

# Chapter 2

## Regulatory T Cells in MS

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### 2.1 Introduction

Multiple sclerosis (MS) is a multifocal demyelinating disease of the central nervous system (CNS), caused by an autoimmune response to self-antigens in a genetically susceptible individual. It is characterized by progressive neurodegeneration and by CNS lesions containing a high number of infiltrating autoreactive B and T cells. In healthy individuals, regulatory T (Treg) cells can control potentially pathogenic autoreactive T cells, while Treg cells in MS patients show insufficient regulatory abilities. The fact that autoreactive T cells can be found in the peripheral blood of healthy individuals without causing any autoimmune diseases underlies the importance of an efficient control of immune responses. Treg cells are key regulators of immune homeostasis and self-tolerance.

Treg cells have been defined as  $CD4^+CD25^+FoxP3^+$  T cells that are capable of modulating the immune function of various effector cells. Other T cells have been described to also possess regulatory activity such as IL-10-secreting type 1 regulatory ( $T_R1$ ) cells and transforming growth factor  $\beta$  (TGF $\beta$ )-secreting T helper 3 ( $T_H3$ ) cells (Roncarolo et al. 2001; Weiner 2001). The population of  $CD4^+CD25^+FoxP3^+$  Treg cells comprises two subpopulations: naturally occurring and induced Treg cells (Curotto de Lafaille and Lafaille 2009). Naturally occurring Treg cells differ from induced Treg cells in being a distinct Treg cell subpopulation specialized for suppressive function that has been determined during its development in the thymus. Studies investigating immunological dysfunctions in autoimmune diseases need to consider the complex composition of the human Treg cell repertoire.

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## 2.2 Treg Cells

### 2.2.1 Cell Surface Characterization and Plasticity of Treg Cells

Treg cells were initially defined as CD4<sup>+</sup>CD25<sup>+</sup> cells in mice. Human Treg cells have been described as a population of CD4<sup>+</sup>CD25<sup>high</sup> T cells in the peripheral blood and the thymus (Taams et al. 2002; Baecher-Allan et al. 2001; Dieckmann et al. 2001; Jonuleit et al. 2001; Levings et al. 2001; Ng et al. 2001; Sakaguchi et al. 1995). The characterization of human CD4<sup>+</sup> Treg cells is complex as the human Treg cell population is heterogeneous (Miyara et al. 2009; Ito et al. 2008). Only 1–2 % of the total CD4<sup>+</sup>T cell population in the human peripheral blood consist of CD25<sup>high</sup> T cells (Baecher-Allan et al. 2001). Moreover, the isolation of Treg cells with high CD25 expression would lead to the exclusion of the naive, CD4<sup>+</sup>FoxP3<sup>low</sup>CD25<sup>mid</sup> Treg cell population.

#### 2.2.1.1 Forkhead Box P3 (FoxP3) Is Essential for Immune Homeostasis

The transcription factor FoxP3 has been established as a specific marker for mouse Treg cells and as a cell surface marker for human Treg cells (Fontenot et al. 2003; Hori et al. 2003; Roncador et al. 2001). The importance of FoxP3 for immune homeostasis can be illustrated by the observation that scurfy mice, X-chromosome-linked mouse mutants, show a defect in the FoxP3 gene and a lack of scurf, the protein that is encoded by FoxP3. This defective gene function results in a lethal disorder in mice similar to the Wiskott–Aldrich syndrome, involving scaly skin, gastrointestinal bleeding, hepatosplenomegaly, and severe anemia. The disorder is mediated by CD4<sup>+</sup>CD8<sup>-</sup> T cells whose activity has been shown to be improperly regulated in scurfy mice (Brunkow et al. 2001; Kanangat et al. 1996; Lyon et al. 1990; Blair et al. 1994). Moreover, mutations in human FoxP3 have been identified to cause the rare, fatal immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). Affected patients show, among other symptoms, a neonatal onset of type 1 diabetes mellitus, immune dysregulation, anemia, eczema, thrombocytopenia, and hypothyroidism (Bennett et al. 2001; Wildin et al. 2001, 2002).

#### 2.2.1.2 Cell Surface Characterization of Treg Cells

In the search for an additional marker for Treg cells, it has been observed by several groups that CD4<sup>+</sup>FoxP3<sup>+</sup> T cells with high suppressive ability downregulate CD127 (IL-7R $\alpha$ ) on the cell surface (Liu et al. 2006; Seddiki et al. 2006a). High CD25 and low CD127 expression can therefore be used to isolate human Treg cells from the peripheral blood. However, it has also been described that nonregulatory CD4<sup>+</sup> T cells downregulate the expression of CD127 after they have been activated, indicating that low expression of CD127 combined with the expression of CD25 cannot be

used to sufficiently distinguish between activated CD4<sup>+</sup> T cells and Treg cells (Maz-zucchelli and Durum 2007). Moreover, the fact that nonregulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells expressing low levels of FoxP3 and CD127 exist, impairs the significance of a staining with CD25 and CD127 in order to isolate Treg cells (Miyara et al. 2009). These nonregulatory Treg cells produce proinflammatory cytokines such as IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ), but do not show suppressive ability *in vitro*. Thus, the methylation status of the FoxP3 gene can be linked to the difference between regulatory and nonregulatory FoxP3<sup>+</sup> T cells, with FoxP3<sup>+</sup> Treg cells being completely and nonregulatory FoxP3<sup>+</sup>CD4<sup>+</sup> T cells being incompletely demethylated (Miyara et al. 2009). Even though CD62L (L-Selectin) is not exclusively expressed on Treg cells, it is considered an effective marker to discriminate Treg cells (CD62L<sup>+</sup>) from activated CD4<sup>+</sup> T cells (CD62L<sup>low</sup>) utilizing the fact that CD62L expression is downregulated after activation (Hamann et al. 2000). Reflecting their suppressive role, CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells also express cytotoxic T lymphocyte antigen 4 (CTLA4 and glucocorticoid-induced TNF-receptor-related protein (GITR)). The expression of these molecules correlates with the expression of FoxP3. Nevertheless, these markers are not solely expressed on Treg cells (Levings et al. 2001; Fontenot et al. 2003; Hori et al. 2003; Khattry et al. 2003).

