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DOI:

[10.1016/j.autrev.2014.10.012](https://doi.org/10.1016/j.autrev.2014.10.012)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Grant, C. R., Liberal, R., Mieli-Vergani, G., Vergani, D., & Longhi, M. S. (2015). Regulatory T-cells in autoimmune diseases: Challenges, controversies and yet-unanswered questions. *AUTOIMMUNITY REVIEWS*, 14(2), 105-116. <https://doi.org/10.1016/j.autrev.2014.10.012>

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Regulatory T-cells in autoimmune diseases: challenges, controversies and - yet - unanswered questions

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Conflict of interest: The Authors have no conflict of interest to declare

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Abbreviations: ADP, adenosine diphosphate; AIH, autoimmune hepatitis; AMP, adenosine monophosphate; APC, antigen presenting cell; ATP, adenosine triphosphate; CFSE, carboxyfluorescein succinimidyl ester; CSF, cerebrospinal fluid; CTLA-4, cytotoxic T lymphocyte antigen-4; FACS, fluorescence activated cell sorting; Foxp3/FOXP3, forkhead box P3; HS, healthy subjects; IBD, inflammatory bowel disease; ICOS, inducible co-stimulator; IPEX, immunodysregulation, polyendocrinopathy, enteropathy X-linked; LAP, latency associated peptide; MS, multiple sclerosis; PBC, primary biliary cirrhosis; PLN, pancreatic draining lymph nodes; PP, primary progressive; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; RR, relapsing

remitting; SLE, systemic lupus erythematosus; SP, secondary progressive; T1D, type-1 diabetes; TIM-3, T cell immunoglobulin and mucin domain-3; Tregs, Regulatory T cells

Abstract

Regulatory T cells (Tregs) are central to the maintenance of self-tolerance and tissue homeostasis. Markers commonly used to define human Tregs in the research setting include high expression of CD25, FOXP3 positivity and low expression/negativity for CD127. Many other markers have been proposed, but none unequivocally identifies *bona fide* Tregs. Tregs are equipped with an array of mechanisms of suppression, including the modulation of antigen presenting cell maturation and function, the killing of target cells, the disruption of metabolic pathways and the production of anti-inflammatory cytokines. Treg impairment has been reported in a number of human autoimmune conditions and includes Treg numerical and functional defects and conversion into effector cells in response to inflammation. In addition to intrinsic Treg impairment, resistance of effector T cells to Treg control has been described. Discrepancies in the literature are common, reflecting differences in the choice of study participants and the technical challenges associated with investigating this cell population. Studies differ in terms of the methodology used to define and isolate putative regulatory cells and to assess their suppressive function. In this review we outline studies describing Treg frequency and suppressive function in systemic and organ specific autoimmune diseases, with a specific focus on the challenges faced when investigating Tregs in these conditions.

Keywords: regulatory T cells, self-tolerance, autoimmune disease, autoimmune liver disease

1. Introduction

Regulatory T cells (Tregs) are a heterogeneous population of lymphocytes central to the regulation of self tolerance and the maintenance of tissue homeostasis [1]. Although ‘suppressor T cells’, as they were originally named, had first been described in 1970 [2], our understanding of this population became clearer in the 1990’s when Hall *et al.* (1990) showed that the CD4^{pos} T cells expressing the α -chain of the IL2 receptor, CD25, were able to promote CD4 and CD8 T cell-mediated allograft unresponsiveness in cyclosporine-A treated mice [3]. Subsequently, seminal studies performed by Sakaguchi and colleagues demonstrated that the 10% of CD4^{pos} T cells expressing CD25 were essential for the maintenance of self tolerance in mice [4-6]. Several groups then reported that, in humans, CD4^{pos}CD25^{pos/high} cells were phenotypically similar to their murine counterparts, and that this population was able to suppress the proliferation of CD4^{pos}CD25^{neg} cells *in vitro* [7-10]. In 2003, the transcription factor forkhead box P3 (Foxp3), was shown to control the generation and function of murine Tregs [11-13] and later, in humans, FOXP3 was shown to be expressed predominantly by the CD4^{pos} cells expressing the highest level of CD25 [14].

Since then, elevated Treg frequencies have been reported in patients with various forms of neoplasia, such as breast cancer [15-19], melanoma [20-22] and leukaemia [23-25], where they are believed to dampen anti-tumour immune responses and promote malignancy. Similarly, Tregs can limit anti-pathogen immunity: high Treg frequency and function are associated with the persistence of infection with hepatitis C virus [26, 27], mycobacterium tuberculosis [28-30] and the malarial parasite [31-33]. Conversely, Treg defects have been shown to contribute to the loss of tolerance in human autoimmune diseases. In this review, current evidence supporting a role for Treg impairments in systemic [systemic lupus erythematosus (SLE)] and organ specific [multiple sclerosis (MS), type-1 diabetes (T1D), rheumatoid arthritis (RA), autoimmune thyroid disease, psoriasis, inflammatory bowel disease (IBD) and autoimmune liver disease] autoimmune disorders will be discussed, with a particular focus on the challenges that might be encountered when investigating human Tregs in these conditions.

