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Regulatory T cells in autoimmune Neuroinflammation

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Summary

Regulatory T cells are the central element for the maintenance of peripheral tolerance. Several subtypes of regulatory T (Treg) cells have been described, and most of them belong to the CD4⁺ T-helper (Th) cell lineage. These specific subtypes can be discriminated according to phenotype and function. Forkhead box protein 3 (FoxP3)-expressing natural Treg cells (Tregs) and IL-10-producing, T-regulatory type 1 cells (Tr1) are the best-studied types of CD4⁺ regulatory T cells in humans and experimental animal models. It was shown that they play a crucial role during autoimmune neuroinflammation. Both cells types seem to be in particular important for multiple sclerosis (MS). Here we discuss the role of CD4⁺ regulatory T cells in autoimmune neuroinflammation with an emphasis on Tregs and Tr1 cells in MS.

Keywords

quetoimmunity; regulatory T cells; Treg; Tr1; FoxP3; multiple sclerosis

Introduction

CD4⁺ T-helper (Th) cells have a central role in adaptive immune responses but are also critically involved in autoimmune reactions. While autoreactive CD4⁺ effector T cells are drivers of autoimmune diseases, several mechanisms evolved to control these effector T cells that escaped central tolerance to prevent autoimmunity. Dominant tolerance by regulatory T cells is one strategy to maintain peripheral tolerance in vertebrates. Regulatory T cells control unwanted immune responses by various mechanisms. Multiple regulatory T-cell subsets have been characterized in rodents and humans, and the majority of them are members of the CD4⁺ T-cell lineage (1, 2). A key feature of CD4⁺ regulatory T cells is that once activated, they specifically modulate immune responses at multiple points. Regulatory T cells can suppress responses of the adaptive immune system as well as regulating innate immune responses. The loss of dominant peripheral tolerance, exerted by regulatory T cells,

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can lead to autoimmune disease, immunopathology, metabolic disease, allergy, and loss of fetal-maternal tolerance during pregnancy. On the other hand, deregulated regulatory T cells may also prevent effective responses against certain pathogens and anti-tumor immunity and could thus be harmful to the host (1–3). Alterations in regulatory T cells are also believed to play an important role in autoimmune neuroinflammatory diseases such as multiple sclerosis (MS) (4). MS is characterized by the autoimmune attack of the host immune system against central nervous system white matter. In particular changes in FoxP3⁺ (forkhead box protein 3) natural regulatory T cells (Tregs) and interleukin-10 (IL-10)- producing, so-called T-regulatory type 1 cells (Tr1) appear to be involved in disease development. In this review, we discuss recent findings about the role of human CD4⁺ regulatory T cells in autoimmune neuroinflammation, with an emphasis on FoxP3⁺ Tregs and IL-10- producing Tr1 cells in MS.

FoxP3⁺ regulatory T cells

Since the discovery that the expression of the IL-2 receptor α chain (CD25) marks regulatory T cells in mice (5), the research on this separate T-cell lineage has grown exponentially. We and others have confirmed the existence of this population in humans, based on a high expression of CD25 (6–11), and a large body of experimental data is now available, which clearly points towards a major role of this regulatory T-cell subset in ensuring peripheral tolerance in rodents and humans. A further breakthrough in Treg research was the identification of FoxP3 as key transcription factor of this T-cell subset (12–14). The importance to maintain peripheral tolerance in humans is in particular evident in IPEX patients (immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome). IPEX is a severe autoimmune disorder, which develops early in life in subjects that carry mutations in the *FoxP3* gene leading to dysfunctional FoxP3 expression. A similar phenotype is observed in *scurfy* mice, which lack functional FoxP3 (15–18).

FoxP3⁺CD25⁺ regulatory T cells develop in the thymus. This was first noted from animal models where postnatal thymectomy led to severe autoimmunity that could be prevented by the transfer of CD4⁺ T cells. Moreover, the depletion of CD25⁺ cells from thymocytes or peripheral T cells could not prevent autoimmunity in co-transfer experiments in immune deficient animals. This led to the terminology of ‘naturally arising’ or ‘natural’ Treg cells (19). Thymic development of natural Tregs is strictly related to the stable induction of FoxP3 and requires high affinity binding of major histocompatibility complex (MHC)-self-peptide complexes from thymic antigen-presenting cells (APCs) to the T-cell receptor (TCR). Moreover, thymic Treg development requires certain costimulatory signals and cytokine environments (in particular IL-2), different from conventional effector T cells, which finally leads to the generation of stable FoxP3-expressing regulatory Tregs in the periphery (20, 21). However, most of the evidence for the thymic selection process of Tregs is based on murine model systems and the exact situation in humans is less well developed (1).