### 2.2.1.3 Treg Cell Plasticity

The human Treg cell population is characterized by a significant heterogeneity that needs to be taken into account when performing and analyzing suppression assays. In addition to the markers noted above, the expression of CD45RA or CD45RO identifies functionally different Treg cell phenotypes, “naive” and “effector” Treg cells (Miyara et al. 2009; Seddiki et al. 2006b; Valmori et al. 2005; Fritzsching et al. 2006). CD45RA<sup>+</sup>CD45RO<sup>-</sup>FoxP3<sup>low</sup>-naive Treg cells express CD31 (PECAM1), a surface marker for cells that have emigrated from the thymus recently, but only low levels of FoxP3 (Miyara et al. 2009; Valmori et al. 2005; Fritzsching et al. 2006; Kimmig et al. 2002). Reflecting the finding that naive Treg cells are more common in the human cord blood, their proportion among the CD4<sup>+</sup> T cells in the human peripheral blood declines with age. In contrast to this, the proportion of effector Treg cells increases, with effector Treg cells being more prevalent in adults and elderly people (Miyara et al. 2009). After TCR stimulation, Treg cells with a naive phenotype proliferate, upregulate their FoxP3 expression, and convert to CD45RO<sup>+</sup>FoxP3<sup>high</sup> effector Treg cells (Miyara et al. 2009). These effector T cells exhibit a strong suppressive activity, but are also highly susceptible to apoptosis after activation and during suppression (Jonuleit et al. 2001; Vukmanovic-Stejic et al. 2006).

The population of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells can be subdivided into two subpopulations: natural, thymus-derived Treg cells and adaptive Treg cells, conventional CD4<sup>+</sup> T cells that have been induced in the periphery (Sakaguchi et al. 2010; Kretschmer et al. 2005; Apostolou and Boehme 2004; Curotto de Lafaille et al. 2004). It has been shown that CD4<sup>+</sup> T cells require TCR stimulation as well as the presence of TGF-beta and IL-2 to convert to CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells

(Chen et al. 2003; Zheng et al. 2007). In a recent publication, Helios, a member of the Ikaros transcription factor family, has been described as a potential new marker for thymus-derived Treg cells. After Helios has been shown to be expressed in FoxP3<sup>+</sup> Treg cells in microarray analyses (Sugimoto et al. 2006), Thornton et al. (2010) have observed that Helios is expressed early in the thymic development. Concurrent with the appearance of FoxP3 on the mRNA level, the expression of Helios is significantly increased in thymic CD4<sup>+</sup>CD25<sup>+</sup> cells. FoxP3<sup>+</sup> Treg cells that were induced in the periphery did not express Helios. Even though Helios does not seem to be involved in the regulation of either FoxP3 expression or Treg cell function, it may be regarded as an important Treg cell marker that allows distinguishing between induced and thymus-derived Treg cells (Thornton et al. 2010).

Moreover, distinct, terminally differentiated subpopulations of Treg cells can be identified based on the level of major histocompatibility complex (MHC) class II determinants (DR) expression. MHC-DR<sup>+</sup> Treg cells express high levels of FoxP3, and account for approximately 20–30 % of human Treg cells and approximately one-third of the circulating MHC II<sup>+</sup>CD4<sup>+</sup> cells. These MHC-DR<sup>+</sup> Treg cells exhibit a FoxP3-associated early contact-dependent suppression and cytokine production, but do not secrete IL-10. In contrast, MHC-DR<sup>-</sup> Treg cells execute their suppressive activity through a late FoxP3-associated cell contact-mediated mechanism as well as IL-10 secretion (Baecher-Allan et al. 2006).

## 2.2.2 *Suppressive Activity*

### 2.2.2.1 Mechanisms of Treg Cell Suppression

Although Treg cells have been shown to play a crucial role in the immune system by controlling immune responses, no single mechanism of suppression used by Treg cells has emerged, suggesting that Tregs are endowed with a number of pathways each contributing to the regulatory functionality of this population of T cells. One possible mechanism of action is cytokine secretion. In vivo, Treg cells secrete the suppressive cytokines TGF- $\beta$  and IL-10 (Powrie et al. 1996; Asseman et al. 1999). TGF- $\beta$  has been shown to be essential for Treg cell-mediated suppression of effector CD4<sup>+</sup> T cells in a murine model for colitis (Fahlen et al. 2005). In this model, Treg cells were not able to control CD4<sup>+</sup> T cells that were not sensitive to TGF- $\beta$ . However, Kullberg et al. (2005) have shown that TGF- $\beta$  production by Treg cells is not essential for in vivo Treg suppression. Other murine models of autoimmune inflammation have shown that IL-10 production contributes to Treg cell-mediated immune suppression (Asseman et al. 1999; Annacker et al. 2001). In vitro, both, TGF- $\beta$  and IL-10, are not required for suppression (Shevach 2009).