2. Definition of regulatory T cells

The term ‘regulatory T cell’ or ‘Treg’ encompasses a range of cell populations specialised to exert cell extrinsic immunosuppression. Tregs regulate the natural course of protective immune responses in order to limit collateral tissue damage and autoimmunity. Several human Treg subsets have been described, including CD8^{pos} Tregs [34], IL10- producing Tr1 cells [35] and Th3 cells, which primarily suppress *via* the secretion of TGFβ [36]. In addition to Tregs, a newly described population of regulatory B cells also contributes to immunosuppression both directly and *via* enhancement of Treg function [37]. The most extensively studied human Treg subset is characterised by high constitutive expression of the IL2 receptor α-chain, CD25 [7-9]. In humans, the CD25^{high} population is enriched for the transcription factor FOXP3 [14], which is responsible for the transcriptional regulation of genes involved in the Treg phenotypic and functional signature, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) [38]. Identifying and isolating human Tregs in the laboratory can be challenging; in contrast to the murine model, the expression of CD25 [5, 39] and FOXP3 [40, 41] increases transiently upon activation also on effector CD4 T cells. Moreover, FOXP3 being an intracellular protein, it cannot be readily used for the isolation of Tregs for functional studies. In humans, within the CD4^{pos}CD25^{high} population, differential expression of the α-chain of the IL7 receptor, CD127, enables distinction between *bona fide* Tregs, which are typically CD127^{neg/low}, and activated effector T cells, which are CD127^{pos}. In the CD4^{pos}CD25^{high} population, CD127 expression is inversely correlated with suppressive function [42-44].

3. Mechanisms of suppression

There are four basic mechanisms that Tregs use to suppress immune responses (Figure 1, A); the modulation of antigen presenting cell (APC) maturation and function, the killing of target cells, the disruption of metabolic pathways and the production of anti-inflammatory cytokines [45].

3.1. Modulation of antigen presenting cell maturation and function

CD80 and CD86, expressed by APCs, provide essential co-stimulatory signals by binding CD28 on T cells during antigen presentation. CD80 and CD86 also bind CTLA-4, expressed by a subset of Tregs.

Direct interactions between APCs and Tregs have been recorded by intravital microscopy *in vivo* [46, 47]. The interaction between CTLA-4 and CD80, leads to CD80 mRNA down-regulation [48] and a reduction in the APC cell surface expression of CD80 and CD86 [48, 49]. In mice and humans, CTLA-4 can also lead to physical removal of CD80 and CD86 from the APC cell surface by trans-endocytosis and degradation [50]. By lowering the expression of the co-stimulatory molecules CD80 and CD86, Tregs limit the ability of APCs to initiate an adaptive immune response.

3.2. Killing of target cells

There is evidence that Tregs from mice and humans express molecules capable of triggering effector T cell apoptosis, such as Granzymes A and B [51-55] and perforin [51, 54]. Granzymes, released from cytolytic granules, enter target cells *via* perforin pores, leading to the activation of the caspase pathway and to apoptotic cell death. Tregs also express Galectin-9 [56, 57] which, in mice, triggers apoptosis upon binding the T cell immunoglobulin and mucin domain-3 (TIM-3) expressed by activated effector T cells [58]. Recent studies have, however, failed to document a direct interaction between the human Galectin-9 and TIM-3 molecules [59], suggesting that Galectin-9 could act independently of TIM-3 in this setting.

3.3. Disruption of metabolic pathways

A key mechanism of metabolic disruption is mediated by CD39. CD39 is an ectonucleotidase expressed constitutively by murine Tregs [60, 61], which catalyses the degradation of adenosine triphosphate (ATP) – which is a predominantly pro-inflammatory molecule – into adenosine diphosphate (ADP) and adenosine monophosphate (AMP). The importance of CD39 expression has been demonstrated using Tregs from CD39 knock-out mice, which have a 50 to 60% reduction in their ability to suppress T cell proliferation compared to those from wild-type mice [60]. CD39 is also expressed by a subset of human Tregs [61, 62]. ADP/AMP, generated by ATP hydrolysis, is subsequently degraded into adenosine by a second enzyme, CD73, which is also expressed constitutively by murine Tregs and by a small proportion of human Tregs [63]. The hydrolysis cascade initiated by CD39 culminates in the production of adenosine, which has multiple anti-

inflammatory properties, including the dampening of effector T cell activation and proliferation [64, 65] and the generation of Tregs [66]. Studies have suggested that CD39^{pos} Tregs are specialised in suppressing IL17 production [62].