The stability of FoxP3 expression by Tregs is guaranteed by epigenetic modifications of the *FoxP3* locus and promoter region, in particular by the Treg-specific-demethylated-region (TSDR) (22, 23). In this context, it is of interest that FoxP3 expression and a Treg phenotype

can be induced in the presence of transforming growth factor- β (TGF- β) or retinoic acid from naive murine T cells, which can give rise to so called 'induced' or 'adaptive' Tregs (iTregs). Induced Tregs might play a more pronounced role in general immune regulation in contrast to natural Tregs that control autoimmunity, since they are generated from conventional CD4⁺ T cells with low affinity for self-antigens (24). Although there are phenotypic and functional overlaps to natural Tregs, iTregs show pronounced differences in stability and gene expression (1, 24). For instance, the TSRD region of iTregs is not fully demethylated compared to natural Treg TSDR, which is fully demethylated (22). However, similar conditions as in mice do lead to the generation of functional iTregs in humans, since the induction of FoxP3 by TGF- β in human T cells is not sufficient to confer a fully suppressive phenotype (25–27). Thus, although it was shown that the forced expression of *FoxP3* by retroviral transduction (28, 29) could lead to some type of regulatory phenotype, these data indicate that iTreg induction in humans may require additional signals compared to murine T cells. This is in line with the fact that the recently identified markers to distinguish iTregs from thymus derived, natural Tregs in mice, including the semaphorin receptor Neuropilin1 and the transcription factor Helios cannot be fully translated into the human system (30). Interestingly, a recent study reported the stable induction of human FoxP3⁺ iTregs from naive T cells by a combination of IL-2, TGF- β , and retinoic acid, which shared many features with human natural Tregs and were suppressive in a xenogeneic graft versus host disease (x-GvHD) model *in vivo* (31). Moreover, there is evidence that Tregs can be induced from the memory T-cell pool *in vivo* (32). However, further research is needed to clarify to what extent this phenomenon plays a role in humans *in vivo* and if these cells can be exploited therapeutically.

Since the expression of CD25 is not restricted to human Tregs, as it is also upregulated on activated conventional T cells, they can be best identified upon the surface expression of CD25 in combination with CD127, the α chain of the IL-7 receptor. Tregs display a high expression of CD25 while they are mainly negative for CD127 (CD25^{high}CD127^{low/neg}) (33, 34). Another method to isolate human Tregs is the combination of CD49d (the α chain of VLA-4) and CD127. We have demonstrated that the majority of human Tregs only express low levels of CD49d and that by depleting CD49⁺, in combination with CD127⁺ cells, the isolation of Tregs is achievable without the use of CD25. Importantly, since most of the cytokine expressing activated CD25⁺ effector cells express higher levels of CD49d, this method removes contaminating cytokine expressing cells from human Treg preparations (35). FoxP3⁺ Tregs further express a set of characteristic markers and molecules, of which some of them are direct targets of FoxP3. Tregs express for instance high levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor-related protein (GITR) (1).

Heterogeneity of human Tregs

Natural Tregs are not a homogeneous population and can be further subdivided into distinct subsets with different functional features. Human Tregs can be classified into CD45RA⁺ naive-like or resting Tregs and CD45RO⁺ activated effector or memory-like Tregs (36). Based on the almost mutually exclusive expression of the two CD45 isoforms, Tregs can be separated into two distinct subsets that display different functional and phenotypic features.

CD45RA⁺ Tregs show a lower FoxP3 and CD25 expression, compared to their CD45RO⁺ counterparts and are less suppressive in *in vitro* suppression assays. Furthermore, these cells can be efficiently expanded in cell culture, whereas the majority of CD45RO⁺ Tregs seems to be terminal differentiated and less able to proliferate *in vitro* (36, 37). However, by deuterium labeling experiments, it was found that the CD45RO⁺ population is highly proliferative *in vivo* but also prone to apoptosis (32). The majority of naive-like Tregs are presumably recent thymic immigrants (38), in line with a high proportion of CD31⁺ cells in between this subset (39–41), whereas the bulk of CD45RO⁺ Tregs consists of antigen-activated Tregs (1). Furthermore, it was shown that the naive-like population differentiates after activation into CD45RO⁺ memory-like Tregs and that the majority of cord blood Tregs expresses CD45RA (1).

This grouping of human Tregs can be further sub-classified, predominantly within the CD45RO⁺ memory-like population. Work from our laboratory has demonstrated that based on the expression of MHC molecule human leukocyte antigen (HLA)-DR, Tregs can be subdivided into DR⁺ and DR⁻ Tregs. All of the DR⁺ Tregs display a memory-type phenotype and make up to 30% of the CD45RO⁺ Treg population (42). Based on *in vitro* observations, the DR⁺ Tregs displayed a different and higher suppressive activity compared to DR⁻ Tregs and can be viewed as a terminally differentiated Treg population, in line with the fact that these cells exhibit the highest expression of CD25 and FoxP3 and derive from DR⁻ cells after activation (1, 43). CD45RO⁺ Tregs can be further divided into functionally different subsets based on chemokine receptor expression. We have shown that the expression of CCR6 identifies regulatory effector-memory-like Tregs (Trem cells) in mice and humans (44). Similar to HLA-DR, the CCR6⁺ subset completely co-segregates with CD45RO expression, comprising more than 50% of the CD45RO⁺ subset. CCR6⁺ Trem cells display a similar phenotype as conventional effector-memory T cells and have a high expression of CTLA-4. We have shown that this population is induced after antigen-specific activation and has a high turnover rate *in vivo* as determined by Ki-67 staining and 5-bromo-2-deoxyuridine (BrdU) incorporation in experimental animal models. Moreover, murine CCR6⁺ Tregs were highly enriched in inflamed tissues and able to produce IL-10 (44, 45). This is in line with the recently observed phenotype in type 1 diabetes (T1D) patients after teplizumab treatment *in vivo* (46). Interestingly, CCR6 is also highly expressed on Th17 cells and the ligands of CCR6, β -defensin and CCL20, can induce migration of both antagonistic CCR6 expressing cell types to inflamed sites, arguing that this Treg subset might be directly involved in the control of effector-memory Th17 cell responses (47). Moreover, since human tolerogenic, indoleamine 2,3-dioxygenase (IDO)-expressing dendritic cells (DCs) also highly express CCR6 (48), the attraction of both subsets might further sustain the suppressive activity of CCR6⁺ Tregs through an interaction with IDO⁺ DC. Of note, IDO expression by DCs is stabilized by IL-10 and induced by CTLA-4 (48, 49), two factors highest expressed by CCR6⁺ Tregs (44). An example for the heterogeneity of the CD45RA⁺ naive-like Treg subset is the expression of the death receptor CD95 (Fas) (50). CD95 is upregulated upon activation of naive-like Tregs and expressed by almost all of the CD45RO⁺ Tregs. However, a subset of CD45RA⁺ Tregs is positive for CD95, even in cord blood, indicating a functional heterogeneity in between CD45RA⁺ naive-like Tregs as well (1, 50).