Other models of Treg suppression require cell–cell contact as one possible mechanism of suppression used by Treg cells. Nakamura et al. (2001) have shown that membrane-bound TGF- $\beta$  contributes to cell–cell contact-mediated suppression. Furthermore, the cell surface molecules Fas, Granzyme B, LAG3, and CTLA-4 have

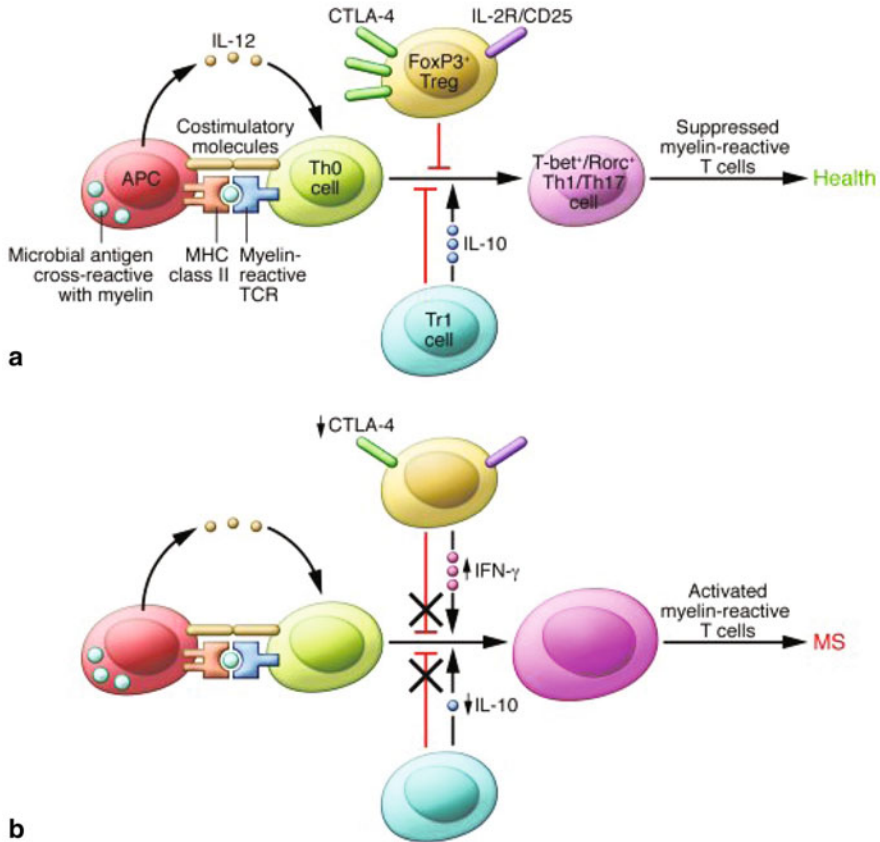
been implicated in suppression (Huang et al. 2004; Janssens et al. 2003; Cao et al. 2007; Read et al. 2000). Despite various reports suggesting that cell surface molecules contribute to Treg-mediated suppression, the role of cell–cell contact may vary depending upon the particular Treg subpopulation and the organ system where the function is being observed.

In vitro, Treg cells can deprive other T cells of IL-2, suggesting that competition for growth factors might contribute to the suppressive capacity of Treg cells (de la Rosa et al. 2004, Barthlott et al. 2005). However, the suppressive function of Treg cells cannot be entirely explained by IL-2 deprivation of effector T cells, as IL-2 receptor-deficient Treg cells are fully able to suppress T cell proliferation in vitro (Fontenot et al. 2005). Furthermore, Treg cells can suppress IL-2 receptor-deficient effector T cells (Fontenot et al. 2005). As no unique mechanism has been identified to be required for suppression, it is very likely that different mechanisms contribute to the suppressive ability of Treg cells.

### 2.2.2.2 Immune Functions Regulated by Treg Cells

Besides modulating the function of CD4<sup>+</sup> T cells, Treg cells also regulate a broad variety of immune cells such as CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, natural killer (NK) cells, natural killer T (NKT) cells, and antigen-presenting cells (APCs), through the suppression of activation, proliferation, and cytokine production (Zhao et al. 2006; Azuma et al. 2003; Ralainirina et al. 2007; Taams et al. 2005; Misra et al. 2004). As a basic prerequisite in order to execute their full suppressive potential, Treg cells must be activated through their T cell receptor (TCR; Baecher-Allan et al. 2001; Dieckman et al. 2001; Jonuleit et al. 2001). Interestingly, after being activated, they do not need to be viable in coculture to be capable to suppress the cell function of responder cells. In addition, the strength of T cell stimulation strongly influences whether suppression or proliferation occurs. It has been shown that effector T cells that have been activated in the presence of strong costimulatory signals seem to be refractory to Treg cell-induced suppression (Baecher-Allan et al. 2001, 2002). Moreover, the resistance of effector T cells to regulation through Treg cells-mediated mechanisms increases when the strength of TCR signals received by the effector T cells is increased (Baecher-Allan et al. 2001).

