T regulatory cells: aid or hindrance in the clearance of disease?

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Abstract

CD4+ CD25+ T regulatory cells (Tregs) are classified as a subset of T cells whose role is the suppression and regulation of immune responses to self and non-self. Since their discovery in the early 1970s, the role of CD4+ CD25+ Tregs in both autoimmune and infectious disease has continued to expand. This review examines the recent advances on the role CD4+ CD25+ Tregs may be playing in various diseases regarding progression or protection. In addition, advances made in the purification and manipulation of CD4+ CD25+ Tregs using new cell markers, techniques and antibodies are discussed. Ultimately, an overall understanding of the exact mechanism which CD4+ CD25+ Tregs implement during disease progression will enhance our ability to manipulate CD4+ CD25+ Tregs in a clinically beneficial manner.

Keywords: CD4+ CD25+ • T regulatory cells • FoxP3 • non-human primates • immune responses
Introduction

The diverse cellular makeup of the immune system enables it to face the daily challenges confronted by both self and non-self antigens. Many of the functional roles of these diverse cell types, however, are only starting to be understood.

T regulatory cells (Tregs) are a subset of T cells that are now increasingly appreciated for their role in immunological defence. Described in the early 1970s by Gershon and Kondo as T cells that were able to suppress immune responses, they were given the early name of suppressor T cells [1]. At that time it was believed that these suppressor T cells were able to mediate their function by secreting antigen-specific factors [2]. However, as research progressed, the inability to isolate and/or demonstrate the function of the suppressor T cells ultimately led to their downfall, with some researchers going so far as to question their existence [3–6]. The interest in suppressor T cells was revived in 1995 when a Japanese group led by Dr Sakaguchi demonstrated that a small sub-population of T cells (≤10% of the total CD4 T cell population) expressing the interleukin (IL)-2 receptor α-chain (CD25) was directly responsible for preventing spontaneous development of autoimmune disease in mice; thus, Balb/c nu/nu mice (CD25) was directly responsible for preventing suppression in vitro, however, the in vivo role of Tregs need to be activated to exert their suppressive function in particular dendritic cells (DCs) [33]. Generally, Tregs seem to act indirectly by involving other cell sub-types, such as CD8+CD4− T cells, Qa-1-restricted CD8+ T cells and natural killer (NK) T cells [15–24] (Table 1). Research is still in the early stages in determining the classification, differentiation and functions of these various regulatory cell types with many of the cells seeming to have redundant capabilities. However, these cellular redundancies may be the key to how the immune system is capable of successfully mounting responses against various antigens.

For the second question, two main concepts have emerged from recent research. The first concept is that there are two distinct populations of CD4+CD25+ Tregs: ‘Natural Tregs’ (CD4+CD25high) which arise directly from the thymus and are programmed to suppress the immune systems response to self anti-gens, and ‘Adaptive Tregs’ (CD4+CD25Variable) which also arise from the thymus, but adopts their suppressive ability in the periphery, after exposure to antigen at a later time (Table 1) [25, 26]. The access to the two different CD4+CD25+ Tregs types is imperative for the immune system to actively suppress both external and internal assaults on the host, as well as protect against self-antigen that may escape from the thymus undetected.

As for the third question, research has shown that both natural and adaptive CD4+CD25+ Tregs are able to suppress the immune system by using both non-specific [27, 28] and antigen-specific responses [29–32]. Many in vivo and in vitro studies have investigated the suppressive functionality of CD4+CD25+ Tregs; however, no general mechanism has emerged to date on how Tregs act on cells. It has been shown that the suppressive function needs direct cell–cell contact and/or release of cytokines (Fig. 1) [27, 29]. Sometimes Tregs seem to act indirectly by involving other cell sub-types, in particular dendritic cells (DCs) [33]. Generally, Tregs need to be activated to exert their suppressive function [34]. When activated, CD4+CD25+ Tregs can secrete various cytokines into the milieu, such as IL-10 and TGF-β [27, 29]. However, TGF-β which is only found cell-bound in non-activated CD4+CD25+ Tregs, seems to be of equal importance for contact-dependent suppression as the secreted form (Fig. 1) [27]. There are conflict-ing data on the impact of soluble cytokines on suppression in vitro, however, the in vivo role of cytokines has been confirmed [35, 36]. Most probably, there is not just one route that the CD4+CD25+ Tregs...
use to exert their functions on their target cells; this complex system could be the result of the CD4+CD25+ Tregs maximizing their efficiency or some kind of specificity of suppressing the immune response by having multiple mechanisms at their disposal. This concept would allow the CD4+CD25+ Tregs to have the ability to express their function on a variety of target cells in different environments as the situation dictates. However, the research to date has not yet led us to a conclusive outcome on how exactly the CD4+CD25+ Tregs exert their suppressive abilities. While the field of Tregs is still very much in flux, in this review we will give a balanced view on the state of Treg research, focusing specifically on CD4+CD25+ FoxP3+ and their characteristics, regulation and potential benefits in the management of various diseases.

### Table 1 Phenotypes of known regulatory cells of the immune system

| T Cell Name | Cell markers | Characteristics /Function | Ref |
|-------------|--------------|--------------------------|-----|
| CD4+CD25<sup>high</sup>FoxP3<sup>+</sup> | ↑GITR, ↑CTLA-4, ↑LAG-3 | Derived from the thymus. Suppression of T cell response to self-antigens. | [25, 26] |
| CD4+CD25<sup>+</sup> | ↓FoxP3, CD25<sub>variable</sub> | Adopts its abilities in the periphery. Suppression of T cell response to self. | [25, 26] |
| Th3 | ↑FoxP3, ↑TGF-β | Important in eliciting oral tolerance | [23] |
| Tr1 | ↑IL-10, ↑TGF-β | Suppression of immune response, mainly found in intestinal mucosa | [253, 254] |
| CD8+CD28<sup>-</sup> | ↑GITR, ↓CD25, ↑CXCR1, ↑FoxP3 | Memory/Effect cells, perforin expression | [255, 256] |
| CD8<sup>*</sup>122<sup>+</sup> | ↑CD44, ↑NK receptors | Respond to both TCR and NR receptor stimulation, may be similar to NK cells | [257] |
| Qa-1 restricted CD8<sup>+</sup> | ↑CD94/NKG2A | Enhances Ag-specific CD4<sup>+</sup> T cell responses, maybe other regulation functions | [258] |
| Natural killer T cells | ↑CD94/NKG2A, ↑CD56, ↑CD25, ↑CD16 | Possibly important in the innate and adaptive immune response??? | [24] |

**Fig. 1** Induced and natural CD4+CD25<sup>+</sup> Tregs have been found to express many different surface markers and cytokines. It is believed that one of the factors involved in Treg induction are antigen-presenting DC (1). Once induced, CD4+CD25<sup>+</sup> Tregs are capable of acting on effector T cells through either cell–cell contact (2) or cytokine secretion (3). TGF-β has been involved in Treg function through both of these pathways.
Characteristics

