How Antigen-Specificity Directs Regulatory T Cell Function: Self, Foreign and Engineered Specificity

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Abstract

Regulatory T cells (Tregs) are a suppressive subset of T cells that have important roles in maintaining self-tolerance and preventing immunopathology. The T cell receptor (TCR) and its antigen-specificity play a dominant role in the differentiation of cells to a Treg fate, either in the thymus or periphery. This review focuses on the effects of the TCR and its antigen specificity on Treg biology. The role of Tregs with specificity for self-antigen has primarily been studied in the context of autoimmune disease, although newer studies have focused on their role in steady state conditions. The role of Tregs that are specific for pathogens, dietary antigens and allergens is much less studied, although recent data suggest a significant and previously underappreciated role for Tregs during memory responses to a wide range of foreign antigens. The development of TCR- or chimeric antigen receptor (CAR)-transduced T cells means we are now able to engineer Tregs with disease-relevant antigen-specificities, paving the way for ensuring specificity with Treg based therapies. Understanding the role that antigens plays in driving the generation and function of Tregs is critical to defining the pathophysiology of many immune mediated diseases and to developing new therapeutic interventions.

1. INTRODUCTION

The ability of the immune system to discriminate between self and non-self is critical for maintaining self-tolerance. CD4⁺ regulatory T cells (Tregs) are essential for maintenance of peripheral tolerance, with a dominant role in prevention of autoimmunity and immunopathology during immune responses to foreign antigens (1). Work to date has focussed on developing cell-based therapies with polyclonal populations Tregs to restore peripheral tolerance, but it is increasingly clear that use of Tregs with defined-specificities will be a more specific and potent approach. Examples of antigens for which specific Tregs could be developed include: self-antigens that drive autoimmune disease, harmless environmental antigens that trigger allergic and inflammatory responses; and allogeneic antigens present on transplanted organs/cells or in recipients of hematopoietic stem cell transplants. Understanding how Tregs specific for these antigens normally function to restrain harmful immune responses will be critical to the design of regulatory cell-based therapies.

Tregs can develop in the thymus or the periphery, with most known about the development of Tregs that express the FOXP3 transcription factor. In mice, it is possible to track the origin of Tregs, enabling precise definition and study of thymically-derived (tTregs) versus peripherally-derived (pTregs). In humans however, it is very difficult to discriminate between pTregs and tTregs (2), with the vast majority of data obtained from mixed populations of FOXP3⁺ cells. In addition to FOXP3⁺ Tregs, there are several other types of regulatory T cells, such as IL-10 producing T regulatory type 1 (Tr1) cells (3).

In this review we will focus on FOXP3⁺ Tregs as most information on antigen specificity in regulatory T cells comes from studies of this subset. We will discuss how the antigen-specificity of CD4⁺ Tregs drives their generation, function and maintenance *in vivo*. We will also review what is known about the biology and possible clinical applications of Tregs with natural specificity for self or foreign antigens, as well as methods to engineer Treg specificity.

2. TREGS WITH SPECIFICITY FOR SELF-ANTIGEN

2.1 Thymic development of self-specific Tregs

During thymic development central tolerance is established by deletion of the majority of self-reactive T cells. However, this process is not completely effective, with circulating self-reactive T cells readily detected in healthy individuals (4). Therefore, in parallel to deletion, thymic selection of auto-reactive Tregs that subsequently mediate dominant regulation in the periphery is essential to prevent autoimmunity. During thymic selection, after initial rearrangement of the α and β chains, T cells interact with major histocompatibility complex (MHC) molecules expressed on cortical thymic epithelial cells (cTECs). If the TCR is non-functional and/or unable to recognize self-MHC, then the absence of TCR signal results in cells 'dying by neglect' (reviewed in (5)). If the TCR is functional, then a positive signal stimulates thymocytes to relocate to the thymic medulla where they interact with a range of antigen-presenting cells (APCs) and medullary thymic epithelial cells (mTECs).

In the medulla, developing T cells encounter a range of tissue specific antigens (TSA) presented by MHC class II, as well as signals from co-stimulatory molecules such as CD80 and CD86. The autoimmune regulator protein (AIRE) is expressed by mTECs and is an important regulator of TSA expression levels. The affinity of the interaction between the TCR and the TSA-MHC class II complexes on mTECs has a major role in determining cell fate: cells with a low affinity TCR interaction are positively selected and survive, whilst high affinity interactions result in cell death (6). It is widely believed that tTregs develop from cells expressing an intermediate affinity TCR that is higher than required for positive selection but lower than the threshold for negative selection (5). This concept has been elegantly demonstrated *in vivo* using a green fluorescent protein (GFP) reporter mouse model, in which GFP fluorescence correlates with TCR signal strength (7). This study observed that Tregs were selected in response to stronger TCR signals than conventional T cells, yet these signals were still below the threshold for negative selection. Epigenetic studies of tTregs have suggested that it is the duration of TCR signalling which initiates hypomethylation of CpG sites within key Treg genes including *FOXP3* and *CTLA4*, an important step in driving thymocytes towards a regulatory T cell fate (8).