3.4. Production of anti-inflammatory cytokines

Tregs also secrete the anti-inflammatory cytokines, TGF β [67-69], IL10 [70, 71] and IL35 [72]. Evidence from murine studies suggests that suppression mediated by cytokines is of particular importance at environmental interfaces, such as the gut, skin and lungs [73-75]. Cell surface TGF β , also known as latency associated peptide (LAP), is also expressed by Tregs [76].

3.5. Additional markers of Tregs

Novel strategies for Treg identification, frequently based on the removal of contaminating activated effector T cells, have been documented. The expression of the IL1 receptor type I/II (CD121a/CD121b) has, for example, been used to distinguish FOXP3^{pos} Tregs from activated FOXP3^{pos} or FOXP3^{neg} effector T cells [77]. Similarly, CD49d has been used to identify contaminating activated effector T cells [78]. Since terminally differentiated effector T cells are negative for CD27, a TNF receptor family member, Tregs have also been defined by the co-expression of CD27 and CD25 [79].

The Treg population has also been subdivided according to the expression of inducible co-stimulator (ICOS) [80, 81]. FOXP3^{pos}ICOS^{neg} cells produce TGF β whereas the FOXP3^{pos}ICOS^{pos} subset secretes both IL10 and TGF β [82]. Miyara *et al.* [83] subdivided Tregs according to the expression of FOXP3 and CD45RA, a marker of naive T cells. FOXP3^{high}CD45RA^{neg} cells are activated/memory Tregs, positive for Ki-67 – a nuclear protein expressed by proliferating cells – whereas FOXP3^{medium}CD45RA^{neg} cells, although suppressive *in vitro*, have the potential to revert to a pro-inflammatory state. The FOXP3^{pos}CD45RA^{pos} population are Ki-67^{neg} resting Tregs that acquire memory Treg phenotype and function after TCR stimulation *in vitro* and *in vivo* [83]. The expression of CD45RO, which defines memory T cells, has also been used to distinguish activated/memory and naive Treg populations [84].

The central role of Tregs in the protection from autoimmunity is exemplified by the observation that *Scurfy* mice, with loss-of-function mutations in the *Foxp3* gene, are afflicted with a fatal, multi-organ lymphoproliferative disease with multiple autoimmune manifestations [11, 85]. Interestingly, in the human equivalent of the disease – the immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome [6] – different *FOXP3* mutations lead to differences in the nature/degree of Treg impairment and, therefore, result in diverse clinical phenotypes. Thus, although in some IPEX cases CD4^{pos}CD25^{high} cells expressing FOXP3 protein are present at normal frequencies, their capacity to suppress is impaired [86]. This observation demonstrates the potential for inherent diversity in terms of clinical phenotypes and, consequently, laboratory findings, highlighting the requirement for careful patient selection and interpretation of experimental findings in this field.

Defective Treg numbers and/or function have been reported in several autoimmune diseases [87-89]. However, because of the technical challenges associated with studying human Tregs, reports are at times inconsistent or even contradictory.

4. Technical challenges

There are several technical challenges in the study of human Tregs. Firstly, no combination of the surface markers described herein enables researchers to identify a homogenous Treg population. In fact, the majority of the defining markers – CD25 [39], FOXP3 [90, 91] and CTLA-4 [92] for example – are also increased during effector T cell activation. For this reason, investigators often choose a combination of different Treg markers in their experimental setting, relying on standard protocols, as well as on personal experience.

The most common way to assess Treg function is based on the ability of these cells to suppress target cell proliferation. The ‘proliferation assay’, pioneered by Thornton and Shevach in 1998 [41], relies on the isolation of the putative regulatory and responder cell populations either immunomagnetically or by fluorescence activated cell sorting (FACS). Responder cells are then activated, commonly under polyclonal stimulation, in the absence or presence of the regulatory population. After a defined period of time, proliferation is examined. Proliferation can be measured using carboxyfluorescein

succinimidyl ester (CFSE) or ^3H -thymidine-based assays. CFSE-based assays have a number of advantages over ^3H -thymidine-based assays. CFSE can be used to label the responder cell population exclusively, whereas ^3H -thymidine measures the proliferation of both regulatory and responder cell populations [93-95]. Moreover, in contrast to CFSE, ^3H -thymidine-based assays only assess proliferation during the short time-span in which ^3H -thymidine is present in the co-culture [93]. CFSE-based assays also allow for cytofluorometry to be performed concurrently, to examine proliferation of more well-defined cell populations. However, CFSE staining requires the use of a cell number larger than ^3H -thymidine-tested proliferation, and therefore this approach is not always feasible when only small numbers of cells are available [96].