CD4+CD25+ Tregs are not the only cells in the body that express CD4 and CD25. In human blood, up to 20% of CD4+ cells express CD25, most corresponding to activated conventional T cells. Although the Treg populations are often confined to the CD25high population, the suppressor cells can also be present in the intermediate sub-population. Moreover, CD25 expression is not always maintained on Tregs [37, 38]. Thus, there is a requirement of additional markers to further differentiate CD4+CD25+ Tregs. Identifying additional surface/internal markers of Tregs will help determine the role that CD4+CD25+ Tregs play in various diseases and will also help improve the design of therapeutic strategies by increasing the purity of isolated CD4+CD25+ Tregs [7, 14, 39–53]. In order to achieve this goal, we will need a more thorough understanding of the known and still to be discovered cellular markers that are solely expressed on the CD4+CD25+ Tregs.

To date, several additional cell markers aside from CD4 and CD25 have been shown to be expressed on CD4+CD25+ Tregs. Table 1 summarizes the known cell markers found on CD4+CD25+ Tregs and their possible or distinct role in the suppression of the immune system.

The forkhead/winged-helix protein transcription factor (FoxP3)

FoxP3 has recently been shown to be predominately expressed in the CD4+CD25+ Tregs [50]. In mice, FoxP3 is expressed mainly in CD4+CD25+ T cells, with a minor population (2–40%) of FoxP3+ cells being CD25neg; conversely, about 10% of CD25+ T cells are FoxP3neg [54, 55]. In humans beings, almost all CD4+CD25high cells are FoxP3+, whereas a variable percentage of CD25int cells express generally lower amounts of FoxP3. FoxP3 is also expressed in other cell populations such as in CD4+ and CD8+ T cells upon activation.

It is becoming more apparent that FoxP3 is not only a phenotypic marker of CD4+CD25+ Tregs, but it is also essential to their development and function [43]. Highly conserved in mammalian species (Fig. 2), the FoxP3 protein belongs to the forkhead/winged-helix FoxP sub-family of transcriptional regulators that have been shown to operate as both transcriptional activators and/or repressors [41]. FoxP3 is a 431 amino acid long protein that encodes for structural domains, such as a leucine zipper, C2H2 zinc finger and a forkhead (FKH) domain (Fig. 3). FoxP3, unlike other FoxP members, encodes its FKH domain at the C-terminus of the protein. Also, the proline rich N-terminus of the FoxP3 protein is considered undefined [48] (Fig. 3).

The gene encoding FoxP3 was discovered in 1949, when a spontaneous mutation was found in an inbred MR strain of mice at the Oak Ridge National Laboratory. These mice, named Scurfy mice due to their phenotype, died at approximately 3 weeks of age from massive lymphoproliferative disease [51]. In human beings, a similar syndrome, IPEX (immune dysfunction/polyendocrinopathy/enteropathy/X-linked), causes infants to die within the first 2 years of life [49]. In both human beings and mice the mutation was traced back to a gene on chromosome X encoding a novel transcription factor, FoxP3 [41, 56, 57]. In scurfy mice, the mutation in the FoxP3 gene was found to be attributed to a 2 bp deletion that caused a premature stop codon to be introduced. To verify that FoxP3 was in fact the gene responsible for scurfy phenotype, the scurfy mutant mice were bred with transgenic mice carrying the FoxP3 gene on a cosmid. The offspring mice, carrying the cosmid, did not display any of the phenotypes brought on by the scurfy disease [41]. This evidence was the first indicating that a defect in the FoxP3 protein alone was involved in the scurfy phenotype and that the intact, functional FoxP3 protein was essential for normal immune homeostasis. In human beings with the IPEX syndrome, 20 different mutations in the FoxP3 gene have been identified, with the majority of the mutations occurring in the regions of the FKH domain and leucine zipper (Fig. 3) [57–61].

Two research groups set out to better understand how the genetic mutations in the leucine zipper of the protein prevents FoxP3 from functioning correctly in human beings [48, 62]. Chae et al. targeted the human JM2 mutation which has been identified as the second glutamic acid residue (AA 251) of the FoxP3 leucine zipper’s EKEK motif [62]. Due to the high homology (90%) between human and mouse FoxP3 (Fig. 2), the glutamic acid deletion was expressed in an inflammatory bowel disease (IBD) mouse model using an engineered retrovirus system. IBD is inducible in RAGneg/neg knockout mice by injecting CD4+CD25neg T cells, which causes...
| Consensus | Homo sapiens | Macaca mulatta | Macaca fascicularis | Bos taurus | Felis catus | Mus musculus |
|-----------|--------------|----------------|-------------------|------------|-------------|--------------|
| A | C | T | C | T | A | C |
| T | A | G | C | A | T | T |
| G | A | C | T | G | C | C |
| C | T | A | G | C | T | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |
| G | A | C | T | G | C | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |

| Consensus | Homo sapiens | Macaca mulatta | Macaca fascicularis | Bos taurus | Felis catus | Mus musculus |
|-----------|--------------|----------------|-------------------|------------|-------------|--------------|
| A | C | T | C | T | A | C |
| T | A | G | C | A | T | T |
| G | A | C | T | G | C | C |
| C | T | A | G | C | T | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |
| G | A | C | T | G | C | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |

| Consensus | Homo sapiens | Macaca mulatta | Macaca fascicularis | Bos taurus | Felis catus | Mus musculus |
|-----------|--------------|----------------|-------------------|------------|-------------|--------------|
| A | C | T | C | T | A | C |
| T | A | G | C | A | T | T |
| G | A | C | T | G | C | C |
| C | T | A | G | C | T | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |
| G | A | C | T | G | C | C |
| T | A | G | C | A | T | T |
| A | C | T | C | T | A | C |
Inflammation and wasting in the animal. Thus, by analysing the animal with either wild type or the mutated FoxP3 along with the CD4+CD25neg T cells, it was demonstrated that the glutamic acid deletion in wild-type FoxP3 was sufficient in preventing the generation of functional Tregs as compared to the fully functional FoxP3 protein.

As described above, in human beings a single codon mutation in the leucine zipper has recently been identified in 4 of 22 immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients studied. The capability of FoxP1, 2 and 4 to homo or heterodimerize through the leucine zipper is essential to their ability to function correctly as transcriptional repressors [63]. Lopes et al. investigated the domains required for FoxP3 to successfully function as a transcriptional repressor and, using a co-immunoprecipitation approach, demonstrated that FoxP3 can homodimerize to itself similar to the other members of the FoxP family. Furthermore, the glutamic acid deletion in the leucine zipper region appeared to be sufficient in pre-venting FoxP3 from homodimerizing with wild type FoxP3 and also abrogated its transcriptional repressor functions [64].