Recent studies demonstrated that Tregs could induce transcriptional programs to suppress specific Th-subtype responses in mice. It was shown that murine Tregs require the expression of T-box transcription factor 21 (TBX21/Tbet), interferon regulatory factor 4 (IRF4) and GATA binding protein 3 (GATA-3), signal transducer and activator of transcription 3 (STAT3) or B-cell lymphoma 6 protein (BCL6) to efficiently control Th1, Th2, Th17 or Tfh (T follicular helper) cell responses, respectively (3, 51). In this respect, it is interesting to note that human Tregs display a great magnitude of plasticity. We and others have demonstrated that Tregs can convert under certain circumstances into potentially pathogenic, cytokine producing Th1-like or Th17-like Tregs (reviewed in 52).

A still unresolved question is if, similar to adaptive responses of conventional T-helper cells, Tregs are able to generate long-lived memory cells (1, 44). So far, no specific markers for 'central-memory' type Tregs have been defined, but some evidence is available from studies in murine animal models that these cells in fact exist and play an important role *in vivo* (53–55). Interestingly, a potential niche where these cells exist *in vivo* was proposed to be inside of hair follicles in murine skin (55, 56). It remains to be seen if similar cells could be identified in humans. The definition of an analogous 'central-memory'-like Treg subset in humans would definitively open up new therapeutic approaches to e.g. 'vaccinate' against autoimmunity.

Mechanisms of action of human Tregs

Several mechanisms have been proposed as to how Tregs exert their suppressive function (57). Since the majority of data on human cells is based on artificial *in vitro* suppression assays, many conclusions drawn so far from experimental animal models have not been validated in humans *in vivo* and have to be interpreted with care. Thus, the knowledge of how human Tregs exactly work *in vivo* is still very limited. Another factor, which has to be carefully reassessed, is that many studies did not take the above-mentioned Treg heterogeneity into account while analyzing suppressive mechanisms of Tregs. This may also lead to conflicting results when analyzing Tregs as a homogenous population.

The suppressive mechanisms of Tregs can be classified into major groups, including cell-cell contact dependent suppression, inhibitory cytokine release, modulation of APC function, cytolysis, metabolic disruption and induction of suppressor cells or 'infectious tolerance'. It was shown that human Tregs could use all of these strategies to exert their suppressive function (1, 57). An important role was for instance demonstrated for the death receptor CD95, which was shown to mediate killing of CD8⁺ T cells via CD95L interaction (58). Furthermore, it was recently reported that different populations of human Tregs, based on inducible T-cell costimulator (ICOS) expression could induce suppressive cytokines like IL-10 and TGF- β to suppress T-cell and DC function (41). Moreover, we have demonstrated that subsets of human Tregs can secrete IL-10 during *in vitro* suppression assays (42). The modulation of APC function seems to work over various mechanisms. Tregs can modulate DC function by CTLA-4 ligation of CD80 and CD86, thereby inhibiting DC costimulation of effector T cells (59). Interestingly, this mechanism might work at least in part by trans-endocytosis, as it was demonstrated that CTLA-4 of human Tregs was able to capture CD86 from interacting DCs by this mechanism (60). Another strategy how Tregs can modulate

APC function is the induction of IDO. The induction and stabilization of the enzyme by T-cell CTLA-4 and IL-10 initiates the tryptophan catabolism and deprives tryptophan and produces inhibitory kynurenins, which blocks proliferation of effector T cells in the proximity of the DC (49, 61, 62). As mentioned above, since human IDO⁺ DCs express CCR6 (63), the IL-10-producing CCR6⁺ effector-memory-like Treg subset may amplify suppression by this mechanism while being attracted to similar sites through CCL20 gradients *in vivo* (44). Cytolysis is another mechanism to suppress ongoing immune reactions. It was reported that human Tregs could mediate cytolysis of effector cells by granzyme A (GZMA) and perforin (64). It was further demonstrated that metabolic disruption is another mechanism by which Tregs can exert suppression of effector T-cell proliferation. Murine CD25⁺ Tregs can deprive IL-2 by their high affinity IL-2 receptor and thereby blocking effector T-cell growth (65, 66). However, it remains to be seen if human Tregs can use a similar mechanism. The discovery that the ectonucleotidase CD39/ENTPD1 is highly expressed on a subset of human Tregs led to the identification of a distinct pathway, the hydrolysis of extracellular adenosine triphosphate (ATP) and the generation of immune suppressive adenosine (1, 67). The enzymatic activity of CD39 is highly induced with Treg stimulation and the hydrolysis of extracellular ATP can extend cell survival of Tregs in high ATP concentrations, prevent APC activation by the depletion of the classical 'danger signal' ATP, as well by the generation of antiproliferative adenosine, generated by a CD39/CD73 cascade (67, 68). However, since both enzymes are only jointly expressed on murine Tregs (67, 68), the latter pathway may only works in *trans* in humans, where CD73 is expressed on activated effector cells (69, 70). Finally, human Tregs have been shown to confer suppression by the induction of other immune-suppressive cells- a mechanism termed 'infectious tolerance'. It was for instance shown that human Tregs have the possibility to induce IL-10 expressing Tr1-like cells (71), a mechanism which was in part mediated by TGF- β (72).