The isolation method utilised is also an important factor to consider. Compared to immunomagnetic isolation, FACS-based protocols give higher purity and also allow for the concomitant use and analysis of several Treg markers, therefore enabling better definition of the Treg population. However, FACS typically gives lower yields than immunomagnetic isolation, and so this method is not applicable to small samples.

One of the most profound challenges associated with Treg research is to assess frequency, phenotype or function at the site of inflammation. In the case of RA or MS, for example, this can be overcome to some extent as the inflamed region is accessible. However, even in these cases, it can be difficult to ascertain whether Treg defects are caused by systemic factors or factors localised to the site of inflammation [96], especially in the absence of properly matched control samples obtained from healthy individuals. Assays are always limited by their ability to reproduce the characteristics of the inflammatory site *in vitro*. Despite this, studies investigating Tregs in autoimmune disease have provided a wealth of information, which collectively point to Treg defects as a key contributor to immune tolerance breakdown in autoimmunity (Figure 1, B).

5. Regulatory T cells in autoimmune disease

In the following sections, studies investigating the frequency and suppressive function of Tregs in the archetypal non-organ specific autoimmune disease SLE, and the organ specific autoimmune diseases

MS, T1D, RA, autoimmune thyroid disease, psoriasis and IBD will be discussed. Information about the findings of individual studies, and details about the markers used to define Treg populations, can be found in Table 1.

5.1. Circulating regulatory T cell frequencies

In SLE [97-109], MS [61, 62, 110-118] and RA [97, 119-125] there are reports documenting decreased, normal and increased circulating Treg frequencies relative to health. Reports are also discordant in autoimmune thyroid disease [126-130] and psoriasis [131-134], in which decreased and normal Treg frequencies have been observed. Similarly, although the majority of reports show numerical Treg defects in IBD [135-138], one study found increased frequencies [139]. While most studies have agreed that T1D patients have a normal frequency of circulating Tregs [43, 140-144], reduced frequencies were found in one recent study [145] (Table 1). In support of a defect of Tregs in the development of autoimmunity, CD4^{pos}CD25^{pos}FOXP3^{pos} Tregs are also reduced in frequency in patients with undifferentiated connective tissue disease, which is regarded as an early-stage connective tissue/systemic autoimmune disease [146]. The reasons behind the reported inconsistencies are not fully understood, but there are a number of non-mutually exclusive explanations, outlined henceforth.

One potential explanation for the observed discrepancies is a lack of standardisation between studies due to the absence of a uniformly recognised marker able to reliably define a homogenous human Treg population. The inevitable result is the use of distinct combinations of Treg markers, which define the real Treg population with varying degrees of accuracy. This phenomenon is apparent in the studies considered in this review (Table 1). In MS, for example, studies defining Tregs as CD4^{pos}CD25^{pos/high} [110-113, 117, 118], CD4^{pos}CD25^{pos}FOXP3^{pos} [114] or CD4^{pos}CD45RO^{neg}CD25^{high} [115] cells have reported increased or equivalent Treg levels relative to health, whereas those using CD39 positivity [61, 62] and/or CD127 negativity [62, 116] as alternative/additional markers have documented decreased Treg frequencies. Similarly, in psoriasis, normal frequencies of CD4^{pos}CD25^{high} [133, 134] or CD4^{pos}CD25^{pos}FOXP3^{pos} [132] cells have been reported in some studies

while reduced frequencies have been described when high CD25 expression is considered in combination with FOXP3 positivity [131]. In one study examining the frequency of CD4^{pos}CD25^{pos} cells in RA, the frequency was increased [119], whereas studies using more sophisticated gating strategies, encompassing high CD25 expression, FOXP3 positivity or CD127 negativity, report normal [97, 120-122] or decreased [123-125] Treg frequencies.

There are two notable ways in which alternative gating strategies could influence study outcome. Firstly, sophisticated gating protocols may more thoroughly eliminate contaminating activated effector T cells, allowing subtle Treg defects to be 'revealed'. Secondly, by focusing on Treg subpopulations, alternative gating strategies may uncover numerical defects unique to specific patterns of autoimmunity. These two explanations could underlie the aforementioned findings in MS; the removal of CD127^{pos} cells eliminating contaminating activated effector T cells, and the use of CD39 as a Treg marker uncovering defects within this specialised Treg subpopulation.