**Fig. 2** Variability between species is shown in the alignment of FoxP3 sequences of different mammalian species. FoxP3 sequences were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/entrez). Sequences were aligned using the CLUSTALW profile alignment option. The resulting alignments were adjusted manually where necessary.

**Fig. 3** A schematic view of the full-length 431 amino acid long human FoxP3 wild-type protein. The structural domains illustrated include those that have been found to be important in the proper functioning of the FoxP3 protein. The exon 2 isoform of the protein has recently been shown to function the same as the wild-type form [53]. In IPEX patients, the majority of genetic mutations found occur in the zinc finger, leucine zipper and FKH domain regions of the protein and this may be attributed to the requirement of these regions in the transcriptional repression capabilities of the FoxP3 protein [44]. Commercially available antibodies that react with FoxP3 from human and non-human primate are diagrammed under the epitopes that the respective antibody reacts against. PCH101, FJK-16s, 236A/E7, 150D/E4 and ebio7979 are available from eBioscience. 150D, 206D and 259D clones are available from Biolegend. ab2481 and ab10563 are available from Abcam.
When similar point mutations occurred in the zinc finger region of the protein, no loss of transcriptional repression activity was observed. This suggests that the leucine zipper and not the zinc finger is imperative for the FoxP3 protein to function efficiently.

The analysis of FoxP3 functions showed that FoxP3 is capable of direct binding to the endogenous IL-2 and interferon (IFN)-γ genes in T cells only upon T cell stimulation [65]. The binding to IL-2 can only occur after the interaction between FoxP3 and the nuclear factor of activated T-cells (NFAT) [65, 66]. The exact pathway by which this cooperation occurs still needs to be resolved. While the full understanding of how FoxP3 functions within the CD4+CD25+ Tregs has not been determined, it has become evident that its expression is essential for the suppressive capabilities of the CD4+CD25+ Tregs.

**Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4)**

In 2000, CTLA-4 was discovered to be expressed on CD4+CD25+ cells from naïve mice [67]. Also known as CD152, CTLA-4 is a type 1 transmembrane glycoprotein of 223 amino acids that is normally found as a covalent homodimer [68, 69]. CTLA-4 contains a single IgV domain that is responsible for B7-1 (CD80) or B7-2 (CD86) ligand binding. As reported for FoxP3 mutations in mice, CTLA-4 deficient mice develop a severe lymphoproliferative disorder and die from autoimmune-like disease within a few weeks of life [70]. CTLA-4 is normally expressed on T cells upon activation and is found intracellularly at high levels in the memory T cells [71]. Both in vivo and in vitro, CTLA-4 overexpression prevents T cell activation by competing with the co-stimulatory molecule CD28 for B7-1/B7-2 or by interfering with CD28 expression or function. This competition may be due to structural similarities between CTLA-4 and CD28 that would allow CTLA-4 to compete for B7-1/B7-2 binding [72]. However, contrary to the initial belief that CTLA-4 acts as a negative repressor of T cell proliferation, research has shown that in vitro antibodies which bind CTLA-4 blocked T cell proliferation and IL-2 production; however, when the CTLA-4 blocking antibody was removed from the cell culture, proliferation of T cells was restored [73]. In addition, by using a T cell line with a mutated B7-1 (B7W) that can not bind CD28 but maintains its CTLA-4 binding capacity, it was demonstrated that the B7W T cells promoted proliferation of both wild type and CD28-deficient T cells [74].

In CD4+CD25+ Tregs, CTLA-4 is constitutively expressed on the cell surface [67]. In human beings, a fraction of CD4+CD25reg Tregs express CTLA-4 even though the frequency of CTLA-4+ cells is higher within Tregs than within conventional T cells. Paust et al. observed that natural CD4+CD25+ Tregs failed to suppress T cells that are deficient in B7, suggesting that the target cells must express the B7 molecule in order for CD4+CD25+ Tregs to function correctly [75]. Later, Oderup et al. showed that CD4+CD25+ Tregs have the ability to down-modulate CD80 and CD86 (B7) on DCs in vitro in a CTLA-4 dependent fashion [76]. This down-modulation further suppresses the immune system by dampening the ability of the DCs to activate T cells. In addition, using a T cell-mediated colitis mouse model, Read et al. showed evidence in vivo that anti-CTLA-4 antibodies were capable of completely inhibiting CD4+CD25+ Tregs function, but did not affect their overall numbers or ability to home to the gut-associated lymphoid tissue (GALT) [77]. Moreover, CTLA-4 deficient mice were able to produce fully functional CD4+CD25+ Tregs suggesting that, in some circumstances, other molecular pathways are able to compensate for the loss of CTLA-4 in order to successfully produce CD4+CD25+ Tregs. In chronically simian immunodeficiency virus (SIV)-infected macaques, the administration of anti-CTLA-4 human antibody resulted in an increase in the effector function of both SIV-specific CD4+ and CD8+ T cells [78]. This CTLA-4 blockade resulted in decreased expression of TGF-β and the indoleamine 2,3-dioxygenase (IDO) enzyme, as well as decreased viral loads in the lymph nodes (LNs). Moreover, a combined treatment of CTLA4-Ig and anti-CD40L antibody into SIV-infected macaques resulted in the inhibition SIV-specific immune responses [79]. Altogether, the current body of evidence suggests that the expression of CTLA-4 on CD4+CD25+ Tregs is important for their ability to suppress the immune system.

**Glucocorticoid-induced TNFR related protein (GITR)**

The tumour necrosis factor receptor (TNFR) family member, GITR, was first identified as a potential surface marker of Tregs in 2002 [80, 81]. GITR was
originally discovered in murine T cell hybridoma cells, and later human GITR and its ligand, GITR-L, were identified by searching an expressed sequence tag (EST) database [82–85]. The idea that GITR is constitutively expressed on Tregs gave rise to the concept that GITR could be used as a unique surface marker to identify Tregs from other T cell types [80, 81]. However, it was eventually found that other T cell subsets express GITR, specifically responder T cells after activation through the TCR receptor. In human peripheral blood mononuclear cells (PBMCs), low levels of GITR transcripts were present and could be dramatically induced after the addition of anti-CD28 and anti-CD3 in vitro [82, 84]. In addition to T cells, GITR has also been shown to be up-regulated on other cell types, such as B cells, macrophages and DCs when these cells were stimulated by lipopolysaccharide (LPS) in vitro and in vivo, thus conflicting with the idea that GITR could be used as a unique CD4^{+}CD25^{+} Treg surface marker, similar to CTLA-4 [80, 81, 86].