The importance of AIRE for thymic Treg development has been controversial, as the study of AIRE-deficient mice suggested it was not necessary for thymic Treg selection (9). However, another study identified that AIRE-dependent antigen-presentation preferentially selects Tregs expressing TCRs that have a low frequency in the overall TCR repertoire (10). Therefore, the

effect of AIRE deficiency on the Treg TCR repertoire is likely being underappreciated if repertoire analysis is performed on the total Treg cell population instead of at a single cell level. Interestingly, in mice, AIRE-dependent generation of tTregs during the perinatal period results in tTregs with distinct gene expression profiles and TCR repertoires compared to those generated during the adult period (11). This is likely due to differences in antigen processing machinery and presenting cells in the perinatal and adult thymus. Studies in humans are still lacking, but should these findings be confirmed then they would have important implications for the use of adult Tregs for treatment of autoimmune diseases. As Tregs with the required specificities for might only develop in the neonatal or paediatric period, adult Tregs might not have the appropriate breadth of specificity for certain disorders.

The size of the tTreg population is limited by the clonal precursor frequency (12), the presence of cognate antigen (13) and the availability of instructive cytokines (reviewed in (14)). Hence, when antigen is abundant due to low precursor frequency or high antigen availability, then IL-2 is limiting (15). In contrast, when antigen is rare, then TCR stimulation is limiting due to feedback competition. Additionally, mature Tregs that are resident in the thymus or recirculating from the periphery – which increases with age in mice – compete with newly developing tTregs for antigen and instructive cytokines (16). Hypotheses for why Tregs are recirculating to the thymus include (i) they provide additional cues that instruct tTreg development or (ii) they suppress developing tTregs that would otherwise dilute the pool of useful Tregs in the periphery or (iii) they prevent the development of tTregs with the duplicate specificities to achieve greater diversity (16). Interestingly, a recent study that investigated the relationship between mTEC numbers and tTreg generation, found that increasing the availability of mTECs leads to increased recirculation from Tregs from the periphery, rather than increased tTreg generation (17).

In addition to TCR-mediated signalling, other cues are required to drive commitment of cells to the Treg lineage. Co-stimulation through CD28 interactions with CD80 and CD86 molecules on APCs and cytokine signalling are both indispensable for the thymic induction of tTregs (14). TGF- β , produced by thymic macrophages, also induces FOXP3 expression and tTreg differentiation in mice (18), although it is unknown if this is also true in humans. TCR signal

transduction through NF- κ B pathways is also required for the induction of FOXP3 expression and subsequent commitment to the Treg lineage (19).

3.2 Maintenance of self-specific Tregs

After development in the thymus, tTregs are maintained in the periphery and receive a range of signals that determines their behaviour. Homeostasis of peripheral Tregs relies on cytokine signalling and TCR stimulation. Continuous interaction of TCR with cognate antigen is required for maintenance of Treg suppressive function and homeostatic proliferation (20-22). Consistent with the hypothesis that tTregs originate from thymocytes with higher TCR-affinities, they also receive stronger TCR signals than conventional CD4⁺ T cells in the periphery, as shown using a reporter mouse for TCR signal strength (7). However, continuous FOXP3 expression and maintenance of a Treg-specific methylation pattern are independent from steady-state TCR signals and mainly rely on cytokine signals (20). IL-2 plays a major role in peripheral Treg homeostasis and is required for Treg survival, regulation of key Treg-signature molecules and controls the size of the Treg pool (14). Additional cytokines involved in the maintenance of murine memory Tregs in the skin, ST2⁺ Tregs in the intestine, and memory Tregs accumulating with age are IL-7, IL-33, and IL-15 respectively (14). Co-stimulatory signals via the CD28 and CD40 pathways are also important for Treg homeostasis in the periphery, with CD28-deficient and CD40-deficient mice both shown to have a severe deficit of Tregs (23, 24).

Although memory CD4⁺ T cells are long-lived, there is a gradual decline in their numbers over time (25). Whilst this decline is not fully understood, there is evidence that T cells with high avidity are preferentially selected for maintenance in the memory pool (26). The benefit of this mechanism would be a rapid, highly effective immune response upon antigen re-exposure. This also implies that Tregs with their high avidity TCRs are maintained at high levels within the memory cell population (27). However, it has also been shown that memory Tregs in the skin can be maintained for long periods without continued antigen-receptor signalling (28).