Human Tregs have the ability to confer suppressive activity through various mechanisms. It is evident that some pathways are in particular only specific for Treg subpopulations and are highly dependent on the microenvironment and type of immune reaction. However, the information on where and under which conditions these strategies are operational or preferentially used by the organism *in vivo* is still very limited.

Type 1 regulatory T cells

The first evidence for the existence of IL-10-secreting CD4⁺ regulatory T cells was based on observations in humans with severe combined immunodeficiency (SCID) who did not develop graft versus host disease (GvHD) after allogeneic stem cell transplants besides the presence of host reactive donor T cells, even in the absence of immune suppressive therapy (2, 73). Alloreactive T-cell clones from patients showed a cytokine profile distinct from Th1 and Th2 cells and were characterized by high-level production of IL-10. Furthermore, the cells were secreting interferon- γ (IFN- γ), TGF- β , and IL-5 but little IL-2 and IL-4 (74, 75). The discovery that regulatory antigen-specific, IL-10-producing cells could be generated under chronic stimulation in the presence of IL-10 in humans and mice led to the term of type 1 regulatory T cells or Tr1 cells (76). Of note, these Tr1 cells were highly effective in suppressing murine colitis (76). Subsequent studies in humans and murine animal models

provided additional evidence that Tr1 cells could be regarded as a distinct CD4⁺ T-cell population of suppressor cells and further shed light on the mechanism of action of these cells. It was demonstrated that antigen-specific activation of T cells by APCs in the presence of IL-10 led to a Tr1 phenotype (76). However, IL-10 alone was not sufficient to induce Tr1 cells in the absence of APCs, suggesting that other factors were required for efficient Tr1 induction. Interestingly, IFN- α was found to be one factor, boosting Tr1 cell induction (77). Other components even proved to be more efficient in the induction of Tr1 cells. The combination of vitamin D3 (1,25 dihydroxyvitamin D₃) and the glucocorticoid dexamethasone in the presence of blocking antibodies for type 1 and 2 cytokines were potent inducers of IL-10-producing Tr1 cells, which did not secrete any type 1 or 2 cytokines and retained proliferative capacity (78). Rapamycin, another immunosuppressant, in combination with IL-10 has been reported to allow efficient Tr1 induction in a murine transplantation model *in vivo* (79). Tr1 cells can be further induced by stimulation with immature DCs (iDCs) or other tolerogenic types of DCs (reviewed in 2). Moreover, specific costimulatory molecules could boost Tr1 induction. It has been shown that T-cell stimulation in the presence of complement receptor CD46 engagement (80) and IL-2 can highly induce IL-10-producing Tr1-like cells (81). Furthermore, costimulation via the CD58-CD2 axis was also an effective inducer of IL-10 production in T cells (82). More recently, another specific inducer of Tr1 cells has been identified. The cytokine IL-27, belonging to the IL-12 family, was able to induce Tr1-like cells and could act as growth factor for Tr1 cells (83–85). IL-27 could be secreted by DCs and could act through the transcription factors musculoaponeurotic fibrosarcoma oncogene homolog (MAF) and the aryl hydrocarbon receptor (AHR) (84). Moreover, it was shown that IL-27 could induce IL-10 production through an early growth response gene 2 (EGR-2)-dependent pathway (86).

Phenotype and suppressive mechanisms of Tr1 cells

Besides the secretion of IL-10 and its dominant mechanism of suppression of Tr1 cells, they can additionally exert their immune suppressive function through several other pathways. Interestingly, some of them are overlapping with mechanisms used by FoxP3⁺ Tregs (2, 73). It has been shown that Tr1 cells can suppress immune responses by cell contact dependent mechanisms using inhibitory co-receptors like CTLA-4, programmed cell death 1 (PD-1), or ICOS (73). Moreover, Tr1 cells showed the ability to kill target cells by granzyme-mediated cytotoxicity (2, 73, 85, 87, 88). Interestingly, similar to Treg subsets, Tr1 cells also exhibited high expression of the ectoenzymes CD39 and CD73, indicating that the generation of immune suppressive adenosine might be another mechanism that is used by these cells (73, 89).

In contrast to Tregs, specific markers for the identification of Tr1 cells are still limited. It was anticipated that Tr1 cells show higher levels of ICOS and PD-1, since the expression segregated with IL-10 production of CD4⁺ T cells. Moreover a correlation with CD49b expression and lymphocyte-activation gene 3 (LAG-3) was proposed as well (73). However, all of these markers showed at best a mild correlation with IL-10 expression and lacked Tr1 cell specificity, since they were expressed by other subpopulations and cell types as well. So far, the best cell surface marker for the identification of Tr1 cells is a combination of some

of the earlier suggested markers. A recent study showed that the combination of CD49b and LAG-3 defines the majority of murine and human IL-10-secreting Tr1 cells (90).