Recent studies have implicated GITR as an essential regulator of immune responses to self and non-self antigens. As frequently seen in Treg research, studies on the specific function of GITR provided mixed results. First, it was shown that GITR functions as a co-stimulatory molecule for conventional T cells in vitro [87–90]. The most compelling research showed that removal of GITR-expressing cells or administration of GITR-specific antibody in mice resulted in autoimmune diseases similar to CD4^{+}CD25^{+} Treg-deficient mice, suggesting that GITR-blocking antibodies may be capable of abrogating the functionality of human CD4^{+}CD25^{+} Tregs and increasing proliferation of human CD4^{+} T cells as wild-type CD4^{+}CD25^{−} T cells, suggesting that GITR may be dispensable for CD4^{+}CD25^{−} suppressor activity or that other suppressive mechanism(s) can compensate for the lack of GITR [90].

Despite its namesake, neither human nor mouse primary T cells require the glucocorticoid hormones to up-regulate GITR [93]. Instead, it seems that GITR is up-regulated via TCR stimulation and is involved in protecting the cell from glucocorticoid-induced apoptosis. The mechanism through which GITR protects the cell from apoptosis remains unclear.

L-selectin (CD62L)

CD62L is essential for T cell entry into the LNs by transition through the high endothelial venules. CD62L is constitutively expressed on the surface of circulating naïve T cells and is involved in their homing to inflammatory sites through the constant attachment/detachment of CD62L with its multiple ligand complex (PNAd) [94]. Once the naïve T cell has been activated by its antigen receptor, down-regulation of CD62L occurs and the T cell is considered active. CD62L is found to be expressed on both CD4^{+}CD25^{+} and CD4^{+}CD25^{−} T cells, so cannot be used as a phenotypic marker for Tregs. However, a series of studies comparing CD4^{+}CD25^{+}CD62L^{+} and CD4^{+}CD25^{+}CD62L^{−} T cells in an acute graft versus host disease (aGVHD) mouse model reported that the CD62L^{+} Tregs were capable of preventing the expansion of donor CD4^{+}CD25^{−} T cells with greater efficiency than their CD62L^{−} counterparts [95]. This evidence suggests that the capability of the CD4^{+}CD25^{+} T cells to enter the priming areas of the immune system is essential for its ability to prevent aGVHD. This work was supported by another GVHD study which reported that the CD4^{+}CD25^{+}CD62L^{+} cells were capable of suppressing the activation and expansion of T effector cells in the secondary lymphoid organs after bone marrow transfer in mice [96].

Chemokine (C-C motif) receptor 7 (CCR7)

CCR7 (CD197) is a member of the G protein-coupled receptor family, which is referred to by numerous other names in the literature, such as Cdw197, Cmkbr7, Ebi1, Ebi1 and MIP-3 receptor [97–100].
Using gene targeted mice, CCR7 was shown to be a necessary chemokine in the primary humoral immune response [101]. Mice lacking the ability to express CCR7 on their lymphocytes were deficient in antibody response and lymphocyte migration, thus causing profound morphological changes in the secondary lymphoid tissues [101].

Since CCR7 and CD62L are required for proper leukocyte trafficking, it was thought that by using these chemokines as cell markers one could differentiate T cell subsets, specifically Tregs. In fact, CCR7^neg T cell subsets displayed other markers that caused the cells to target inflamed tissue, while CD4^+CCR7^+ T cells were routed to the lymphoid tissues [102, 103]. In a more recent study, CD45RA^CD4^+CD25^+ (naïve Tregs) taken from adult peripheral blood were shown to constitutively express CCR7, CD62L and FoxP3 even after the cell lines were maintained for several weeks, while the CD45RA^negCD4^+CD25^+ (memory-like Tregs) cells lost their ability to express CCR7, CD62L and FoxP3 expression after weeks of continuous culturing [104]. These results clearly demonstrate that CD4^+CD25^+ are not exclusive to the CD45RO^+ memory cells, as previously reported [105, 106]. More importantly, this study showed that only naïve CD45RA^+ T cells give rise to homogeneous Treg cell lines, thus opening a new clinical avenue on the prevention of T cell-mediated diseases through the adoptive cell transfer of CD4^+CD25^+ Tregs that have been selectively expanded from CD45RA^+ cell lines.

**Alpha E integrin (CD103)**

CD103 (alpha E integrin) is responsible for homing of the immune cells to sites of inflammation by controlling the adherence of conventional T cells to epithelium in the gut by binding to E cadherin [94]. CD103 has been shown to be expressed on DCs, B cells and both CD4^+ and CD8^+ T cells [107, 108]. CD103 was also found to be expressed on approximately 25% of mouse Tregs. The subset of CD25^+ CD103^+ T cells appears to exert more potent suppressive effects in vitro and to circulate preferentially to inflammatory sites [109]. CD4^+CD25^+CD103^+ Tregs may thus be involved in tissue-specific homing, such as mucosal areas of the body, while CD4^+CD25^-CD103^neg Tregs are thought to be more involved in systemic regulation. However, current research is somewhat conflicting on the exact role of CD103 in defining Tregs [109, 110]. One report has shown that the adoptive transfer of CD4^+CD25^-CD103^negneg natural Tregs was just as capable of preventing colitis in immunodeficient mice as wild-type CD4^+CD25^+ Tregs, suggesting that CD103 may not be as essential as previously thought for the suppressive activity of CD4^+CD25^+ Tregs in mucosal inflammation [111, 112]. Another study analysed CD103 expression on large intestinal DC aggregates in the colitis mouse model. In this study, T cell grafts depleted of CD103 T cells produced similar numbers of colonic CD103 T cells as the unfractonated T cells, which suggests that these DCs are responsible for the expansion of CD4^+CD25^neg FoxP3 Tregs in the colitis mouse model [113]. In contrast, CD103 was shown to be a critical component of the successful retention of Tregs at the site of *Leishmania major* infection in a mouse model. Genetically susceptible mice that lacked the ability to express CD103 were found to be resistant to the bacterial infection. This suggested that CD103 is absolutely required for proper activation of the immune system during *Leishmania* infection [114]. CD103 is potentially incorporated into Treg function; however, the requirement for CD4^+CD25^+ Tregs to express CD103 may ultimately be shown to be organ-specific.

**Other cell markers with potential**

Several other cell markers, such as OX40, LAG-3 and neuropilin have been demonstrated to be expressed constitutively or specifically in Tregs. OX40 and its ligand OX40-L were recently described to be important in the regulation of a specific subset of Tregs, termed type 1 Tregs (Tr1), for which production of high levels of IL-10 is characteristic. Tr1 cells are capable of suppressing antigen-driven proliferation of naïve CD4^+ T cells both *in vivo* and *in vitro* [115, 116]. While several studies successfully induced Tr1 cells through various methods, their negative regulation is still poorly understood [117–119]. Recently, it was shown that OX40-L has the ability to prevent IL-10 production from Tr1 cells and also to inhibit the Tr1 cells suppressive abilities [120].