3.3 Function of self-specific Tregs

Different mechanisms exist to keep self-antigen-specific non-regulatory T cells from inducing autoimmunity. They are either negatively selected in the thymus, regulated by Tregs in the

periphery or remain ignorant of their target antigen by seclusion of the antigen in immune-privileged zones. Depending on expression of TSA in the tissues and in the thymus (via AIRE), one or several of these mechanisms will be critical to ensure tolerance. Two publications recently investigated these mechanisms by using Cre recombinase as a neo self-antigen (22) or eGFP, eYFP or CFP as model self-antigens (29) expressed in a tissue-restricted manner. It was observed that tolerance to TSA expressed only in secluded tissues and absent in the thymus was maintained by T cell ignorance, whereas tolerance to ubiquitously expressed TSA was achieved through negative selection (deletion) of self-reactive T cells in the thymus. Immunity to TSA expressed at low to intermediate levels was controlled by increased tTreg development in the thymus. Therefore, the level of thymic expression of self-antigen negatively correlates with self-antigen-specific tTreg development.

Tregs continuously suppress auto-reactive T cells in both steady state conditions and active autoimmune disease. It has been shown that Tregs expressing pSTAT5 co-aggregate with and suppress IL-2-producing auto-reactive effector T cells in the lymph nodes (30). This clustering of Tregs and effector T cells involves TCR-dependent mechanisms, indicating that antigen-specificity plays an important role in directing Treg-mediated suppression (30). The continuous regulation of auto-reactive effector T cells demonstrates the importance of self-specific Tregs and helps explain why, after deletion of functional Tregs, the onset of autoimmune disease is so rapid (31).

4. TREGS WITH SPECIFICITY FOR FOREIGN ANTIGEN

Although most Tregs are thought to be specific for self-antigens, a substantial portion recognizes foreign antigens (32). These foreign antigen-specific T cells are important for maintaining tolerance to harmless dietary, environmental and commensal antigens, controlling immune responses towards pathogens to limit immunopathology, and aiding in the development of robust memory responses.

4.1 Development of foreign-antigen-specific Tregs

Considering the mechanism of their development through a TCR-instructive pathway, tTregs are mostly specific for self-antigens (33), although foreign antigen-specific Tregs can also develop

in the thymus (see section 2.1 above). Most foreign antigen-specific tTregs are therefore pTregs that are generated during an immune response to antigen in the periphery. Although both tTregs and pTregs appear to be preferentially induced as a result of higher avidity TCR interactions (27), it appears that there is minimal overlap between their TCR repertoires (2). This suggests that tTregs and pTregs are distinct populations that originate from different precursor cells.

It is still debated whether pTregs arise from a pre-committed cell lineage, or if any T cell can convert into a pTreg depending on the cytokine milieu. It has been hypothesised that CD4+FOXP3⁻ conventional T cells thymically imprinted with a Treg 'epigenome' are pre-disposed to become pTregs upon receiving TCR signals of appropriate strength in the periphery (34). In contrast, peripheral conversion of pTregs in mice has been shown not to require pre-activation in the thymus and most pTregs were converted from recent thymic emigrants, with cell maturation limiting susceptibility to pTreg conversion (35). It has also been demonstrated that antigen dose is a factor in determining pTreg conversion, with lower doses favouring the generation of FOXP3⁺ pTregs (36). In addition to low doses of antigen, pTreg conversion requires a high avidity TCR interaction, the strength of which is below that required for activation of conventional T cells (27). These data suggest that the nature of the TCR expressed may, to some extent, pre-dispose a T cell towards Treg fate in the periphery.

Due to a lack of unique surface markers, pTregs have often been indirectly studied through *in vitro* induction of Tregs, termed iTregs. There is evidence that unstable FOXP3 expression in iTregs, generated in culture with TGF- β , is due to incomplete demethylation of the FOXP3 Treg-specific demethylated region (TSDR) (37). Therefore, extrapolating data from experiments with iTregs should be treated with caution, as they may not represent a truly committed Treg population.

The TCR repertoire of foreign-specific Tregs provides clues regarding their origin and function. Studies of FOXP3⁺ Tregs from mice and humans indicate that these cells have a diverse TCR repertoire, which is required for their optimal function *in vivo* (38, 39). However, there are few studies which specifically examined the TCR repertoire in foreign-antigen specific Tregs. Studies with transgenic mice found that Tregs and conventional T cells specific for the same

foreign haemoglobin antigen have distinct TCR repertoires (40). In contrast studies in humans found that CMV-specific regulatory and effector T cell clones shared TCRs (41), suggesting they arose from a shared precursor. If Tregs and conventional T cells indeed express the same TCRs then they would have the same affinity for antigen. Therefore, the observed differences in functional avidity between these cell subsets would be due to the actions of other molecules that stabilise the TCR/pMHC interaction, such as CTLA-4 (27, 42).