CD4⁺ regulatory T cells in multiple sclerosis

MS is an inflammatory multifocal demyelinating disease of the central nervous system (CNS). MS is characterized by progressive neurodegeneration mediated by an autoimmune response to self-antigens in genetically predisposed individuals. The disease is frequently described to begin with an initial clinically isolated syndrome (CIS), resulting in demyelination and edema, which is followed by several clinical relapses with spontaneous remission where patients usually return to almost normal neurologic function. This phase of the disease is termed relapsing remitting MS (RRMS). In a small number of patients, the disease progresses directly without remissions, leading to severe clinical manifestations. This type of the disease is called primary progressive MS (PPMS). However, many patients with RRMS suffer over time from progression of the disease causing irreversible damage and disability as well, referred to as secondary progressive MS (SPMS) (91).

Significant progress has recently been made in deciphering the genetic basis of MS. Besides the association with the MHC gene region, over 100 allelic variants have been discovered that are associated with the disease, mostly being immune response genes (92, 93). These studies identified enrichment in associations to gene regions linked to immune function (4). However, besides genetic factors, there must be environmental triggers, driving the disease in relationship to the genetic variations. This is suggested by epidemiologic studies on MS, including the observation of a constant increase of disease incidence over the past decades (91, 94, 95). Environmental risk factors for MS have been extensively investigated and associations with disease have been described for instance for specific infections, low serum vitamin D levels 25-hydroxyvitamin D), smoking, obesity, and dietary components (96, 97).

Proinflammatory myelin-reactive CD4⁺ T cells, in particular pathogenic Th17 and Th1 cells, are believed to be involved in disease pathogenesis (4, 52). However, a single causative antigen has not been elucidated, though myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and heat shock protein α B-crystallin (HspB5) have been found to be important in MS. Of note, the presence of myelin self-reactive T cells can be found in healthy individuals at lower frequencies as well, indicating that the presence of self-reactive T cells is not sufficient for disease induction (4, 98). This observation indicates that regulatory mechanisms are available in healthy individuals that control these autoreactive T-cell specificities. Accumulating evidence indicates that regulatory CD4⁺ T cells play a major role in this process.

FoxP3⁺ Tregs and MS

The critical involvement of regulatory CD25⁺ T cells in MS was initially indicated by studies in experimental autoimmune encephalomyelitis (EAE), the murine animal model of MS, covering many aspects of the human disease. Most importantly, the adoptive transfer of CD25⁺ Tregs was able to ameliorate disease, whereas the depletion of Tregs worsened disease in different models of EAE (reviewed in 15, 99). In humans, no obvious differences in frequency of Tregs were initially noted between healthy subjects and MS patients when

analyzing Tregs in peripheral blood based on the high expression of CD25 (100, 101). In contrast, MS patients showed an enrichment of Tregs in cerebral spinal fluid (CSF) compared to peripheral blood with increased expression of markers associated with an effector memory-like phenotype (102, 103). However, when comparing the suppressive capacity of Tregs from RRMS patients to healthy controls *in vitro*, we noticed that the Treg function of patients was severely impaired (101). In line with this study, other groups reported similar findings on disturbed Treg function in MS patients (102–108). Interestingly, the effect was not observed in patients with SPMS (106, 107). Moreover, the loss of Treg function in MS patients correlated with a decrease in FoxP3 and CTLA-4 expression (103, 109, 110) and an altered distribution of distinct Treg subsets in RRMS patients (40). This altered distribution was in particular due to the impaired generation of naive Tregs in the thymus as demonstrated by lower numbers of CD31⁺CD45RA⁺ recent thymic emigrant Tregs and a higher frequency of CD45RO⁺ memory-like Tregs in the peripheral blood of MS patients (40, 107, 111). Of note, the subset alterations and suppressive function were partially reversed upon IFN- β or glatiramer acetate (GA) therapy, in line with a higher thymic output of naive Tregs (112–115) in contrast to treatment with natalizumab (116, 117). Intriguingly, a higher frequency of CD45RO⁺ Treg-like cells can also be found in IPEX patients with *FoxP3* mutations, which harbor nonfunctional Tregs. IPEX patients showed defects in peripheral B-cell tolerance, indicating a role for FoxP3⁺ Tregs in controlling autoreactive peripheral B cells (118). Interestingly, we observed a similar phenotype of defective peripheral B cell tolerance in MS patients. This implies that, in line with the impaired Treg function *in vitro*, Tregs of MS patients are unable to control autoreactive peripheral B cells *in vivo*, similar to IPEX patients that lack functional FoxP3 (119). Human Tregs could also act by interfering with Ca²⁺ signaling of effector T cells after TCR-mediated activation during cell-contact dependent *in vitro* suppression assays (120). Interestingly, a recent study indicated that in particular naive-like CD45RA⁺ Tregs efficiently reduced Ca²⁺ signaling in effector T cells. Based on the lower frequency of CD31⁺CD45RA⁺ Tregs in MS patients, it was suggested that the loss of Treg function in MS patients is due to the decreased ability to interfere with Ca²⁺ signaling in effector T cells (121).