Lymphocyte activation Gene-3 (LAG-3) is related to CD4 in structure and function suggesting to some that they may be involved in direct competition for MHC class II molecules [121]. LAG-3 is selectively
up-regulated on the cell surface of CD4⁺CD25⁺; however, its precise role remains unknown.

Neuropilin-1 (Nrp1), a receptor involved in the activation of T cells, was discovered as a CD4⁺CD25⁺ surface marker via global expression studies [122]. This work identified Nrp1 as a marker specific in mice to CD4⁺CD25⁺FoxP3⁺ T cells, in contrast to CD4⁺CD25⁻ T cells, in which Nrp1 is down-regulated. Nrp1 was also shown to be associated with CD4⁺ CD25⁺ Tregs in Schistosoma mansoni murine infections [123].

Expression of CD127, the chain of the IL-7 receptor, has been reported to allow a flow cytometry-based distinction between CD127(lo) Tregs and CD127(hi) conventional T cells within the human CD25⁺CD45RO⁺ effector/memory and CD45RA⁺ naive compartments in peripheral blood and LN [124, 125]. However, memory/effector T cells can also be CD127⁺.

Expression of the programmed cell death-1 inhibitory receptor (PD-1) was shown to discriminate between naïve CD4⁺ CD25⁺ FoxP3⁺ Tregs and acti-vated CD4⁺ T cells [126]. CD4⁺ CD25⁺ FoxP3⁺ Tregs isolated from the LNs and spleens of normal mice and from healthy human PBMCs did not express PD-1 on their surfaces; however, expression of this marker was maintained intracellularly [123]. In contrast, activated CD4⁺ T cells significantly expressed PD-1 on their surface, thus allowing for the ability to sort CD4⁺ CD25⁺ FoxP3⁺ Tregs from activated T cells [123]. However, once activated through their TCR receptor, CD4⁺ CD25⁺ FoxP3⁺ Tregs also expressed PD-1 on their surface similar to the activated CD4⁺ T cell population.

Altogether, these studies report alternative cell surface markers that may eventually be used in combination to distinguish CD4⁺CD25⁺ Tregs. However, the identification of specific markers for CD4⁺CD25⁺ Tregs is necessary in order to fully understand the power and capabilities of these T cell subsets.

Manipulation of CD4⁺CD25⁺ Tregs

The natural expression of CD4⁺CD25⁺ Tregs during disease manifestation has been shown to have either beneficial or detrimental outcomes to the infected individual (Table 2). Studies have suggested that disease treatment will largely benefit from the manipulation of this T cell subset. However, for CD4⁺CD25⁺ Tregs to be of use in clinical settings, the ability to regulate Treg numbers and function needs to be made more efficient. For this effort, several drugs, cytokines and antibodies that induce or deplete Tregs have been examined in animal and cell models. In this section, we will look at these recent advances in the ability to induce and delete CD4⁺CD25⁺ Tregs in vitro and in vivo.

Induction and regulation of CD4⁺CD25⁺ Tregs

It is understood that thymus-derived CD4⁺CD25⁺ T cells have the ability to educate some populations of CD4⁺CD25⁻ T cells to develop suppressive activity [127, 128]. A laundry list of drugs and pro-inflammatory cytokines, such as IL-2, IL-4, IL-10, IL-13, IL-15, IFN-γ, TGF-β, CD52, cyclophosphamide, G protein-coupled receptor 83, LPS, rituximab, co-polymer 1, rapamycin, anti-thymocyte globulin, ovalbumin(OVA)/cholera toxin B subunit (CTB) conjugate and FK778 have all been shown to be capable of inducing the expression of CD25 and FoxP3 from naïve CD4⁺CD25⁻ T cells [128–141].

Interleukin-2 (IL-2)

Prior to activation Tregs bind IL-2, but generally do not produce this cytokine [142, 143]. IL-2 is not required for the generation of natural Tregs in the thymus, but low doses of IL-2 are required for maintaining the suppressive functions of Tregs [144, 145]. However, too high levels of IL-2 can actually reverse their suppressive function [67]. IL-2 levels may fluctuate during the immune response. During bacterial invasion, the bacterial lipoprotein (BLP) seems to play a direct role in the induction and function of Tregs [146]. BLP binds to the toll-like receptor 2 (TLR-2) causing an increase in IL-2 production. This IL-2 production causes an inhibition of the CD4⁺CD25⁺FoxP3⁺ suppressive activity and an increase in the population of CD4⁺CD25⁻ T cells. As the CD4⁺CD25⁻ T cells combat the invading bacterial infection, IL-2 levels will decrease, causing the CD4⁺CD25⁺ cells to slowly increase their suppressive ability [146]. Therefore, IL-2 produced by antigen-presenting cells (APC) may be responsible not only in the regulation of CD4⁺CD25⁺ Tregs, but also CD4⁺CD25⁻ cells. Other studies further confirmed that TLR-2 is important in the regulation/induction of...
### Table 2 A short list of diseases in which CD4⁺CD25⁺ regulatory cells have been associated