It is clear, however, that the use of different Treg markers cannot fully explain the discrepancies observed in these autoimmune conditions. In SLE, for example, the same Treg markers (CD4^{pos}CD25^{pos/high}FOXP3^{pos}) have, in different studies, yielded results consistent with increased [104, 107] and decreased [102, 109, 147] Treg frequencies. Similar inconsistencies are apparent when considering CD4^{pos}CD25^{pos/high} cells in RA [97, 119-123, 125] and autoimmune thyroid disease [127-129].

In light of this, it should be noted that flow cytometry gating is, to some extent, subjective. Even with gold standard staining techniques, appropriate experimental controls and sophisticated analysis software, the placing of gates still depends partly on researcher selection. Studies using the same defining Treg markers are, therefore, not necessarily comparable. An excellent example of this is the placing of CD25 gates; there is currently no consensus about what constitutes 'high' or 'bright'. The interpretation of Treg research is further complicated in the absence of clear and detailed information about gating methodology.

It is also important to point out that regulatory T cell frequencies in autoimmune disease are influenced by disease stage and treatment regimen. In SLE, for example, Tregs have been reported to be less numerous in patients with active [99, 101, 105] or newly diagnosed [100] disease compared to those with inactive disease. In MS, disease duration is correlated with the frequency of memory Tregs, defined by the expression of CD45RA. Patients with short disease duration have a lower frequency of both naïve and memory Treg subsets, while the frequency of memory Tregs in age-matched subjects with chronic disease is equivalent to that seen in health [116]. In the context of RA, while a normal frequency of Tregs was found in patients with well managed disease, a lower frequency was found in patients with early/active disease [125]. Additionally, although Ehrenstein et al. (2004) did not find numerical Treg defects in RA, the frequency of Tregs increased significantly over time in patients responding to anti-TNF α therapy [120]. Treg frequency is also influenced by disease stage and treatment regimen in psoriasis. Tregs are found at lower frequencies in untreated patients compared to health. Moreover, in patients responding to anti-TNF α therapy, Treg frequencies increase significantly during treatment, while in non-responders Treg frequency remains stable or decreases during therapy [131]. Additionally, photo(chemo)therapy treatment for psoriasis increases Treg frequency in patients who originally had a numerical Treg impairment [132]. Similarly, in IBD, Treg frequencies are lower in patients with active disease compared to those with inactive disease [137]. Treatment with the anti-TNF α monoclonal antibody Infliximab treatment increases Treg frequency in IBD, particularly in patients responding to therapy [138, 148]. These observations demonstrate that Treg frequencies in autoimmune diseases are heavily influenced by disease stage and treatment regimen, highlighting the need to carefully consider these variables when interpreting published reports.

5.2. Circulating regulatory T cell function

In SLE [100-104, 106, 107, 109, 147, 149], T1D [140-144, 150], RA [119-123, 125] and autoimmune thyroid disease [127-130], studies have reported either normal or decreased Treg suppressive abilities compared to health. Studies are in agreement that Tregs are impaired in MS [62, 110-112, 114-118,

151] and psoriasis [131-134], whereas in IBD Tregs have suppressive abilities comparable to healthy subjects (HS) [137, 152].

Conflicting reports about Treg function in autoimmune disease can be accounted for using the explanations discussed in relation to Treg frequency. The lack of a universal human Treg marker has led to the use of diverse Treg gating strategies for the purposes of Treg isolation by FACS (Table 1). In RA, for example, although no defects were noted when $CD4^{pos}CD25^{pos/high}$ cells were used as Tregs [119-121, 123, 125], a reduced ability to suppress proliferation was apparent when CD127 negativity was used as an additional Treg marker [122]. The use of different Treg markers cannot adequately account for all of the discrepancies noted when considering Treg function; in SLE [100, 101, 103, 106, 149], T1D [140, 141, 143, 144, 150] and RA [119-121, 123, 125], the same markers ($CD4^{pos}CD25^{pos/high}$) have, in some instances, yielded results consistent with Treg functional impairment, while in others a preserved function was reported.

Experimental findings can also be influenced by patient selection. This is particularly apparent in MS, which has four distinct pathological patterns that differ in terms of clinical course: acute, relapsing remitting (RR), primary progressive (PP) and secondary progressive (SP) disease. For example, Tregs from acute [115] or RR-MS [114] patients, but not SP-MS patients [112], are less able to suppress responder cell proliferation compared to those from HS, and defects are more pronounced during episodes of relapse compared to periods of remission [114]. Additionally, Venken *et al.* (2008) isolated $CD4^{pos}CD25^{high}CD127^{low}CD45RA^{pos}$ naïve and $CD4^{pos}CD25^{high}CD127^{low}CD45RA^{neg}$ memory Treg subsets, and found that in RR-MS, naïve and memory Tregs were impaired in their ability to suppress proliferation, while in the chronic disease state, the suppressive ability of memory Tregs was equivalent to that found in health [116]. This study highlights the importance of patient selection but also demonstrates that functional defects can be specific to particular Treg sub-populations.