In line with an altered distribution of Treg subsets in MS, we observed a lower frequency of CD39⁺ Tregs in patients with RRMS (67). As outlined above, CD39 expression defines a subset of effector memory-like Tregs with specialized function in humans and the enzymatic activity of Treg expressed CD39 is essential for the hydrolysis of extracellular ATP and for the generation of suppressive adenosine, delineating an important mechanism for Treg function (67). Another group detected a similar reduction of CD39⁺ Tregs in RRMS patients compared to controls (122). Moreover, the study demonstrated that the CD39⁺ Treg population is in particular important for the suppression of Th17 cells but is functionally impaired in MS patients. Interestingly, only CD39⁺ Tregs were able to efficiently suppress IL-17 secretion of effector T cells, while being unable to produce IL-17 by themselves, whereas CD39[−] Tregs were actively secreting IL-17 after stimulation in mono- or co-cultures with effector T cells (122). This is in line with another report, analyzing human T cell CD39⁺ expression in detail (69) and indicates that in particular the CD39⁺ Treg population is crucial for controlling pathogenic Th17 responses in human MS. As mentioned

above, in contrast to mice, most human CD39⁺ Tregs do not co-express the ectonucleotidase CD73 (69), which converts adenosine monophosphate (AMP) to immune suppressive adenosine. In this context, it is of interest that CD73 is highly expressed on human Th17 cells (70) and on a subset of Tregs capable of producing IL-17 (43). This indicates that the generation of immune suppressive adenosine might play a role when both, CD39⁺ Tregs and CD73⁺ Th17 cells, come into close physical contact. Interestingly, CD73 is also expressed by endothelial cells of the blood-brain barrier (BBB) and astrocytes and is highly induced upon IFN- β treatment in MS patients, implicating a potential role of the enzyme in T-cell BBB transmigration (123). Another mechanism as to how CD39⁺ Tregs might contribute to control pathogenic T cell and in particular Th17 responses is the hydrolysis of extracellular ATP. Extracellular ATP is released by damaged cells upon trauma and cell death and can for instance be found in high concentrations at sides of inflammation and tissue damage. Extracellular ATP is an activator of the inflammasome, which can lead to IL-1 β release by APCs (124). Of note, IL-1 β is a key pro-inflammatory cytokine that drives Th17 cell differentiation and Th17-type Treg plasticity in humans (52). Moreover, it has been shown that extracellular ATP can induce IL-23 secretion by DCs, the key survival factor for pathogenic Th17 cells (125). CD39⁺ Tregs therefore might additionally suppress the induction of Th17 cells by removing extracellular ATP and thereby lowering the release of pro-inflammatory IL-1 β and IL-23 by APCs (67, 126). Thus, the expression of CD39 by Tregs may be involved in the regulation of pathogenic effector cells in MS.

Defects in Treg function of MS patients might also be related to an enhanced plasticity of Tregs towards a proinflammatory, cytokine-producing effector phenotype. Data from our laboratory indicated that particularly the skewing towards an IFN- γ -producing Th1-like Treg phenotype might play a role in MS. By analyzing FoxP3⁺ Tregs of RRMS patients compared to healthy controls we noticed a significant increase of Th1-like Tregs, secreting IFN- γ (127). Interestingly, IFN- γ production of Tregs was dependent on IL-12 and could also be induced *in vitro* in Tregs from normal subjects. Besides the expression of IFN- γ , the subset of Th1-like Tregs displayed high expression of TBX21 and CXCR3, sharing similarities with a conventional Th1 phenotype (127). IFN- γ ⁺ Tregs did not show differences in the *FoxP3* TSDR methylation status compared to IFN- γ ⁻ Tregs, indicating that these Tregs stably expressing FoxP3. Th1-like Tregs showed defective suppressive activity *in vitro* and this phenomenon was related to IFN- γ secretion, since the blockade of IFN- γ partially restored the suppressive activity of IFN- γ ⁺ Tregs. Importantly, the blocking of IFN- γ also partially restored the suppressive activity of Tregs from RRMS patients. Of note, the frequency of Th1-like Tregs was found to be similar to healthy controls in MS patients treated with IFN- β , indicating an influence of this treatment on Th1-like Tregs, potentially through its effect on IL-12 (127). Thus, the increased frequency of Th1-like Tregs in RRMS may partly explain why Tregs of MS patients display a lack in suppressive function. The phenomenon might be important for other autoimmune diseases where a lack of Treg function is evident as well (15, 128, 129). A similar Treg phenotype was for instance observed in patients with T1D (130). In addition to the Th1-like phenotype, human Tregs can also differentiate into Th17-like Tregs characterized by the secretion of IL-17. Th17-like Tregs can be induced in the presence of IL-1 β and IL-6 and are characterized by the expression of CCR6 and high levels of CD49d and were found to be enriched in CD73

expressing cells (35, 43, 52, 131). Although an association of Th17-like Tregs was reported with autoimmune hepatitis (132, 133), systemic sclerosis (134) and psoriasis (135, 136), we so far did not find a direct correlation of this subset with MS (127).