| Disease or organism | Role of CD4⁺CD25⁺ regulatory cells (if known) | Ref. |
|--------------------|------------------------------------------------|------|
| *Helicobacter pylori* | Shift to Th2 immune response accompanied by severe gastritis after depletion of Tregs cells. 100-fold increase in FoxP3 after infection. | [179, 185] |
| *Mycobacterium tuberculosis* | Bacterial loads unaffected when mice were depleted of CD4⁺CD25⁺ Tregs. CD4⁺CD25⁺FoxP3⁺ Tregs were significantly increased in the blood of infected human beings. | [183, 184] |
| *Listeria monocytogenes* | Possible dual role for Tregs: Assist in expansion of cytotoxic CD8⁺ T cells during initial infection, but suppress CD8⁺ T cells during chronic phase of disease. | [180] |
| *Candida albicans* | TLR-2 pathway controls Treg expansion during infection. 100-fold decrease in C. albicans in TLR-2⁻/₋ mice. | [147] |
| *Leishmania major* | CD4⁺CD25⁺ Tregs maintain low-level infection in the dermis via IL-10 mechanisms. | [29] |
| *Plasmodium falciparum* | Rapid CD4⁺CD25⁺FoxP3⁺ Treg and TGF-β upregulation during infection, decreased pro-inflammatory cytokine production and antigen-specific immune responses. | [190, 191] |
| *Shistosoma mansoni* | CD4⁺CD25⁺ T cells cause a Th1 to Th2 shift in infected mice by preventing IL-12 secretion. Inability to control DC activation, increased levels of IL-10 and IFN-γ | [159] |
| Friend virus | CD4⁺CD25⁺ Tregs isolated from infected mice suppress the function of activated CD8⁺ T cells in vitro. | [205, 206] |
| HSV-1/HSV-2 | CD4⁺CD25⁺ Tregs isolated from human patients suppress CD4⁺ cells. Depletion of murine Tregs resulted in increases in immune response, viral clearance and corneal damage | [13, 221, 223] |
| HCV | CD4⁺CD25⁺ Tregs suppress virus-specific CD8⁺ T cells in vitro. IL-10 levels increased in chronically infected patients. | [197, 199, 200] |
| HIV/SIV | Depletion of Tregs causes immune hyper-activation. CD4⁺CD25⁺FoxP3⁺ Tregs are susceptible to HIV infection. Immediate immune response may prevent chronic T cell hyper-activation in non-pathogenic SIV infection | [202, 209, 210, 212–215] |
| HBV | CD4⁺CD25⁺ Tregs directly linked to progression of HBV. Tregs levels higher in liver of chronic patients. Tregs capable of suppressing HBV-specific immune responses. | [201, 204] |
| Ovarian cancer | Tregs suppress tumour-specific T cell immunity in patients. Tregs have increased secretion of TGF-β, IL-2, TNF-α, and IFN-γ. TGF-β can convert CD4⁺CD25⁻ T cells to Tregs. | [242-244] |
| Prostate cancer | CD4⁺CD25⁺ Tregs elevated in blood and LNs of cancer patients. Malignant tissue harbors a significantly higher level of Tregs compared to benign, indicates poor prognosis. | [245–247] |
| Breast cancer | CD4⁺CD25⁺ Tregs elevated in blood, one study found no difference. Patients older than 50 have lower levels of CD4⁺CD25⁺ Tregs as compared to younger patients. | [248–250] |
CD4+CD25+ Tregs by demonstrating that CD4+CD25+ cells are diminished in the circulation of TLR-2<sup>neg/neg</sup> but not TLR-4 neg/neg mice, directly linking the TLR-2 receptor to the number of CD4+CD25+ Tregs [142]. It was further shown that adoptively transferred wild-type CD4+CD25+ Tregs into TLR-2<sup>neg/neg</sup> mice were neutralized after systemic TLR-2 ligands were administered during the acute phase of a *Candida albicans* infection. This neutralization of wild-type CD4+CD25+ Tregs caused a 100-fold decrease in the growth of the yeast [147], which confirms that IL-2 has a direct effect on diminishing the suppressive actions of the Tregs, thus allowing effector T cells to combat the ongoing infection. However, it is also possible that competition is contributing to the suppressive mechanism. Indeed, under conditions of limited IL-2 supply, the efficient capture of IL-2 by Tregs causes IL-2 deprivation of responder T cells. In case of sufficient IL-2, Tregs would be unable to inhibit responder T cells’ access to IL-2 [143]. This competition can also explain some of the currently discussed discrepancies between in vivo and in vitro activity of Tregs.

**Interferon gamma (IFN-γ)**

IFN-γ has been well-documented to be one of the pro-inflammatory cytokines responsible for orientation of the immune response to Th1 versus Th2. However, recent research has shown that IFN-γ is also capable of converting CD4+CD25<sup>neg</sup> T cells to CD4+CD25<sup>+</sup> Tregs in vitro as evidenced by the increased expression of FoxP3. In addition, adoptive transfer of IFN-γ treated T cells to experimental autoimmune encephalomyelitis (EAE) mice (multiple sclerosis [MS] model) resulted in disease suppression [139].

In contrast, peptide-specific IFN-γ producing CD8+ T cells were capable of controlling the generation of CD4<sup>+</sup> T cells including CD4<sup>+</sup>CD25<sup>+</sup> Tregs [148]. In addition, mice receiving IFN-γ along with serological identification of antigens by recombinant cDNA expression cloning (SEREX)-self antigens were also found to prevent the generation of CD4<sup>+</sup>CD25<sup>+</sup> cells [149]. Wild-type BALB/c mice treated with a DNA J-like 2 plasmid mixed with a plasmid encoding IFN-γ showed no increase in pulmonary metastasis after 28 days post-inoculation. Furthermore, naïve BALB/c mice inoculated with CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from mice treated with the DNA J-like 2 plasmid and the plasmid encoding IFN-γ also prevented the metastasis of the pulmonary tumours. While the exact reasoning for IFN-γ ability to interfere with the generation of CD4<sup>+</sup>CD25<sup>+</sup> Tregs remains elusive, this finding suggests that the development of anti-tumour vaccines should consider the use of IFN-γ in order to prevent the generation of self-antigen responding CD4<sup>+</sup>CD25<sup>+</sup> Tregs that could further enhance cancer metastasis.

**Transforming growth factor β1 (TGF-β1)**

TGF-β1 has also been implicated in the regulation of CD4+CD25<sup>+</sup> T cells *in vivo*. Indeed, TGF-β triggers the expression of FoxP3 in CD4<sup>+</sup>CD25<sup>neg</sup> precursors [150]. These FoxP3<sup>+</sup> cells act like Tregs. However, studies conducted in the TGF-β1<sup>neg/neg</sup> mouse model suggest that TGF-β is not required for the development of the naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell. Thus, FoxP3 levels are normal in the thymic CD4<sup>+</sup>CD25<sup>+</sup> precursors for regulatory T cells in TGF-β1<sup>neg/neg</sup> mice, but decreased in peripheral CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Therefore TGF-β1 is important for the maintenance of this class of regulatory cells in the periphery [151].