Moreover, various groups have described the occurrence of human Treg cells that secrete the proinflammatory cytokine IL-17 (Koenen et al. 2008; Beriou et al. 2009; Ayyoub et al. 2009). These cells have been shown to express ROR $\gamma$  t, a specific transcription factor for T<sub>H</sub>17 cells (Koenen et al. 2008, Ayyoub et al. 2009). Beriou et al. (2009) have identified a subset of Treg cells within a CD4<sup>+</sup>CD45RA<sup>-</sup>CD25<sup>high</sup>CCR6<sup>+</sup>HLA-DR<sup>-</sup>FoxP3<sup>+</sup> population that is capable of producing IL-17. The presence of IL-1beta and IL-6 during activation of the cells was required for the production of IL-17. Interestingly, IL-17<sup>+</sup>/FoxP3<sup>+</sup> clones from this subpopulation were able to alternately suppress Treg cells or secrete high levels of IL-17, depending on the stimuli.



**Fig. 2.1** Role of FoxP3<sup>+</sup> Treg cells in immune regulation. **a** In healthy individuals, Treg cells modulate the immune function through the suppression of activation, proliferation and cytokine production, thereby interacting with various cell types. **b** In patients with MS, the immunoregulatory function of Treg cells is impaired. The frequency of IFN- $\gamma$  producing T<sub>H</sub>1-like cells is increased (Nylander and Hafler 2012)

In recent publications, Eos, a zinc-finger transcription factor of the Ikaros family, has been identified as an important element of FoxP3-mediated suppressive activity of Treg cells. Pan et al. (2009) have demonstrated that Eos induces gene silencing in Treg cells by directly interacting with FoxP3. Eos has been shown to coimmunoprecipitate with FoxP3. The secretion of IL-2 by transduced primary CD4<sup>+</sup> T cells was inhibited by the expression of full-length FoxP3. In contrast, the expression of  $\Delta$ FoxP3 (a 51-amino acid fragment of FoxP3 that is necessary to bind to the C-terminal region of Eos) reversed this effect. After a knockdown of Eos in the same cells, the FoxP3-dependent suppression of IL-2 was abrogated. Moreover, the same group investigated the effect of an Eos-knockdown on a FoxP3-dependent gene set from Treg cells (Pan et al. 2009). Strikingly, more than 70 % of genes affected by

a Eos-knockdown in Treg cells were FoxP3-dependent, and 90 % of the genes that are usually downregulated by FoxP3 expression were no longer inhibited after a knockdown of Eos.

Another cell type that executes regulatory effects is the  $T_{R1}$  T cell.  $T_{R1}$  cells are induced through activating signals such as CD3/CD46 cross-linking or IL-27 and TGF $\beta$ . They are characterized by the secretion of high levels of the highly immunosuppressive cytokine IL-10 as well as the lack of IL-4 expression and no or only low expression of IL-2.  $T_{R1}$  cells modulate immune function through IL-10 secretion by inhibiting effector function and activation of various cell types (Roncarolo et al. 2001; Fig. 2.1).

## 2.3 Treg Cells in MS

The role of Treg cells in the development and in the course of MS has been in the focus of intensive clinical and basic research in the past years. These studies have investigated the frequency as well as the immune-modulating function of Treg cells, thereby considering disease activity and therapy status.

### 2.3.1 *Frequency of Treg Cells in the Peripheral Blood of MS Patients*

The frequency of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells in patients with MS has been investigated by various groups. Viglietta et al. (2004) first reported that while there were no significant differences in the frequency of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells in the peripheral blood of 15 untreated relapsing remitting MS (RRMS) patients and 21 healthy controls, there were differences in function that are discussed below. A similar result has been reported by a different group, which has analyzed CD4<sup>+</sup>CD25<sup>high</sup> Treg cells from 73 RRMS patients and 73 healthy controls (Haas et al. 2005). Moreover, Feger et al. (2007) have observed no differences in the frequency of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the blood of 40 healthy controls and 36 untreated patients with different disease subtypes, including clinically isolated syndrome (CIS), RRMS, secondary progressive MS (SPMS), and primary progressive MS (PPMS).

### 2.3.2 *Frequency of Treg Cells in the CSF of MS Patients*

Treg cells have been identified in the cerebrospinal fluid (CSF) of MS patients. It has been observed by Haas et al. (2005) that the number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the CSF and the peripheral blood of 15 untreated RRMS patients did not differ significantly. Nevertheless, in another study, the CSF of 14 untreated CIS

and RRMS patients and 9 patients with other, nonautoimmune neurological diseases has been analyzed. The frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells in the CSF of the MS patients was significantly increased compared with peripheral blood of the MS patients. In contrast to this, patients with other neurological diseases did not show elevated Treg cell levels in the CSF compared with the blood. These data suggest that Treg cells are selectively enriched in the CSF of MS patients in order to fight autoimmune processes (Feger et al. 2007). In a recent report, Fritzsching et al. (2006) have compared the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells in the CSF of 17 treatment-naïve MS patients with the number of Treg cells in the peripheral blood. CD45RO<sup>high</sup>CD95<sup>high</sup> Treg cells have been shown to be highly sensitive to CD95L-induced apoptosis. Strikingly, this subpopulation was increased in the CSF of MS patients compared with the peripheral blood. The Fritzsching group therefore hypothesizes that Treg cells may be eliminated in the CNS through CD95L-mediated apoptosis. Interestingly, the same group has also examined brain biopsies of 16 untreated RRMS patients. No Treg cells were detectable in 30 % of the biopsies; the number of FoxP3<sup>+</sup> T cell was generally low in the analyzed brain tissue (Fritzsching et al. 2011).