4.2 Tregs with specificity for intestinal antigens

An important role for foreign-antigen specific Tregs is maintenance of tolerance to intestinal antigens such as dietary antigens and commensal microbiota. Most intestinal antigens are harmless and do not trigger immune responses, however, if this tolerance is altered then acute allergic responses or chronic inflammatory disease can develop. For example, in inflammatory bowel disease, loss of regulatory responses to bacterial flagellin is thought to have a major role in triggering pathogenic inflammation (43). It is widely assumed that Tregs specific for these intestinal antigens develop in the periphery – a process that seems to be facilitated by commensal bacteria (14). However, when Cebula et al investigated the origin of commensal-specific Tregs, they used single-cell high-throughput sequencing of TCRs and found the vast majority of TCRs expressed by colonic and intestinal Tregs were shared with CD4⁺ FOXP3⁺ Tregs in the thymus, suggesting that the intestinal Tregs were thymically-derived. These data contrast to those in another study, which found most colonic Tregs were peripherally-derived (44). Of interest is whether these commensal-specific tTregs are truly selected as microbiota-specific cells or whether they arise due to cross-reactivity with a self-antigen selected TCR (45).

In the setting of food allergy, the triggering peptides and their HLA restriction are often unknown, making it difficult to study pathogenic versus regulatory antigen-specific T cells. One example of a dietary antigen-triggered disease where peptides and HLA restriction are precisely mapped is celiac disease, making this an excellent model to examine the role of foreign antigen-specific Tregs. Celiac disease is a chronic inflammatory disorder of the intestinal system induced by exposure to dietary gluten, a prolamine found in wheat, rye and barley sources (46). Upon ingesting gluten the protein is modified by the gut enzyme tissue transglutaminase 2 (TG2), which deamidates neutral glutamine residues to negatively charged glutamate (46). These

modified peptides can then be presented in complex with HLA-DQ2 or DQ8 triggering a pathogenic CD4⁺ T cells response. Notably, only a small fraction of individuals with these HLA types develop celiac disease, so there are clearly other genetic and/or environmental factors that trigger the full loss of tolerance.

Several groups have analyzed the frequency of peripheral and mucosal Tregs and found that they seem to be increased in untreated disease, but return to normal or lower proportions in individuals on a gluten-free diet (47, 48). Increased proportions of Tregs at sites of inflammation may seem counterintuitive, but this finding is consistent with many studies in other diseases and is likely due to unsuccessful attempts to enhance regulation to limit inflammation (reviewed in (49)). Functional studies of polyclonal Tregs isolated from the lamina propria of celiac disease patients found these cells could suppress heterologous effector T cells, yet were unable to suppress autologous T effector cells (50, 51). Collectively, these studies of antigen-non-specific Tregs suggest that, similar to type 1 diabetes and multiple sclerosis (52), there are sufficient functional Tregs in celiac disease but that effector T cells have become 'resistant' to Treg-mediated suppression (53).

In contrast, we recently studied gluten-specific Tregs and found that gluten-specific, but not polyclonal, Tregs had impaired suppressive function. Surprisingly we also found that CD39⁺FOXP3⁺ Tregs comprise a major proportion (>80%) of gluten-specific CD4⁺ T cells that return to circulation upon oral gluten challenge in celiac disease patients on a gluten free diet (Cook *et al*, 2016, unpublished data). By comparison, the proportion of circulating antigen non-specific memory Tregs were decreased in these subjects compared to healthy controls. These findings highlight that study of disease-relevant antigen-specific Tregs, rather than polyclonal Tregs, is be critical to fully understand the immunopathology of autoimmune and allergic disease and to develop appropriate therapies.

4.3 Tregs with specificity towards pathogens

Tregs have been identified with specificity to bacterial (54) and viral antigens (1) and play an important role in modulating immune responses against invading pathogens and parasites to prevent immunopathology. Clonotypes of these Tregs have been found in the pre-infection pool,

indicating that those are not pTregs developing upon infection but tTregs that have developed in the thymus (55). This notion is supported by the observation that, in mice, thymus-derived Tregs proliferate in response to influenza virus-antigen, whereas there is minimal peripheral pTreg induction from naïve CD4⁺ T cells (56). In contrast, other studies suggest that Treg expansion upon acute viral infection is driven by antigen-nonspecific mechanisms such as cytokines, enhanced presentation of self-antigens or activation of receptors for pathogen-associated molecular patterns (PAMPs) on Tregs themselves or other immune cells (57).