Most studies have analysed Treg suppression by assessing the ability of Tregs to limit effector CD4 T cell proliferation. It is, however, becoming increasingly clear that Tregs can suppress a range of effector cell functions mediated by several cell populations. In MS, studies have shown that

CD4^{pos}CD25^{pos/high} Tregs are impaired in their ability to suppress IFN γ production [110, 111] while CD4^{pos}CD25^{pos}CD127^{neg}CD39^{pos} Tregs have a reduced ability to suppress IL17 production [62]. Similarly, in RA, although CD4^{pos}CD25^{high} Tregs are able to suppress effector T cell proliferation, they are impaired in their ability to suppress IFN γ and TNF α production by effector T cells and monocytes. Interestingly, treatment with the anti-TNF α agent Infliximab enhances the ability of Tregs to suppress the production of these pro-inflammatory cytokines [120]. The inability of Tregs to suppress pro-inflammatory cytokine production has been associated with low CTLA-4 expression, and an inability of Tregs from RA patients to recruit CTLA-4 to the immunological synapse [153]. In this study, blockade of CTLA-4 in Tregs from HS abrogated suppression of T cell-derived IFN γ , leaving unchanged the ability to suppress proliferation [153]. These studies again demonstrate that functional defects can be limited to specific Treg subpopulations, while highlighting the importance of considering multiple readouts of suppression assays.

Studies have also suggested that an inability to detect Treg functional defects [142] might be related to inappropriately low levels of effector T cell activation *in vitro*. Lawson et al. (2008) found that lowering the concentration of anti-CD3/CD28 beads, a polyclonal T cell activation stimulus mimicking T cell receptor triggering, masked the Treg defect they observed in T1D patients when using higher concentrations [143]. This emphasises another potential hurdle when examining Treg function in autoimmune disease.

Another important point to address is the fact that the prototypical Treg suppression assay cannot distinguish impaired Treg suppressive ability from resistance of effector T cells to Treg control. To overcome this, a number of studies have used cross-culture experiments in which Tregs isolated from patients are cultured with effector T cells from healthy subjects and *vice versa*. Tregs from MS patients are unable to suppress the proliferation of effector T cells from healthy subjects, whereas Tregs from healthy subjects effectively suppress the proliferation of T cells from MS patients, demonstrating that the immunoregulatory defect is Treg intrinsic [110, 111]. Similar findings have been reported in the context of psoriasis [133]. In other autoimmune conditions, the results from cross-culture experiments are inconclusive. In SLE, while one study suggested that defects were Treg

intrinsic [149], in another study the immunoregulatory defect was attributable to the effector T cell compartment [107]. While the reasons for these discrepancies remain unclear, the cross-culture approach involves major histocompatibility complex mismatches that can influence the results.

5.3. Regulatory T cell frequency and function at the site of inflammation

Tregs from the pancreatic draining lymph nodes (PLN) of T1D patients are both reduced in frequency and impaired in their ability to suppress the proliferation or IFN γ production of effector cells compared to Tregs from matched peripheral blood or from the PLN of non-diabetic brain dead multi-organ donors [144]. This implies that defects can be localised to the target organ, highlighting an important limitation of studies focusing solely on the peripheral blood.

On the other hand, studies examining Tregs in the cerebrospinal fluid (CSF) of MS patients have found elevated frequencies compared to matched peripheral blood, while this difference was not observed when Treg frequencies in the CSF and peripheral blood of patients with non-autoimmune neurological defects were compared [115, 151]. Similarly, studies investigating the frequency of Tregs in the synovial fluid of RA patients have documented increased frequencies relative to matched peripheral blood [119, 121, 123, 154].

These observations could suggest that Tregs migrate to the inflamed site in an attempt to dampen autoimmune damage, failing to accomplish this due to functional defects. Alternatively, they may reflect increased contamination of gated Treg populations with effector T cells, due to activation-induced CD25 and FOXP3 expression. Studies examining the suppressive function of synovial fluid Tregs, reporting normal [119, 121] or impaired [122] Treg suppressive function, have suggested that both explanations may be valid. In the study reporting impaired Treg function, CD127 negativity was used as an additional Treg marker, therefore enabling more thorough exclusion of CD127 positive activated effector T cells. Interestingly, synovial fluid Tregs from HS were able to suppress the proliferation of effector T cells from RA patients, suggesting that the observed immunoregulatory defect was Treg-intrinsic. However, when Tregs from HS were pre-treated with RA synovial fluid, they were also functionally impaired, suggesting that factors present in the inflammatory

microenvironment promoted Treg dysfunction. Indeed, the addition of anti-TNF α reversed this effect. The presence of TNF α in the RA synovium altered FOXP3 transcriptional activity and induced Treg defects [122].