### 2.3.3 *Function of Treg Cells in MS*

#### 2.3.3.1 **Function of CD4<sup>+</sup>CD25<sup>+</sup> Treg Cells Is Impaired**

Even though frequency of Treg cells in the peripheral blood of MS patients does not differ in comparison to healthy controls, the immunomodulatory function of Treg cells has been shown to be impaired in MS patients (Kumar et al. 2006, Baecher-Allan et al. 2004). Viglietta et al. (2004) have analyzed samples from untreated RRMS patients. When cocultured with CD4<sup>+</sup>CD25<sup>-</sup> responder cells, CD4<sup>+</sup>CD25<sup>high</sup> Treg cells from healthy subjects effectively suppressed the proliferation of the responder cells. In striking contrast to this, CD4<sup>+</sup>CD25<sup>high</sup> Treg cells from MS patients showed an inadequate suppressive ability and inhibited proliferation only poorly. A similar observation has been made after coculturing CD4<sup>+</sup>CD25<sup>high</sup> Treg cells from MS patients with CD4<sup>+</sup>CD25<sup>-</sup> responder cells from either patients or healthy controls. The Treg cells were incapable of suppressing responder cell proliferation, while CD4<sup>+</sup>CD25<sup>-</sup> responder cells from MS patients could be suppressed by CD4<sup>+</sup>CD25<sup>high</sup> Treg cells from healthy controls, indicating that a defect in the regulatory function of the Treg cells themselves is responsible for the impaired suppressive ability. Moreover, single-cell cloning experiments were performed. Strikingly, the cloning frequency of CD4<sup>+</sup>CD25<sup>high</sup> T cells was significantly decreased in MS patients compared with healthy controls. Furthermore, Huan et al. (2005) have shown that the levels of FoxP3 mRNA and protein expression are reduced in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from untreated MS patients compared with healthy controls, indicating a correlation between the reduced FoxP3 expression and the impaired suppressive capacity of Treg cells in MS patients.



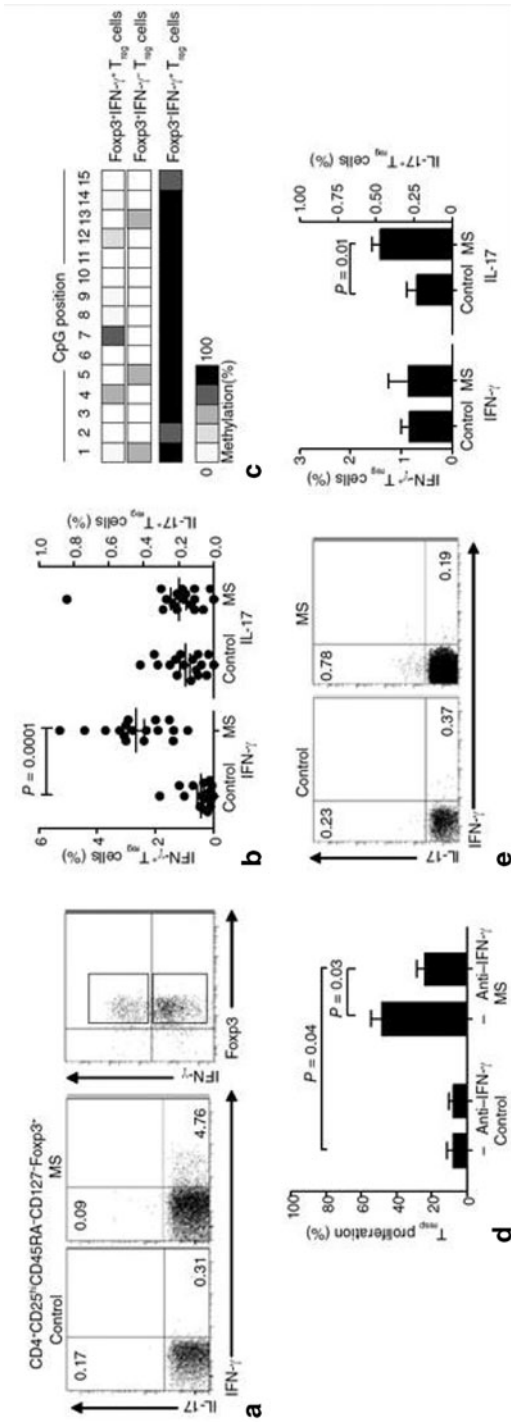
### 2.3.3.2 Frequency and Suppressive Function of CD31<sup>+</sup>-Naive Treg Cells Are Altered

An altered Treg cell subpopulation has been described by Haas et al. (2007) CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells that enter the circulation and coexpress CD31 are defined as recent thymic emigrants (RTEs). The number of RTEs decreases with age and is significantly diminished in RRMS patients compared with healthy controls. The reduced de novo generation of CD31<sup>+</sup>-naive Treg cells was compensated by an increase in the amount of memory Treg cells so that the total Treg cell number was stable. The differences in the functional capacity of Treg cells between MS patients and healthy controls were neutralized by depletion of CD31<sup>+</sup> T cells, indicating that RTEs contribute to the functional characteristics of the Treg cell population.