In the case of chronic viral infections, such as HCV and HIV, the effect of Tregs may not be entirely beneficial, although this is an area of controversy. Some studies of chronic viral infections suggest Tregs inhibit crucial anti-viral responses (58, 59), whilst others argue they beneficially limit bystander tissue damage by reducing chronic immune activation (60, 61). Studies of encephalitis in mice show that virus-specific Tregs reduce disease severity through suppressing effector T cell responses during the priming and effector stages of the immune response (62). A murine study of influenza A virus infection also demonstrated that, during recall responses, viral antigen-specific memory Tregs are critical for moderating memory CD8⁺ T cell proliferation and reducing lung inflammation (63). Pathogen-specific Tregs also appear to be an important part of memory immune responses in humans, with *in vivo* studies of varicella zoster virus demonstrating that virus-specific effector and regulatory CD4⁺ T cells accumulate in parallel at the site of antigen challenge (64). The same study also observed that the amount of virus-specific Tregs in the skin inversely correlated with clinical responses to antigen, indicating they are modulating effector responses *in vivo*.

Several studies investigating chronic hepatitis C virus (HCV) or human immunodeficiency virus (HIV) infection support a detrimental role for Tregs. A study of HIV-infected patients found that the percentage of regulatory cells within HIV-Gag specific CD4⁺ T cells pre-antiretroviral therapy was associated with a worse recovery of CD4⁺ T cell after commencing therapy (32). Studies in HCV have found clear evidence for HCV-specific Tregs (65, 66), including identification of dominant Treg-specific epitopes (66, 67). There does not appear to be a functional defect in HCV-specific Tregs, with *in vitro* assays determining they can suppress HCV-specific CD8⁺ T cell responses (65). These data suggest that finding ways to limit

suppression mediated by HCV-specific Tregs during acute HCV infection, or vaccination, could be beneficial. This concept is supported by pre-clinical studies where the efficacy of HCV vaccination in mice, utilising recombinant adenovirus expressing the viral protein NS3, was significantly enhanced (from 60% to 100%) by co-administering a peptide inhibitor of FOXP3 (68). This peptide inhibitor, p60, prevents nuclear translocation of FOXP3 and inhibits Treg suppressive function in both mice and human cells (68). Similarly, alternative strategies to deplete Tregs to enhance vaccination efficacy (e.g. with anti-CD25 and anti-CTLA-4 antibodies) are being explored (69), but it will be critical to develop methods to ensure that these approaches only deplete disease-relevant Tregs to limit the risk of developing systemic autoimmunity. Moreover, it has also been reported that in some settings Tregs actually enhance the avidity of primary CD8+ T cell responses (70), so in order to apply these concepts in humans, a better understanding of the phenotype and epitope specificity and function of virus-specific human Tregs is required.

4.4 Using Tregs to generate tolerance to ingested antigens

The suppression of immune responses to antigen that is administered via the oral route is known as 'oral tolerance'. This is a continuous process that has evolved to prevent immune responses to harmless dietary antigen and maintain tolerance to commensal bacteria (71). Two proposed mechanisms of oral tolerance are: (i) the induction of pTregs in response to low doses of antigen; or (ii) the induction of clonal deletion, or anergy, of effector T cells through high doses of antigen (72). Involvement of pTregs in the induction of tolerance to ingested antigen was first proposed after observing that oral delivery of chicken ovalbumin (OVA) to tTreg deficient mice generated FOXP3+ Tregs in the periphery (73). Additionally, it was found that mice with an inability to generate pTregs were unable to control Th2 cell-mediated gastrointestinal and airway inflammation (74). The knowledge that orally administered antigens can induce pTreg-mediated tolerance has been utilised in several human clinical trials of autoimmunity, with the outcomes proving the safety and moderate efficacy of the administered antigens (75, 76). A proof-of-principle study with oral administration of a *Lactococcus lactis* strain engineered to secrete deamidated DQ8-restricted gliadin epitopes, which is able to induce gluten tolerance in DQ8 transgenic mice, has also shown promising results in celiac disease (77). This tolerance was due to generation of FOXP3+ pTregs in the spleen and gut-associated

lymphoid tissue (77). Interestingly, the same approach, with *L. lactis* engineered to secrete proinsulin, has been utilised to cure diabetes in NOD mice (78).

It has been hypothesised that the ability of pTregs to respond to very low doses of antigen could be a key mechanism behind their ability to induce oral tolerance, including establishing tolerance to allergens in sensitive individuals through low-dose therapy (79). This therapeutic approach involves administering gradually increasing doses of soluble antigen, typically through subcutaneous delivery, over a period of months or years to induce tolerance (79). Therefore, it is feasible that this process could also be used to re-establish tolerance towards self-antigens in autoimmune disease. Consistent with this notion is the findings from a phase1/2 clinical trial of children with genetic susceptibility for type 1 diabetes. This study found administering high dose oral insulin generated immune responses with approximate 1:1 ratio of effector to regulatory T cells without any hypoglycaemia (80).