CD8+ T cells expressing a dominant negative receptor for TGF-β1 (in which no signaling can be conducted through TGF-β1) were able to escape the suppressive control of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the diabetic mouse model [152]. TGF-β signaling therefore seems to be required in order for CD4<sup>+</sup>CD25<sup>+</sup> T cells to successfully suppress self-reactive cytotoxic CD8+ T cells in the diabetic mouse model. TGF-β has also been shown to be involved in tumour growth in mice, in which TGF--insensitive, tumour-reactive, CD8+ T cells were able to successfully destroy, via apoptosis, transgenic adenocarcinoma mouse prostate cancer cells (TRAMP-C2) that have been shown to produce high levels of TGF-[153]. This suggests that TGF-β produced by prostate cancer cells inhibits the growth and effect of CD8+ T cells possibly by mimicking the expression of TGF-β produced by Tregs. In contrast, TGF-β derived from CD4+CD25<sup>+</sup> cells is not necessary for the CD4+CD25<sup>+</sup> Tregs to mediate their effects in the IBD. Thus, CD4+CD25<sup>+</sup> Tregs from either TGF-β1<sup>+/+</sup> or neonatal TGF-β1<sup>neg/neg</sup> mice can equally control the incidence and severity of IBD [154]. As for other mechanisms, TGF-β expression from CD4<sup>+</sup>CD25<sup>+</sup> T cells may be disease specific and not essential for the elimination of all disease types.
As discussed above, FoxP3 is considered essential for CD4+CD25+ Tregs to function properly. Recently, transduction of CD4+CD25neg T cells with a FoxP3 expression vector has resulted in increased levels of CD4+CD25+ Tregs. In human beings, two isoforms of the FoxP3 protein were identified by Western Blot and reverse-transcription (RT)-PCR analysis, in contrast to only one isoform found in mice [64, 155–157]. Our lab has also observed by RT-PCR two isoforms of the FoxP3 protein in non-human primates (NHPs) (Fig. 4). In human beings, one isoform closely resembles the mouse FoxP3 while the other isoform is apparently encoded by mRNA lacking exon 2 (Amino Acids 71–105). In addition, Smith et al. reported a third isoform of FoxP3 in human beings that is lacking both exon 2 and 7 [156]. The significance of the isoforms in human beings is not immediately apparent. Recent evidence using a retrovirus-based overexpression strategy showed that overexpression of either isoform resulted in similar phenotypes in transfected CD4+ T cells [155, 156]. Co-expression of FoxP3 and FoxP3 (Δexon2) isoforms in transfected CD4+ T cells resulted in elevated suppression of the cytokines IL-2 and IFN-γ and also increased expression of CD4+CD25+ Tregs surface markers as compared to the expression of either individual isoform alone [155]. However, CD4+ T cells transfected with either FoxP3 or FoxP3 (exon2) isoforms did not have increased suppressive capabilities as compared to naturally occurring CD4+CD25+ Tregs, suggesting that either isoform of FoxP3 is adequate for inducing the suppressive abilities of CD4+CD25+ Tregs.

Cytokines and drugs with potential

Several new studies postulated that IL-4, IL-13, IL-10 and Copolymer-I (COP-I) are involved in the induction and regulation of CD4+CD25+ Tregs [133, 137, 158, 159]. IL-4 and IL-13, which function through the same IL-4 α-chain-binding receptor, were shown to induce CD4+CD25+ Tregs from peripheral CD4+CD25neg naïve T cells. The IL-4 induced Tregs are similar to naturally derived Tregs, with high expression levels of FoxP3, suppressive capabilities and the expression of the surface markers GITR and CTLA-4 [137].

Copaxone or Co-Polymer 1 (COP-1) is a random polymer of four amino acids (glutamic acid, lysine, alanine and tyrosine) which mimic a myelin protein. This combination gives the drug unique immune regulatory properties. COP-1 has been approved since 1995 as one of the primary treatment options for MS, but the mechanism by which it acts is yet to be determined. A recent study showed that COP-1 caused in vivo and in vitro transformation of CD4+CD25neg T cells to CD4+CD25+ Tregs and an increased expression of FoxP3 [133]. In MS patients treated with COP-1, CD4+CD25+ Tregs levels increased when compared to baseline levels prior to treatment, providing for at least one mechanism by which COP-1 acts.

Depletion of CD4+CD25+ Tregs

Several options are currently available to deplete CD4+CD25+ Tregs in vivo and in vitro including
monoclonal antibodies, pharmaceutical drugs, immuno-toxins and knockout animal models.

The monoclonal antibody PC61 [160], originally identified as a monoclonal antibody against murine IL-2R (CD25), has been used in many antibody-depletion experiments described to determine the impact of depleting the CD4\(^+\)CD25\(^+\) Tregs in mice after a pathogenic infection. Interestingly, a recent report suggests that in vivo treatment with CD25 monoclonal antibodies does not deplete CD4\(^+\)CD25\(^+\) Tregs, rather it was found to functionally inactivate the cells. Thus CD25 monoclonal antibody-treated mice had normal levels of CD25 mRNA and no change in CD4\(^+\)FoxP3\(^+\) T cells [161]. These results suggest that conclusions drawn from previous in vivo studies may need to be revisited.

In human beings, the development of the recombinant IL-2 diphtheria toxin conjugate DAB389IL-2 (also known as denileukin diftitox or ONTAK) was seen as a major breakthrough in some cancer patients. Designed to target cells expressing CD25 (IL-2R), ONTAK once bound to CD25 causes internalization of the diphtheria toxin fragment which halts protein synthesis and ultimately causes the death of the cell [162]. ONTAK has been used in conjunction with other therapies to successfully treat several diseases including non-Hodgkin lymphoma, T cell leukaemia, graft-versus-host disease and chronic lymphocytic leukaemia [163–167]. Due to the promising preliminary data in cancer research, ONTAK has been incorporated in several other disease models with mixed results. ONTAK administration successfully abolished CD25-expressing cells from healthy donor PBMCs; however, foxp3 expression was not reduced, suggesting that ONTAK is unable to specifically target CD4\(^+\)CD25\(^+\) Tregs [168]. Experiments comparing CD25 depletion options showed that ONTAK was inferior to anti-CD25 immunotoxin or anti-CD25 microbeads at depleting alloreactive CD4\(^+\)CD25\(^+\) Tregs [169]. In other studies, ONTAK has been shown to successfully deplete CD4\(^+\)CD25\(^+\) Tregs. Thus, in metastatic renal cell carcinoma patients ONTAK treatment resulted in lower levels of CD4\(^+\)CD25\(^+\) Tregs in the peripheral blood and decreased suppressive abilities [170]. Our lab showed that ONTAK is capable of depleting CD4\(^+\)CD25\(^+\) Tregs in NHPs in vitro (data not shown).

Another available drug, cyclophosphamide, has been shown to successfully deplete CD4\(^+\)CD25\(^+\) Tregs in human beings, mice and rats [171–173]. At a low dose (20 mg/kg) cyclophosphamide depleted CD4\(^+\) and CD8\(^+\) T cells by 50%; however, at higher concentrations (200 mg/kg) the drug depleted all T cell subsets by greater than 90% [171]. In end-stage cancer patients, low dosing of cyclophosphamide was capable of selectively reducing circulating CD4\(^+\)CD25\(^+\) Tregs and restoring T cell proliferation [173].

One additional drug, daclizumab, is a blocking humanized monoclonal antibody to the \(\alpha\)-chain of CD25 [174]. Daclizumab is given after transplant surgery to effectively prevent acute graft rejection; however, its long-term side effects are unknown [174, 175]. In a 2 year post-kidney-transplant study, patients receiving a single dose of daclizumab were shown to have decreased numbers of circulating CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Tregs, however, after clearance of the drug Treg levels returned to normal within 8 weeks [175]. In RMs, daclizumab was shown to successfully protect against inflammation and joint degradation in collagen-induced arthritis [176]. In addition, we also showed that daclizumab is capable of blocking CD25\(^+\) simian T cells in vitro (Fig. 5).