The assumption that Tregs play a critical role in MS is further supported by the analysis of the genetic variants associated with the disease. For instance, genome-wide association studies (GWAS) identified strong associations with disease susceptibility for the *IL2RA* and *IL7RA* loci, with both genes strongly implicated in Treg function (4, 137). In addition to several other T-cell-related loci, GWAS studies on MS patients further discovered an association with the *CD58* gene encoding for the costimulatory molecule lymphocyte function-associated antigen 3 (LFA-3). Since CD58-CD2 costimulation is important for Treg function (42), we have analyzed this variant in more detail (138). Interestingly, the protective allelic variant of *CD58* leads to increased CD58 expression and this could potentially lead to FoxP3 upregulation and enhanced Treg function through CD2 engagement. In line with these findings, increased mRNA expression of *CD58* correlated with clinical remissions of MS patients, suggesting a relation to restored Treg function, potentially based on increased CD58-CD2 co-stimulation (138) (Fig. 1). Additionally, a recent study described an essential role of the transcription factor BTB and CNC homology 2 (*BACH2*), previously primarily associated with B cells, for Treg cell development and function in mice (139). *BACH2* was recently discovered as a new risk variant for MS (92) and is further associated with T1D, asthma, Crohn's disease, coeliac disease, and vitiligo (139). Interestingly, besides genetic factors, there are a few studies available which link the suppressive capacity of Tregs indirectly to environmental factors. A recent study found for instance a positive correlation of the suppressive activity of Tregs in RRMS patients with high serum vitamin D levels (140). In this context, the growing field of the analysis of microbiota and immune system interactions is likely to continue to contribute new views on adaptive immune regulation including that of autoimmune neuroinflammation (reviewed in 141–146). Moreover, it remains to be seen if recently identified environmental cues influencing proinflammatory CD4⁺ T cells during neuroinflammation also play a role in immune regulation by FoxP3⁺ Tregs (147, 148).

Type 1 regulatory T cells in MS

Similar to FoxP3⁺ Tregs, evidence that Tr1 cells might play a role in MS came first from studies in experimental animal models. Numerous studies have demonstrated the importance of IL-10 in murine EAE (149, 150). Moreover, in particular the induction of Tr1 cells by dexamethasone and vitamin D3 were beneficial in the treatment of EAE (78). Vitamin D3 alone is highly effective in the amelioration of EAE and is a potent immunomodulator, which can influence various immune cells. Furthermore, it was shown that the effect of vitamin D3 is directly connected to the IL-10 pathway including a potential effect on regulatory T cells (2, 151, 152). Of note, serum vitamin D3 levels are strongly associated with MS, and vitamin D3 is directly connected to sunlight exposure and thus may provide a link to the geographical variations in MS incidence (94, 97, 153). Furthermore, variants in *CYP27B1*, encoding for a vitamin D3 converting enzyme are strongly associated with MS (92, 153). The link that vitamin D3 might be an inducer of IL-10-producing Tr1 cells is intriguing, although direct evidence that Tr1 cells are affected in MS is sparse (150).

However, we have demonstrated that IL-10-producing Tr1 cells of MS patients, similarly to FoxP3⁺ Tregs, are functionally impaired (154). When comparing MS patients to normal healthy subjects, we observed a striking defect in IL-10 secretion of Tr1 cells from RRMS patients (154). The effect was only evident when the cells were differentiated into Tr1 cells by anti-CD46 mediated co-stimulation but not under anti-CD28 stimulatory conditions. The effect was not due to changes in proliferation or altered surface expression of CD46 and could not be restored by modifying the strength of stimulation. Of note, the effect observed in MS patients was specific for IL-10, since we did not observe changes in IFN- γ secretion (154). To test the Tr1 response after treatment, we analyzed the CD46-induced IL-10 secretion in RRMS patients with IFN- β therapy. However, while increased IL-10 levels were reported to be associated with remissions (150), we could not detect a restoration of the Tr1 response in treated patients, although another study could identify slight changes in a subset of patients receiving IFN- β (114). Of note, we found evidence that the impaired function of Tr1 cells in untreated RRMS patients is correlated with an altered isoform expression of CD46. MS patients showed increased expression of the isoform Cyt2 (CD46.2), which carries a cytoplasmic tail that leads to proinflammatory responses and blocks IL-10 secretion (154–156). The defective Tr1 response in RRMS was replicated in a larger cohort of patients, linking the reduced IL-10 induction to abnormal IL-10 signaling in MS patients (157). Moreover, in line with the functionally impaired Tr1 population in RRMS patients, similar findings were observed in a model of multiple sclerosis in Cynomolgus monkeys (158). Interestingly, a recent study indicated that the defective CD46 triggered Tr1 response of RRMS patients could be partly restored *in vitro* in the presence of vitamin D3 (159).

Since IL-10-secreting Tr1 cells can be in particular induced by CD58-CD2 costimulation (82), it is likely that the protective allelic variant of *CD58*, which is associated to MS, is related to Tr1 cells as well. Similar to FoxP3⁺ Tregs, the increased CD58 expression could potentially influence the induction of Tr1 cells and thus anti-inflammatory IL-10 secretion in humans. Moreover, the increase of CD58 mRNA in MS patients during remission (138) might be correlated to higher IL-10 levels and enhanced Tr1 function as well.

Information on a direct involvement of other mediators or mechanism of suppression by Tr1 cells besides IL-10 in human autoimmunity is still sparse and other defects of Tr1 cells have not been demonstrated in MS patients yet. However, the fact that human Tr1 cells were reported to express for instance the enzymes CD39/CD73 in line with the ability to generate immune suppressive adenosine in a cancer model (73, 89) indicates that this mechanism might play a role in MS as well. Moreover, similar to FoxP3⁺CD39⁺ Tregs, it is possible that CD39 expression by Tr1 cells could further act through removing extracellular ATP, by preventing DC maturation and by potentially blocking inflammasome dependent pro-inflammatory IL-1 β release (67), as recently shown for CD39-expressing DCs in murine EAE (160). Similar associations to MS might exist for granzyme B (GZMB)-mediated killing or the inhibitory receptors CTLA-4, PD-1, and ICOS as well, which have shown to be highly expressed by Tr1 cells and being used for suppression by Tr1 cells under certain conditions (73). However, at this point there is no direct proof that these pathways, similar to IL-10 secretion, are impaired in Tr1 cells of MS patients. The cytokine IL-27 might be