Overall, current data suggest that within recall responses to foreign antigens, the balance between antigen-specific effector and regulatory T cells governs outcomes, with the optimal ratios varying depending on the nature of the disease. The growing recognition of previously unappreciated roles of Tregs in primary and secondary immune responses to foreign antigens implies that antigen-specific Tregs are a critical immune parameter to consider when assessing the effectiveness of vaccination and T cell-based therapies.

5. CLINICAL APPLICATIONS OF IN VIVO DIFFERENTIATED TREGS

Boosting the numbers of Tregs is an attractive therapeutic option for many diseases, with several clinical trials having examined the safety and feasibility of polyclonal Treg-based therapies, including for T1D (81, 82) and for graft-versus-host disease (83-85). The results from completed trials are promising, revealing that Treg therapy is safe and possibly effective in reducing inflammation. Additionally, trials using polyclonal Tregs to induce tolerance after solid organ transplantation are currently ongoing for liver (Treg trial NCT01624077, ThRIL trial NCT02166177) and kidney transplantation (TRACT Trial NCT02145325). **Table 1** provides a summary of the therapeutic approaches with human FOXP3⁺ Tregs that are discussed in this review.

The major risk associated with polyclonal Treg based therapy is that of non-specific suppression of important immune functions. As such, Brunstein et al. reported that patients treated with polyclonal Treg cell therapy had increased cumulative density of opportunistic infections compared to historical controls within the first 30 days post cell-infusion (86). Therefore there is a strong rationale to pursue antigen-specific approaches. Studies of alloantigen-specific Tregs have shown they offer superior protection for skin allografts in mice (87, 88) and reduce graft versus host disease (GVHD) pathology (89). When compared to polyclonal Treg populations, Tregs isolated from pancreatic lymph nodes or pulsed with islet antigen were significantly better at preventing and curing type 1 diabetes in mice (90-92). Furthermore, it has been demonstrated in preclinical humanized mouse models that antigen-specific Tregs are more effective compared to polyclonal Tregs in preventing graft rejection (93, 94) and can therefore be used at lower doses thus lowering the risk of pan-immunosuppression.

In humans, donor-specific expanded Tregs are been tested in the context of liver transplantation (delta Trial NCT02188719, ARTEMIS Trial NCT02474199). Furthermore, GMP-compatible protocols have been developed to generate clinically relevant numbers of donor-specific Tregs using CD40L-activated allogeneic B cells (94) and anti-CD3/anti-CD28-coated microbeads (82). Therefore, as we identify the self-antigens that drive disease autoimmune diseases and the allogeneic antigens that cause graft rejection and graft versus host disease in transplantation, the subsequent development of antigen-specific Treg-based treatments may also be a safer and more efficacious approach than using polyclonal Tregs for these indications.

6. TREGS WITH ENGINEERED SPECIFICITY

The major challenge to antigen-specific Treg-therapy is that it requires methods to isolate and expand sufficient numbers of rare *in vivo* generated antigen-specific Tregs without loss of specificity or function. Therefore, several groups have investigated methods to engineer their specificity either by transducing Tregs with either a TCR or chimeric antigen receptor (CAR).

6.1 Transgenic TCR Tregs

Over-expressing the α and β chains of a desired TCR has long been used as an approach to engineer T cell specificity, with a substantial body of work from the field of cancer immunotherapy (reviewed in (95)) which can be readily applied to Tregs. As an example, in a study of murine experimental autoimmune encephalitis (a model of multiple sclerosis) adoptive transfer of Tregs that expressed a transgenic T cell receptor (TCR) specific for a disease-relevant autoantigen (myelin basic protein) were superior to polyclonal Tregs at suppressing central nervous system inflammation (96). Similar results have been obtained in mouse studies of solid organ transplantation. Tregs expressing a transgenic TCR with direct alloreactivity, i.e. recognises a donor MHC class II molecule on the surface of donor cells, were superior to polyclonal Tregs at prolonging allograft survival, with their suppressive function dependent on the presence of alloantigen (97). Transducing Tregs with direct allospecificity with a transgenic TCR that has indirect allospecificity, i.e. recognises donor alloantigen when presented in complex with recipient MHC on the surface of recipient APCs further enhanced these protective effects (98).

Human Tregs have also been successfully transduced with TCRs that recognise HLA-A2-restricted peptides from either HIV or melanoma antigens (99, 100). These Tregs could suppress effector T cell responses regardless of stimulating antigen, although this function appeared to require the presence of dendritic cells (101). Interestingly, the affinity of the transduced TCR had no effect on the antigen-specific suppressive function of the generated Tregs, indicating that the function of fully differentiated *ex vivo* Tregs is not affected by strong TCR signals (100). Furthermore, CD4⁺ Tregs can be effectively redirected with a low affinity HLA Class I-restricted TCR, suggesting less dependency on CD4 signaling for Treg function, and illustrating that engineered specificity of Tregs can be distinct from that of CD4⁺ T effector cells.