Although a defect in Treg cells function has been observed in patients with RRMS, the suppressive capacity of Treg cells from patients with SPMS does not seem to be impaired (Venken et al. 2006). The reduced suppressive ability of the Treg cells has been correlated with a decreased FoxP3 expression in RRMS patients (Venken et al. 2008a). Moreover, the Venken group has compared the frequency and function of naive (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>CD45RA<sup>+</sup>) and memory (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>CD45RO<sup>+</sup>) Treg cells from untreated RRMS and SPMS patients with short and long disease duration, respectively. The suppressive capacity of naive Treg cells was decreased in all groups compared with healthy controls; in contrast, the suppressive function of memory Treg cells was similar to healthy controls and was increased in SPMS patients and RRMS patients with a long disease duration ( $\geq 10$  years) compared with RRMS patients with a short disease duration ( $< 10$  years). When comparing the frequency of naive and memory Treg cells of early (disease duration  $< 10$  years) and chronic (disease duration  $\geq 10$  years) untreated MS patients, they observed that both groups showed decreased numbers of naive Treg cells. The frequency of memory Treg cells was increased in chronic patients. Interestingly, the proportion of CD31<sup>+</sup> memory Treg cells was diminished in early MS patients, suggesting a high cell turnover in early disease (Venken et al. 2008b).

### 2.3.3.3 IFN- $\gamma$ -Secreting T<sub>H</sub>1-Like T Cells Are More Prevalent in MS Patients

CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> Treg cells isolated ex vivo from patients with untreated RRMS show a T helper type 1 (T<sub>H</sub>1)-like phenotype after stimulation with PMA and ionomycin. The frequency of these IFN- $\gamma$ -secreting T<sub>H</sub>1-like T cells was higher in untreated RRMS patients compared with healthy controls (Fig. 2.2). Treg cells and responder T cells from patients with RRMS have been cultured with an IFN- $\gamma$ -specific antibody showing that blocking IFN- $\gamma$  leads to a significantly elevated suppressive capacity of the Treg cells, thereby confirming that the suppressive ability of Treg cells from RRMS patients is decreased by IFN- $\gamma$  production. Moreover, a comparison of Treg cells from IFN- $\beta$ -treated RRMS patients with Treg cells from healthy controls has shown a similar frequency of IFN- $\gamma$ -secreting FoxP3<sup>+</sup> T cells in both groups. Comparable results have been reported for type 1 diabetes (Dominguez-Villar et al. 2011, McClymont et al. 2011).



**Fig. 2.2** Treg cells from individuals with RRMS secrete IFN- $\gamma$  ex vivo. **a** The frequency of FACS-sorted IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> Treg cells in healthy control individuals (*left*) and untreated individuals with RRMS (*middle*,  $n = 17$ ) gated on Foxp3<sup>+</sup> Treg cells. *Right*, purity analysis of the sorted IFN- $\gamma$ <sup>+</sup>Foxp3<sup>+</sup> and IFN- $\gamma$ <sup>-</sup>Foxp3<sup>+</sup> populations from subjects with RRMS used for methylation analysis in (c). **b** Percentage of IFN- $\gamma$ <sup>+</sup>Foxp3<sup>+</sup> and IL-17<sup>+</sup>Foxp3<sup>+</sup> Treg cells ( $n = 17$ ) as a proportion of total Foxp3<sup>+</sup> Treg cells. **c** Representative example of methylation analysis of the TSDR region of the FOXP3 locus in sorted IFN- $\gamma$ <sup>+</sup>Foxp3<sup>+</sup> and IFN- $\gamma$ <sup>-</sup>Foxp3<sup>+</sup> Treg cells from subjects with RRMS. An analysis of IFN- $\gamma$ <sup>+</sup>Foxp3<sup>-</sup> memory T cells from subjects with RRMS is shown as a control. **d** Proliferation of Tresp cells cultured with ex vivo FACS-sorted Treg cells from healthy control subjects and untreated subjects with MS (Treg cell:Tresp cell ratio of 1:2) in the presence or absence of an IFN- $\gamma$ -specific antibody ( $n = 4$ ). **e** The frequency of IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> Treg cells in healthy control subjects (*left*) or IFN- $\beta$ -treated patients with RRMS (*right*) as assessed by intracellular cytokine staining and FACS analysis. The bar diagram (*right*) shows the percentage of IFN- $\gamma$ <sup>+</sup>Foxp3<sup>+</sup> and IL-17<sup>+</sup>Foxp3<sup>+</sup> cells as a proportion of total Foxp3<sup>+</sup> Treg cells in healthy controls or IFN- $\beta$ -treated patients with RRMS ( $n = 12$ ). (Dominguez-Villar et al. 2011, Copywright 2011)

### 2.3.3.4 CD46-Activated T Cells Have an Impaired Immunomodulatory Function

A defect of suppressive function has also been described for other regulatory cells, such as  $T_R1$  cells.  $T_R1$  cells are characterized by the secretion of high levels of IL-10 (Roncarolo et al. 2001; Groux et al. 1997). It has been observed that the immunoregulatory function of CD46-activated T cells is impaired in patients diagnosed with MS. CD46 is a costimulatory membrane molecule with two cytoplasmatic isoforms, Cyt1 (16 amino acids) and Cyt2 (23 amino acids). CD46-costimulated T cells acquire a  $T_R1$ -like phenotype in the presence of IL-2 with IL-10 and granzyme B production, but can also show a  $T_H1$ -like response, characterized by elevated levels of IL-2, IL-10, and IFN- $\gamma$  and decreased secretion of IL-5. CD4<sup>+</sup> T cells from healthy controls and from untreated or IFN- $\beta$ - treated patients with RRMS were stimulated with anti-CD3 and either anti-CD28 or anti-CD46 antibodies in the presence of IL-2. The IL-10 secretion of CD46-activated T cells from treated and untreated MS patients was significantly diminished compared with healthy controls, while INF- $\gamma$  production did not significantly change in any of the groups. This effect could not be observed for CD28- stimulated T cells, and is therefore specific to CD46. Moreover, it has been detected that the CD46 isoform Cyt2 is significantly higher expressed in T cells from MS patients compared with healthy controls. In a murine model, Cyt2 is associated with an enhancement of inflammatory processes, while the other isoform, Cyt1, seems to inhibit inflammation (Astier et al. 2006; Marie et al. 2002).

