in patients (Eggenhuizen, Ng & Ooi, 2020). The results of a clinical trial in patients with type 1 diabetes produced safety data of autologous *ex vivo* expanded polyclonal T_{reg} (Bluestone *et al*., 2015), prompting the development of T_{reg} therapies for other autoimmune diseases, including MS. In addition, several studies with UCB-derived T_{reg} have demonstrated that they displayed superior phenotypic stability and greater TCR repertoire diversity compared to PB-derived T_{reg} (Seay *et al*, 2017; Motwani *et al*., 2020). As UCB biobanks expand, the use of HLA-matched allogeneic T_{reg} population for the treatment of GvHD, and possibility certain autoimmune conditions, becomes a realistic possibility (Motwani *et al*., 2020).

iPSC is an attractive source for producing large numbers of 'off the shelf' T_{reg} with defined specificity and phenotypic and functional features. However, T_{reg} differentiation from iPSC is still at an early stage of development, and TCR+FoxP3 transduction combined with Notch-1 ligand stimulation was required to achieve T_{reg} differentiation (Haque *et al*., 2012; Haque *et al*., 2016). The use of iPSC-derived T_{reg} as 'off the shelf' medicines would require additional genetic engineering to

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avoid host-mediated recognition and rejection of the adoptively transferred cells (Raffin, Vo & Bluestone, 2019).

8. The Risks and Challenges of Antigen-Specific T_{reg} Cell Therapy

The challenges of T_{reg}-based adoptive cell therapy include common limitations of currently available techniques, and challenges specific to the treatment of MS. ***The first challenge is the yet inconclusive markers for the isolation of*** T_{reg} cells with high ***purity, phenotypic stability, and continued functionality. At present the markers CD4+ CD25+ CD127-/low are frequently used to purify*** T_{reg}***. Unfortunately, staining for FoxP3, an intracellular protein, requires cell permeabilization and is therefore not suitable for the purification of life cells for adoptive therapy, although the vast majority of CD4+ CD25+ CD127-/low cells are FoxP3-positive and thus largely overcomes the inability of using FoxP3 for cell purification. However,*** T_{reg} ***isolation is further complicated by the observation that FoxP3-positive*** T_{reg} ***consist of distinct subsets with distinct functionality. For example, it was shown that*** tT_{reg} ***cells are more resistant*** to phenotypic plasticity than pTreg cells (Miyao et al., 2012), yet any differentiating phenotypic markers remain to be elucidated in humans (Raffin, Vo & Bluestone, 2019). At present, we do not know which Treg subset is most suitable for dampening the neuroinflammation in the CNS in the context of MS.

9. Off-Target Toxicities

Retro and lentiviral gene transfer, which so far has been used in most human applications of CAR or TCR-T cell therapy, can cause genome toxicity by insertion mutagenesis, which has resulted in fatal side effects with gene engineered stem cells (Hacein-Bey-Abina et al., 2003), while similar side effects have not been seen with gene engineered T cells. Owing to the heterodimer nature of TCR alpha and beta chains, mis-paring between transgenic TCR and endogenous TCR is a risk associated with TCR but not CAR engineering. Experiments in murine T cell transfer models have shown that TCR mis-pairing can result in severe side effects in lymphodepleted recipient mice (Bendle et al., 2010). In patients, toxicities caused by TCR mis-pairing have not yet been described, although a large number of patients have been treated over the past years. Nevertheless, strategies for modifying transgenic TCR to avoid pairing with endogenous TCR, such cysteine-modification (Cohen et al., 2007), domain-swapping (Bethune et al., 2016), constant region modification (Sommermeyer & Uckert, 2010) and variable region modification (Thomas et al., 2019), all reduce the risk of mis-pairing. The CRISPR/Cas9 precision gene-editing tool changed the landscape, as it allows specific disruption of TRAC and TRBC loci, which encode the TCRα and TCRβ chain, respectively. As recently demonstrated, the disruption of endogenous TCR expression and insertion of therapeutic CAR or TCR constructs into the TRAC locus has improved the in vivo functionality of engineered T cell products in preclinical models (Eyquem et al., 2017; Roth et al., 2018).

10. Systemic Immunosuppression

Inevitably, any drugs with immunosuppressive activity afford a risk of systemic immunosuppression, whereupon the likelihood of opportunistic infection or malignancy will be elevated. Being a cell therapy, adoptively transferred Treg cells are expected to persist long-term and therefore require careful assessment of possible side effects.

Safety features developed for TCR/CAR engineering for cancer cell therapy can be translated into controlling unwanted systemic immunosuppression of Treg-based therapy. One strategy is to use a suicide gene to control the in vivo survival and/or functionality of the adoptively transferred Treg cells. Numerous 'suicide switched' have been designed and assessed for their efficacy and safety in preclinical studies and in patients, exemplified by truncated EGFR (Paszkiewicz et al., 2016) and inducible Caspase 9 (Mestermann et al., 2019). Another approach to regulating Treg function in vivo is the introduction of synthetic receptors. The requirement of IL-2 for Treg proliferation, survival and function makes IL-2 a promising manipulative pathway (Chinen et al., 2016). Recently, an elegant study has demonstrated that a genetically modified IL-2 receptor binds selectively to a synthetic IL-

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2 molecule that contains a 'matched' modification, while wild type IL-2 does not bind to the modified receptor. Hence, engineered T cells expressing the modified IL-2 receptor responded in vivo only to administered of synthetic IL-2 containing the 'matched' modification but not to endogenous IL-2 (Sockolosky et al., 2018). This technology could be used to engineer Treg whose survival and function in vivo is controlled by the time and dose of synthetic IL-2 administration.

11. Plasticity of Functions

The functional plasticity of Treg cells has been demonstrated repeatedly both in vitro and in vivo following adoptive transfer. Upon cues from inflammatory cytokines and IL-2 deficiency, antigen-specific Treg cells can adopt an alternative transcriptional program and become effector T cells that may contribute to tissue pathology (Sawant et al., 2014). For example, in the murine EAE model researchers identified a population of exFoxP3-Treg that had lost expression of FoxP3 which was associated with the production of the effector cytokine IFNg (Bailey-Bucktrout et al., 2013). However, genetic engineering can be used to reinforce the Treg identity by forcing FoxP3 expression. This can be achieved by delivering a genetic construct that drives FoxP3 expression from a constitutively active promotor (McGovern et al., 2022), or by inserting a constitutively active enhancer/promotor into genome proximal to the FoxP3 gene (Honaker et al., 2022). Both approaches lead to sustained level of FoxP3 expression which 'locks' Treg cells into a stable phenotype. Both approaches can also be utilized to force FoxP3 expression in conventional CD4+ T cells and convert them into Treg-like cells that display suppressive activity in vitro and in vivo (Wright et al., 2009).

12. Conclusions

With its multifaceted immunosuppressive functions and remyelinating capacity, T_{reg} cells are undoubtedly an ideal cellular candidate for MS immunotherapy. Adoptive therapy with engineered T_{reg} is now at the exciting stage where the first human clinical trials are ongoing, and will soon provide eagerly awaited feasibility, safety and efficacy data. Success of T_{reg} therapy will depend on the selection of suitable target antigens that stimulate the suppressive activity and the tissue repair function of T_{reg} at the local site of disease, without any systemic impairment of the immune system.

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