Another recent study focused on generating antigen-specific Tregs to treat hemophilia A patients that suffer from factor VIII (FVIII)-specific T- and B- cell (102). In this study, a TCR was cloned from a FVIII-specific effector T cell clone, and then transduced into human CD4⁺CD25^{hi}CD127^{low} Tregs, followed by polyclonal or antigen-specific expansion *in vitro*. The

transduced Tregs had increased FOXP3 and GARP expression after antigen-specific expansion and could suppress antigen-specific T effector cell proliferation and antibody production by B cells more effectively than mock-transduced Tregs.

As an alternate approach to transducing *ex vivo* Tregs, it is also possible to transduce induced pluripotent stem cells (iPSCs) with both a transgenic TCR and FOXP3. In this study murine iPSCs were retrovirally transduced with a construct containing genes for an ovalbumin(OVA)-specific TCR and FOXP3 and the transduced cells were then differentiated using notch signalling (103). The iPSC-derived transgenic Tregs could suppress OVA-specific effector T cells *in vitro* and *in vivo* by reducing inflammation and joint destruction in an antigen-induced arthritis mouse model during OVA challenge. These studies have provided proof-of-principle for several protocols to generate therapeutically relevant numbers of transgenic TCRs and work is needed to validate these approaches with human cells and human disease-relevant antigens.

6.2 CAR Tregs

An alternative strategy for generating antigen-specific Tregs is to transduce them with a chimeric antigen receptor (CAR). CARs are synthetic proteins created by combining an extracellular antigen recognition domain with trans-membrane and signalling domains derived from the TCR and T-cell-relevant co-stimulatory molecules (104). The antigen-binding domain of a CAR is commonly derived from an antibody by generating a single-chain variable fragment (scFv) fusion of the complementarity determining regions of the heavy and light chains of a monoclonal antibody. A major advantage of scFvs is that they are not MHC-restricted, meaning that the relevant patient population is much larger than with the approach of TCR overexpression.

Expression of CARs to engineer specificity for tumour antigens is now a major area of focus in cancer immunotherapy (105), paving the way for similar approaches to be used in regulatory cells. For example, the surface protein CD19, which is expressed on B cells and some malignancies arising from B cells, has been successfully targeted by CAR T cell therapy in lymphoma patients. Other cell-surface antigens that have been targeted include, but are not limited to, CD20 (B cell lymphomas), Her2 (breast cancer), CEA (colorectal cancer), GD2

(neuroblastoma) and mesothelin (mesothelioma). Importantly, the use of a single chain antibody does not exclude targeting peptides that would normally be presented in MHC. For example, a melanoma peptide in the context of HLA-A1 has been targeted with a CAR (106).

The potential that CARs can boost Treg cell therapy the same way they boost cell therapy for cancer is exciting; this simple way of introducing antigen specificity could reduce generalized immunosuppression, activate a larger proportion of Tregs. In addition T cell signalling pathways could be specifically manipulated to increase the suppressive activity and longevity of transferred Tregs. Because CAR Tregs would not be targeting a particular cell type, i.e. a cancer cell as in CAR T cell therapy, and because their off-target effect are less likely to be lethal compared to cytotoxic T cells, the choice of target antigens for Tregs is likely to be broad. For example, an entire tissue may be targeted rather than a protein restricted to a cancer cell. One such example is MOG-CAR FOXP3-transduced Tregs, which protect mice against EAE (107). Although MOG is a model antigen that drives expansion of auto-reactive cells, it is not clear that MOG-CAR Tregs are reacting to antigen in the same way as T cells. Specifically, MOG-CAR Tregs traffic to the CNS where they are activated and retained, potentially suppressing immune responses through local anti-inflammatory cytokine production and IL-2 consumption. Similarly, targeting an antigen in the colon via carcinoembryonic antigen(CEA)-CAR Tregs allows for potent suppression of CEA-CAR conventional T cells after trafficking to the site of antigen (108).

Recently we developed CAR technology to engineer the specificity of human Tregs towards a transplant-relevant antigen: HLA-A2. We found that over-expression of the HLA-A2-CAR in Tregs did not affect their phenotype or function, with full preservation of suppressive function even when expanded via stimulation through the CAR rather than their endogenous TCR. Using a mouse model of graft versus host disease, we also found that HLA-A2-CAR expressing Tregs were significantly better than polyclonal Tregs at preventing xenogeneic GVHD (109). These promising findings suggest that expression of CARs in Tregs may be just as powerful at turning off undesired immune responses as CAR expression in effector T cells is at turning them on. Nevertheless, more research is needed to fully understand the mechanisms of suppression

induced by CAR stimulation, and to investigate their specificity and possible undesired non-specific immunosuppressive effects.