### 2.3.3.5 Allele of CD58 Locus Has a Protective Effect

In the past years, genetic analyses such as genome-wide association studies (GWAS) have contributed greatly to the expansion of our knowledge of MS. In several GWAS, multiple susceptibility loci have been identified that are associated with the risk of developing the disease. Some of these loci, such as IL2RA (CD25), IL7RA, and CD58, are of particular interest as they have been shown to be linked to the function of Treg cells (Jager et al. 2009; Sawcer et al. 2005, 2011).

Recently, the protective effect of the rs2300747<sup>G</sup> allele of the CD58 locus has been described. CD58 (LFA-3) influences TCR signaling through the engagement of its receptor CD2. CD2 enhances the suppressive function of human Treg cells through costimulation and, moreover, by promoting FoxP3 expression. An enhanced CD58 mRNA expression has been observed in lymphoblastic cell lines and peripheral blood mononuclear cells (PBMCs) from patients with RRMS or CIS. Furthermore, it has been shown in a different set of patients that CD58 mRNA expression levels were higher in those patients who were in clinical remission, suggesting that elevated levels of CD58 RNA expression may play a role in minimizing inflammation in MS patients by enhancing the function of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells through CD2-mediated upregulation of FoxP3 expression (Jager et al. 2009).

### **2.3.4 Treg Cells in Experimental Autoimmune Encephalomyelitis (EAE)**

In the investigation of immunological mechanisms, which contribute to the pathogenesis of MS, the use of EAE, and the mouse model of MS, has led to important observations. Many groups have described the complex role of Treg cells in the development and control of EAE. The CNS shows an increased proliferation of Treg cells during inflammation (O'Connor et al. 2007). It has been shown by several groups that murine CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells accumulate in the CNS, and that this accumulation correlates with recovery (Korn et al. 2007, McGeachy et al. 2005). Nevertheless, the accumulated cells do not have the functional capacity to effectively suppress effector T cells during the peak of EAE (Korn et al. 2007). As mentioned above, a subset of CD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells with the ability to produce IL-17 under certain inflammatory conditions has been identified in the human peripheral blood. Treg cells taken from the CNS of mice with EAE did not secrete IL-17 in the presence of IL-6. It has been observed that Treg cells from the CNS of these mice were lacking the IL-6 receptor chains, CD126 and gp130, suggesting a reduced responsiveness to IL-6 (O'Connor et al. 2012).

#### **2.3.4.1 Adoptive Transfer of Treg Cells Has a Protective Effect**

In 1994, Lafaille et al. (1994) have shown that the development of spontaneous EAE is increased in immunodeficient TCR myelin basic protein (MBP) transgenic mice. For these experiments, TCR transgenic mice were crossed with recombinant activating gene (RAG)-1 gene-deficient mice in order to generate mice with no lymphocytes but CD4<sup>+</sup> T cells expressing TCRs specific for MBP. All TCR transgenic RAG-1-deficient (T/R<sup>-</sup>) mice developed spontaneous EAE, while only some mice with an intact RAG-1 gene (T/R<sup>+</sup>) were affected, suggesting that nontransgenic lymphocytes have a protective effect. Moreover, it has been observed that an early transfer of total splenocytes or CD4<sup>+</sup> T cells from normal donor mice into T/R<sup>-</sup> mice can prevent EAE in T/R<sup>-</sup> mice. The same group has also described that CD4<sup>+</sup> T cells expressing endogenous  $\alpha$  and  $\beta$  TCR chains are required to protect T/R<sup>+</sup> from developing spontaneous EAE. These observations have been interpreted in favor of the immunomodulatory effect of Treg cells on the development of EAE (Olivares-Villagomez et al. 1998).

These conclusions have been supported by a study of Hori et al. (2002) on the effect of CD25<sup>+</sup>CD4<sup>+</sup> Treg cells on the development of EAE in TCR MBP RAG-1-deficient mice. In contrast to T/R<sup>-</sup> mice, T/R<sup>+</sup> mice contain CD25<sup>+</sup>CD4<sup>+</sup> T cells. The adoptive transfer of CD25<sup>+</sup>CD4<sup>+</sup> T cells from either wild-type or T/R<sup>+</sup> mice into T/R<sup>-</sup> mice prevented T/R<sup>-</sup> mice from developing EAE. Moreover, Kohm et al. (2002) have observed that the adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells results in a decreased CNS infiltration and in protection from myelin oligodendrocyte glycoprotein (MOG)- induced EAE in C57BL/6 mice, underlining the protective

effect of Treg cells. In addition to this, Treg cells have been shown to have the potential to inhibit the cytokine production and proliferation of MOG<sub>35–55</sub>-specific Th1 cells in vitro.

Passive transfer of CD4<sup>+</sup>CD25<sup>+</sup> CNS-derived Treg cells in low numbers that were isolated from mice that were in the recovery phase of MOG- induced EAE has been shown to protect C57BL/6 mice from the development of EAE. In contrast, the same number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from naive lymph nodes did not have the same effect, indicating that antigen-specific Treg cells that have been isolated from the inflamed tissue seem to be more potent to suppress inflammation (McGeachy et al. 2005).