Other studies have suggested that the pro-inflammatory milieu present at the site of inflammation may modulate Treg function. In IBD, the percentage of CD4^{pos}CD25^{high} or CD4^{pos}FOXP3^{pos} cells in the lamina propria or mesenteric lymph nodes is elevated, particularly within the inflamed tissue [135, 137, 139, 152]. Moreover, Tregs isolated from the mucosal lymphoid tissue (143) or lamina propria [139, 152, 155] have normal suppressive activity. Although it is currently unclear why IBD persists despite the presence of a high frequency of Tregs with suppressive potential in the target organ, it should be noted that *in vitro* assays cannot always reflect all of the characteristics of the inflammatory site *in vivo*. In the setting of IBD, it is possible that the inflammatory milieu of the inflamed gut alters Treg functional properties, rendering them non-suppressive or even pathogenic [156]. Support for this hypothesis comes from the observation that, in the intestinal mucosa of IBD patients, the expression of the IL1 β and IL6 genes is higher compared to that of control subjects [136]. These cytokines have previously been implicated in the development of Th17 cells [157, 158]. In line with this, the numbers of IL17^{pos} cells and IL17 mRNA expression are higher in the lamina propria of IBD patients compared to controls [136, 159], suggesting that pro-inflammatory skewing of intestinal Tregs could be a pathogenic feature of IBD.

6. Regulatory T cells in autoimmune liver disease

There are three liver diseases with recognised autoimmune component to their pathogenesis; primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH).

PBC is characterised by progressive destruction of the small intrahepatic bile ducts, leading to cholestasis, portal inflammation, fibrosis and potentially cirrhosis and liver failure [160].

The first indications that Tregs may be defective in PBC originate in the 1980s [161]. More recently, studies have shown a reduced frequency of circulating CD4^{pos}CD25^{pos/high} [162, 163] or

CD4^{pos}FOXP3^{pos} [164] cells in PBC patients, although in another study, no numerical CD4^{pos}CD25^{pos} Treg defect was observed [165].

Magnetically isolated CD4^{pos}CD25^{pos} cells from PBC patients have no notable impairment in suppressive function [162, 165]. At variance with CD4^{pos}CD25^{pos}, the CD8^{pos}CD28^{neg} Treg subset was found to be less able to suppress proliferation in PBC compared to health [165]. Studies examining the livers of PBC patients have reported increased frequencies of FOXP3^{pos} cells compared to controls [163, 166]. Moreover, the frequency of FOXP3^{pos} cells positively correlates with the degree of portal inflammation [166]. These observations could be indicative of elevated activation-induced FOXP3 expression on non-regulatory T cell subsets or of increased migration of Tregs to the site of inflammation. Although functional CD4^{pos}CD25^{pos} Treg defects in PBC have not been observed *in vitro*, it remains possible that the microenvironment of the inflamed liver renders Tregs unable to adequately suppress. In support of this, studies have demonstrated increased IFN γ expression in the bile ducts of PBC patients [163]. An imbalance between Treg and Th17 cells has also been reported in PBC; in peripheral blood mononuclear cells, the gene expression of IL1 β , IL6, IL23, ROR γ t and IL17A were higher in PBC compared to health, whereas TGF β and FOXP3 gene expression was lower in PBC patients compared to HS. Similarly, the serum IL1 β , IL6 and IL23 concentrations were higher in PBC compared to health [164, 167].

PSC is characterised by diffuse inflammation and fibrosis of the intra- and extra-hepatic bile ducts, eventually progressing to biliary cirrhosis, portal hypertension and hepatic failure. Sebode *et al.* (2014) have recently reported that circulating CD4^{pos}CD25^{high}FOXP3^{pos}CD127^{low} cells are reduced in frequency in PSC patients, demonstrating that these cells are also less able to suppress proliferation. Similarly, a reduced number of intrahepatic CD3^{pos}FOXP3^{pos} cells were found in biopsies taken at diagnosis from PSC patients compared to patients with PBC [168]. In line with this, a number of studies, focusing on Tregs in AIH, have included a subset of patients with the autoimmune hepatitis/sclerosing cholangitis overlap syndrome, autoimmune sclerosing cholangitis. These studies have reported decreased circulating Treg frequencies and impaired suppressive function [57, 169, 170]. On the other hand, Oo et al. (2010) examined explanted livers from patients with autoimmune or

chronic inflammatory liver diseases, some of whom had PSC, and reported a higher frequency of liver-infiltrating CD4^{pos}CD25^{pos}FOXP3^{pos}CD127^{neg} Tregs relative to HS. Moreover, isolated CD4^{pos}CD25^{pos} Tregs from these patients were able to suppress proliferation *in vitro* [171].