7. CONCLUSION

The endogenous TCR expressed by Tregs influences all aspects of their biology: from their development in either thymus or periphery, to their function and maintenance. As well as refining our understanding the role for self-specific Tregs in preventing autoimmunity, we are also discovering that Tregs comprise a substantial proportion of the CD4⁺ T cell memory response to most foreign antigens. The ability to induce Tregs through the oral route also has potential to be harnessed as a way to induce tolerance towards ingested antigens of choice. There is emerging technology enabling us to engineer the specificity of Tregs through generating cells that express transgenic TCRs or CARs, with recent work sets the stage to use CARs to redirect the antigen specificity of Tregs towards self- or foreign antigens in multiple immune mediated diseases.

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Table 1. Human FOXP3+ Treg-based therapeutic approaches

Cell Type	Clinical setting	Antigen-Specificity	Therapeutic Approach	References
<i>Ex vivo</i> expanded polyclonal Tregs	Type 1 Diabetes	NA	- Infusing autologous <i>ex vivo</i> polyclonally expanded Tregs	(81, 82)
	GVHD	NA	- Adoptive transfer of donor Tregs to prevent GVHD in hematopoietic stem cell transplantation	(83-85)
	Transplantation	NA	- Infusing autologous Tregs in solid organ transplant recipients	NCT02166177 NCT02145325
<i>Ex vivo</i> expanded antigen- specific Tregs	Liver Transplantation	Donor alloantigen	- Infusing Tregs to induce tolerance and reduce immunosuppressant use in transplant recipients	NCT01624077 NCT02188719 NCT02474199
	Hemophilia A - <i>in vitro</i> proof-of-principle study	Factor VIII	- Transduced Tregs were expanded with factor VIII and retained function and phenotype	(102)
	Humanized mouse transplantation model	Donor alloantigen	- Human Tregs expanded with alloantigen protected against alloimmune-mediated injury of human skin graft i	(93, 94)
TCR-transduced <i>ex vivo</i> Tregs	Melanoma - <i>in vitro</i> proof-of-principle study	Tyrosinase (melanoma antigen)	- Polyclonal human Tregs were transduced with a tyrosinase-specific TCR, antigen-expanded and displayed <i>in vitro</i> suppression	(99)

CAR-transduced ex vivo Tregs	Mouse transplantation model	HLA-A2	- Human HLA-A2-CAR Tregs retained suppressive function <i>in vitro</i> and could preventing xenogeneic GVHD	(109)
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Review HLA “How Antigen-Specificity Directs Regulatory T Cell Function: Self, Foreign and Engineered Specificity”, by R.E. Hoeppli et al.

Short Biosketch of authors

Romy E Hoeppli completed a BSc in biology followed by a MSc in human biology at the University of Zurich, Switzerland, graduating in 2012. Her master’s research focused on signaling pathways in antigen presenting cells. She then worked as a research associate and clinical trials assistant at the University Hospital Zurich until 2013. She joined Megan Levings’ laboratory in 2013 to commence her PhD in immunology, which is focused on developing regulatory T cell therapies.

Katherine G MacDonald obtained a BSc in Microbiology from the University of Victoria, Canada, in 2010. She then completed a PhD in Experimental Medicine at the University of British Columbia, Vancouver, Canada, in 2015. Her graduate studies focused on the role of human FOXP3+ T regulatory cells in autoimmunity and transplantation. She is currently working on immunotherapy within the Biologics Division at the Centre for Drug Research and Development in Vancouver.

Megan K Levings completed a BSc at Simon Fraser University, Vancouver, Canada, in 1993 and obtained a PhD in genetics at the University of British Columbia, Vancouver in 1998. She then joined Maria Grazia Roncarolo’s laboratory at the San Raffaele Scientific Institute, Milan, Italy where she completed a postdoctoral research in the field human regulatory T cells, and was the first to show that human regulatory T cells could be expanded and used as a cell therapy in transplantation. She returned to the University of British Columbia, Vancouver, Canada in 2003 as a Canada Research Chair in Transplantation. She currently heads the Childhood Diseases Theme at the Child & Family Research Institute. Her research is focused on studying where, why and how regulatory T cells mediate their effects in the contexts of transplantation, diabetes and inflammatory bowel disease.

Laura Cook graduated in 2009 with a BSc (Hons) and the University Medal in Medical Microbiology and Immunology from the University of New South Wales, Sydney, Australia. She then completed a PhD in Immunology in the laboratory of Dr Anthony Kelleher, Kirby Institute, Sydney, Australia, graduating in 2014. Her PhD work focused on isolating and characterizing human antigen-specific CD39+ regulatory T cells. She commenced a postdoctoral research fellowship in Megan Levings’ laboratory in 2014 where she is studying antigen-specific effector and regulatory T cells in a range of autoimmune and infectious diseases.

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