another factor that could play an important role for Tr1 cells in MS. IL-27 was recently identified as inducer of IL-10-secreting Tr1-like cells in mice and humans and seems to play a crucial role in EAE (83, 85, 161–164). Of note, IL-27 appears to be related to responsiveness of MS patients to IFN- β therapy (85), and it was demonstrated that IFN- β treatment increases IL-27 secretion by human DCs *in vitro* (165, 166). Although IL-27 has pleiotropic effects on different CD4⁺ T-cell populations, it is clearly inducing IL-10 in a STAT3-dependent manner in CD4⁺ T cells (73, 85). Moreover, it was also demonstrated that IL-27 induces granzyme B expression (85). Thus, the induction of IL-10-secreting Tr1 cells by IL-27 might play a role in humans as well. The relation of IL-27 to IFN- β treatment is intriguing, although direct data demonstrating that this is affecting IL-10 secreting Tr1 cells in MS patients is missing (Fig. 2).

Conclusions

There is clear evidence that CD4⁺ regulatory T cells play a crucial role in autoimmune neuroinflammation. Both, defects of FoxP3⁺ Tregs and of IL-10-producing Tr1 cells, have been observed in patients with RRMS. Moreover, genetic factors associated with the disease are directly or indirectly linked to regulatory T-cell phenotype and function. The available data are furthermore pointing towards an involvement of regulatory T cells in successful treatments such as IFN- β therapy and indicates an active role of regulatory T cells during remission phases. The reestablishment of changes in the subset distribution of Tregs, an increased thymic output and the restoration of suppressive capability are associated with clinical remission phases or successful treatment. Similarly, a shift in the cytokine profile towards Tr1-promoting conditions, with upregulation of IL-10 and IL-27, is evident under these settings.

The available data on CD4⁺ regulatory T cells in MS is complex and indicates that several mechanisms and subsets of Tregs or Tr1 cells could contribute to the observed effects of defective regulatory T-cell function and its association to disease. It will be a challenge to finally integrate all of the genetic and environmental cues contributing to disease development. Moreover, although there is a large amount of studies analyzing peripheral blood of patients, the studies on regulatory T cells analyzing phenotype and function in the CNS or CSF are still sparse. Here, most of the information can only be extrapolated from animal models. Thus, future research is definitively needed to clarify the role of CD4⁺ regulatory T cells in multiple sclerosis. The dissection of several, potentially overlapping mechanism and the detailed analysis of the relation and interplay of Treg and Tr1 subsets, in particular during treatment is warranted. Since the available data points towards a key role of Tregs and Tr1 cells in MS, a better understanding of CD4⁺ regulatory T cells and the affected mechanisms will certainly lead to new avenues in therapy.

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Fig. 1. Impaired function of FoxP3⁺ Tregs in multiple sclerosis

Indicated are the main functional and phenotypic differences between Tregs from healthy donors (healthy) versus Tregs from RRMS patients (multiple sclerosis). Potential deregulated molecules and pathways in RRMS during antigen-presenting cell (APC) and CD4⁺ effector T-cell (Teff) interactions are highlighted. APCs from patients express less CD58, potentially leading to diminished CD58-CD2 costimulation. Thereby inducing lower FoxP3, CD25 and CTLA-4 expression in Tregs of RRMS patients, possibly leading to defects in suppression via IL-2 deprivation, decreased CTLA-4-CD80/86 interaction and reduced IDO induction by APCs. Moreover, a subset of Tregs in patients has lower CD39 expression, possibly resulting in impaired ATP hydrolysis and adenosine generation in concert with Teff CD73. Moreover, higher expression of CD95 and lower levels of CD31, in line with decreased thymic output, indicate changes in subset distribution of Tregs from MS patients. The loss of suppression could lead to an enhanced proinflammatory environment with increased IL-12 and IL-1 β secretion by APCs. Increased levels of IL-12 can further induce Tregs to secrete IFN- γ after induction of TBX21. The proinflammatory environment and enhanced CD80/86-CD28 costimulation of Teffs could potentially lead to the induction of pathogenic Th1 and Th17 cells, indicated by TBX21 and ROR γ c transcription and higher expression of CD25.

Fig. 2. Functional changes of Tr1 cells in multiple sclerosis

Indicated are the major functional and phenotypic differences between IL-10-producing Tr1 cells of healthy donors (healthy) versus Tregs from RRMS patients (multiple sclerosis). Possibly involved molecules and pathways in the interaction with antigen-presenting cells (APCs) and CD4⁺ effector T (Teff) cells are highlighted and explained in the text. Tr1 cells are characterized by the co-expression of CD49b and LAG-3. Tr1 cells from healthy donors can secrete large amounts of IL-10, induced for example by IL-27, CD46, or CD2 costimulation. Tr1 cells also have the potential to secrete granzyme B (GZMB) and express CTLA-4 and are equipped with the molecules CD39/CD73 to hydrolyze ATP and generate suppressive adenosine. The induction of IL-10-secreting Tr1 cells may be impaired in MS patients as result of lower APC CD58 expression, less IL-27 and increased expression of the CD46.2 isoform, associated with proinflammatory responses. If other mechanisms of suppression besides IL-10 secretion are functionally impaired in MS patients is not fully resolved yet. Less IL-10 secretion by Tr1 cells could potentially lead to an increased proinflammatory environment and enhanced Teff induction as indicated in Figure 1 (CD46.1= CD46 isoform Cyt1, CD46.2= CD46 isoform Cyt2).