#### 2.3.4.2 Differences in Disease Susceptibility

Not all mouse strains are equally or at all susceptible to the induction of EAE. Although B10.S mice are highly resistant, SJL mice show a high susceptibility to PLP139-151-induced EAE. Reddy et al. (2004, 2005) have demonstrated that after using PLP139-151 tetramers, both mouse strains do not show significant differences in the frequency of tetramer-positive T cells in the naive compartment. Interestingly, most tetramer-positive T cells in SJL mice were CD4<sup>+</sup>CD25<sup>-</sup>; while PLP139-151 tetramer-reactive T cells in B10.S mice have been shown to be CD4<sup>+</sup>CD25<sup>+</sup>. After depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells through administration of anti-CD25 antibody, the susceptibility of B10.S to the induction of EAE has been shown to be increased. This observation indicates that B10 mice have strong Treg cell population and underlines the importance of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the regulation autoimmune diseases. Interestingly, the depletion of Treg cells before induction of PLP139-151-mediated EAE leads to a greater degree of susceptibility to EAE in male mice compared with nontransgenic controls and female mice.

#### 2.3.4.3 Depletion of CD25<sup>+</sup> Increases Disease Severity

This observation is supported by another study, in which it has been demonstrated that the depletion of CD25<sup>+</sup> Treg cells in C57Bl/6 mice by anti-CD25 antibodies has been shown to inhibit the recovery from the MOG- induced EAE. Furthermore, the resistance to reinduction of EAE is removed when Treg cells are depleted (McGeachy et al. 2005). Another important study reports that SJL mice show an increased severity of PLP139-151-induced EAE after administration of anti-CD25 antibodies in order to reduce CD25<sup>+</sup>CD4<sup>+</sup> T cells. Although cells from the lymph nodes of anti-CD25 antibody-treated mice produced more IFN- $\gamma$  after stimulation with PLP139-151 in vitro, the IL-10 secretion of these cells was significantly reduced. The adoptive transfer of CD25<sup>+</sup>CD4<sup>+</sup> T cells from naive SJL mice into mice before induction of EAE decreased disease severity, illustrating the suppressive *capacity* of Treg cells (Zhang et al. 2004).

### 2.3.5 *Treg Cells and MS Therapy*

A therapeutic strategy to recover immune function in MS patients could be the restoration of impaired Treg cell function. Some immunomodulatory MS treatments, such as glatiramer acetate (GA) and IFN- $\beta$ , have been described to partially execute their therapeutic effect through Treg cells.

Treatment with GA has been shown to increase the FoxP3 expression of Treg cells from MS patients (Hong et al. 2005). In a recent study, the blood of 15 RRMS patients has been analyzed before and after long-term treatment with GA. Before treatment with GA (baseline) the proportions of naive and recent thymic emigrant Treg cells within the total Treg cell population were decreased compared with healthy controls. Interestingly, this effect was reversed after the patients had received treatment with GA for up to 6 months. Moreover, the impaired suppressive function of the total Treg cells was improved compared with the baseline results (Haas et al. 2009). In addition to this, it has been observed that treatment with GA leads to an upregulation of specific CD8<sup>+</sup> T cells responses (Karandikar et al. 2002). GA-reactive CD8<sup>+</sup> T cells have been shown to execute suppressive function. Although the suppressive ability of CD8<sup>+</sup> T cells was reduced in untreated MS patients compared with healthy controls, it was significantly enhanced in GA-treated MS patients, suggesting that treatment with GA has the potential to modulate immune responses directly during ongoing therapy (Tennakoon et al. 2006).

Another potent therapeutic agent is IFN- $\beta$ . Blood samples of 22 untreated RRMS patients were analyzed before and after treatment with IFN- $\beta$ -1a. The suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells was impaired at baseline, but was restored after 6 months of treatment (Andres et al. 2007). Furthermore, Vandembark et al. (2009) have reported elevated FoxP3 mRNA levels in RRMS patients that have been treated with IFN- $\beta$ -1a for 12 months compared with their own baseline results and to untreated RRMS patients and healthy controls. Nevertheless, the FoxP3 protein levels did not differ significantly from those observed in untreated RRMS patients and healthy controls. Interestingly, one group has observed no differences in the frequency of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells in the blood patients treated with GA and/or IFN- $\beta$ -1a compared with untreated MS patients and healthy controls (Putheti et al. 2004).

Natalizumab, a monoclonal antibody against the  $\alpha$ -4 chain of very late activation antigen 4 (VLA-4), is an efficient drug in the treatment of MS. However, it does not seem to influence the suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells (Stenner et al. 2008, Ramos-Cejudo et al. 2011).

## 2.4 Conclusion

MS is an autoimmune disease caused by uncontrolled autoreactive T and B cells. The failure of Treg cells to suppress these immune cells in MS patients plays an important role in disease onset and progression. Although the frequency of CD4<sup>+</sup>CD25<sup>high</sup> Treg

cells is not altered in MS patients as compared with healthy controls, the suppressive capacity of Treg cells is impaired in MS patients. Recent studies have shown that Treg cells in MS patients can exhibit effector T cell functions, such as IFN- $\gamma$  production. Modification of Treg cell activity is therefore a promising approach for the development of new experimental therapies for MS and other autoimmune diseases.

Treg cells are key regulators of the immune system. Imbalance of Treg cells and effector immune cells contributes to the development of various autoimmune diseases. Lessons learned from Treg studies in MS patients and EAE mouse models might therefore not only contribute to a better understanding of MS development and progression, but also provide insights into other autoimmune diseases.

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