In contrast to the two cholestatic autoimmune liver diseases, PBC and PSC, the targets of the autoimmune attack in AIH are the hepatocytes themselves. Associated with AIH are a histological picture of interface hepatitis, a biochemical pattern characterised by elevated aspartate aminotransferase levels, and positivity for circulating autoantibodies.

The first studies describing ‘suppressor cell’ defects in AIH were performed in the 1970s and 80s [172-174]. Since then, lower circulating frequencies of CD4^{pos}CD25^{pos} [170, 175] or CD4^{pos}CD25^{high} [57, 176] Tregs have been reported in AIH patients compared to health, and this defect is associated with lower FOXP3 expression [169, 176]. Moreover, during immunosuppressive therapy, the circulating Treg frequency has been shown to increase, though not reaching levels seen in health [170, 176]. In one study, however, the circulating frequency of CD4^{pos}CD25^{high}CD127^{neg} FOXP3^{pos} cells was equal in AIH patients and healthy subjects, and the frequency of this population was higher in patients with active disease compared to those in remission [177]. This discrepancy is likely to be due to differences in technical and methodological approaches [178, 179].

In co-culture with CD4^{pos}CD25^{neg} cells, magnetically isolated CD4^{pos}CD25^{pos} Tregs from AIH patients were less able to generate an anti-inflammatory cytokine milieu rich in TGFβ, suggesting that Tregs from AIH patients are ineffective promoters of linked-suppression [169]. Magnetically isolated CD4^{pos}CD25^{pos} Tregs from AIH patients have been shown to be impaired in their ability to suppress the proliferation of CD8^{pos} [175] and of CD4^{pos}CD25^{neg} [57, 169, 176, 180] cell populations compared to health. CD4^{pos}CD25^{pos} Tregs from AIH patients are also ineffective suppressors of IFNγ production by CD8 T cells [175]. Additionally, Tregs from AIH patients, but not HS, enhance the activation of pro-inflammatory monocytes, by elevating the level of spontaneous migration and by increasing the production of TNFα and the expression of TLR4 [181]. Magnetically isolated CD4^{pos}CD25^{pos}CD127^{neg} Tregs from AIH patients were also less able to suppress proliferation than

from HS [57], although this defect was not observed in FACS isolated CD4^{pos}CD25^{high}CD127^{neg} cells [177]. The discrepant results reported in AIH are likely to derive from differences in methodology, patient demography, disease activity and type of treatment [182, 183].

Tregs from AIH patients have also been shown to express lower levels of Galectin-9, and this defect is mirrored by reduced expression of the Galectin-9 ligand, TIM-3, by CD4^{pos}CD25^{neg} cells, suggesting that AIH may also be associated with effector cell resistance to Treg control [57].

We have recently observed that AIH is associated with lower frequencies of CD39^{pos} Tregs. In this condition, Tregs fail to adequately hydrolyse pro-inflammatory nucleotides and to suppress the production of IL17 by effector CD4 T cells. CD39^{pos} Tregs from AIH patients were also unstable upon pro-inflammatory challenge [184], suggesting that defective immuno-regulation in AIH might result not only from reduced Treg number and function but also from increased conversion of Tregs into effector cells.

7. Conclusion

Treg defects are frequently reported in autoimmune disease. There are, however, often discrepancies in the literature, which can be accounted for by the choice of study participants and the techniques used to study this challenging population of cells. The search for new markers that could unequivocally identify *bona fide* human Tregs – for the purposes of both phenotypic and functional analysis – will greatly facilitate our understanding of the role of Tregs in autoimmune disease. Studies suggest that the nature of the Treg impairment differs according to the autoimmune disease under investigation. There are reports of numerical and functional Treg impairments, of resistance of effector T cells to Treg suppression and of conversion of Tregs to effector cells (Figure 1, B). It is, therefore, important to consider numerical, phenotypic and functional defects affecting a range of Treg subsets. Moreover, current evidence strongly implies that systemic or regional factors can confine Treg impairments to the target organ. Treg studies would, therefore, benefit from more thorough investigation of the inflammatory site.

Acknowledgements: CR Grant is supported by an Alex P Mowat PhD Studentship, King's College Hospital Charity, UK. R Liberal is supported by a Research Grant from King's Health Partner Research and Development Challenge Fund, UK. MS Longhi is supported by a Clinician Scientist Fellowship from the Medical Research Council, UK.

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