Other agents were reported to have depleting abilities; pertussis toxin (Ptx) has been used for many years to enhance the clinical signs of EAE in mice strains. Recently, Ptx has been shown to decrease the number of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells in the spleen of mice, but the residual CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells were shown to have no loss in suppressive abilities [177].

Role of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Tregs in disease

Since their discovery, CD4\(^+\)CD25\(^+\) Tregs have been reported to be involved in a variety of diseases as diverse as bacterial, fungal, viral, autoimmune and cancer. While some studies have shown that CD4\(^+\)CD25\(^+\) Tregs are absolutely required to protect the host from various diseases, others have shown that Tregs may actually be responsible for the symptoms of particular diseases (Table 2). Below, we will review some of these studies and the roles that CD4\(^+\)CD25\(^+\) Tregs can play in disease suppression or progression.
Bacterial and fungal

CD4⁺CD25⁺ Tregs have been shown to be involved in several bacterial and fungal infections, such as Candida albicans, Chlamydia trachomatis, Helicobacter pylori, Listeria monocytogenes and Mycobacterium tuberculosis (Table 2) [178–183].

Some bacteria might be capable of exploiting the suppressive activities of CD4⁺CD25⁺ Tregs for their own needs, thus preventing the immune system’s ability to clear the infection from the host. Using mice depleted of CD25, a recent study showed that M. tuberculosis bacterial loads were unaffected as compared to wild-type mice during the acute phase of infection [183]. Small increases in IFN-γ and IL-2 were observed in the CD25 neg depleted mice prior to mycobacterial infection; however, these cytokine increases had no affect on the pathogen clearance. CD4⁺CD25<sup>low</sup>FoxP3⁺ cells, which are found in higher numbers in the lung and were unaffected by the CD25 depletion experiments, may ultimately play a role in the control of the mycobacterial infection.

In the blood and pleural fluid of infected human beings, CD4⁺CD25⁺FoxP3⁺ Tregs were found to be significantly increased as compared to healthy volunteers [184]. Also, Tregs isolated from infected patients were capable of suppressing the M. tuberculosis-specific IFN-γ and IL-10 production, suggesting that Treg expansion in infected individuals may contribute to pathogenesis. However, additional work needs to be done on the role of CD4⁺CD25⁺ Tregs in the chronic phase of infection in order to confirm these results.

Helicobacter pylori infects over half the world’s population, but only 10–15% of the infected individuals actually display any symptoms of the infection. This suggests that a compromise has been reached between the host and microbe over time allowing the microbe to colonize the host without inducing any sign of disease. CD25 depletion in mice prior to or during chronic Helicobacter infection did not affect overall bacterial loads or the severity of the disease [179]. Chronic Helicobacter infection is characterized by the secretion of Th1 cytokines; however, after depletion of CD25, the immune response shifts to a Th2 response, as suggested by the increased levels of IL-4 and IL-5. This shifted response lasts several weeks, despite only a transient depletion of CD25; however, it has no effect on the gastric inflammation. In the same study, depletion of CTLA-4 also caused a Th1 to Th2 shift and resulted in a decrease in the severity of the gastritis. This response was probably due to the multiple cellular pathways that CTLA-4 is involved in and not just simply the loss of function from the CTLA-4-expressing Tregs. Furthermore, FoxP3, IL-10 and TGF-β all increased 1 month after H. pylori infection in C57BL/6 mice as compared to non-infected control mice; in the case of FoxP3 the
increase was over 100-fold [185]. In human gastric biopsies, FoxP3-expressing cells were found to be 50-fold higher in *H. pylori* infected individuals than in non-infected subjects [185]. In contrast to the studies conducted by Kaparakis et al., where no increase in gastric inflammation was observed in CD25 depleted mice, Rad et al. reported severe gastritis 4 weeks after *H. pylori* infection, whereas non-depleted infected mice showed only scarce mononuclear cell infiltrates at the same time point [185]. While these two studies reported conflicting results, the common idea is that a compromise has been made over centuries of evolution between the host immune system and the microbe pertaining to the function of CD4+CD25+ Tregs.

*Listeria monocytogenes* is a Gram positive bacterium that is responsible for meningitis and spontaneous abortions in human beings. In mice depleted of CD4+ cells and infected with *L. monocytogenes*, the loss of the CD4+ cell population allowed for an enlarged population of *L. monocytogenes* antigen-specific CD8+ T cells [180]. The antigen-specific CD8+ T cells were further increased 10-fold after a boost immunization with a *L. monocytogenes* DNA vaccine and CD4+ depleting antibody. A similar increase in antigen-specific CD8 T cells was also seen when a CD25+ depleting antibody was administered to mice [180]. In addition, in vitro suppression assays confirmed the ability of CD4+CD25+ Tregs to suppress memory CD8+ T cells isolated from vaccinated mice [180]. This increase in antigen-specific CD8+ T cells in the absence of CD25+ T cells suggests that CD4+CD25+ Tregs may play a dual role in protection from *L. monocytogenes*. During initial bacterial infection, CD4+ T cells assist in the expansion of antigen-specific CD8+ T cells, while during secondary infection CD4+CD25+ Tregs control the expansion of CD8+ T cells.

Work also has been conducted using *L. monocytogenes* as a vaccine vector due to its ability to target the innate and cellular immune system functions, enhancing antigen presentation and cytokine response [186, 187]. Recently two *L. monocytogenes* vaccine vectors, Lm-LLO-E7 and Lm-E7 were created that express the HPV-16 E7 protein. Mice with established E7-expressing tumours were vaccinated with either of the two vaccines with vastly different results. Mice inoculated with the Lm-LLO-E7 vaccine showed marked tumour regression, while mice vaccinated with the Lm-E7 showed no tumour improvement. Further investigation showed that when compared to Lm-LLO-E7 vaccinated mice, Lm-E7 vaccinated mice had high induction of CD4+CD25+ Tregs that were suppressive in vitro and secreted IL-10 and TGF-β, suggesting that Tregs generated during a vaccine treatment can have debilitating results on the desired outcome.

*Candida albicans* is a cause of vaginal and oral infections in healthy human beings, while in immunocompromised patients it can lead to acute disseminated candidiasis. Protective immunity to the pathogen is mediated mainly by an antigen-specific Th1 response and the role that CD4+CD25+ Tregs play in controlling this immune response is currently being elucidated [142, 182, 188]. One of the earliest studies used BALB/c mice with CD28, B7-1 or B7-2 gene knockouts [182]. While no differences in susceptibility to *C. albicans* was detected between the different mouse backgrounds, in the mice with CD28 or B7-2 knockouts CD4+CD25+ Tregs were not generated. Differences were also observed in the ability of mice to survive disseminated infection, with survival of B7-1 and demise of B7-2 and CD28 knockout mice. The pathogen loads in the kidneys and stomach were significantly lower in the B7-2 and CD28 knockout mice, but the amount of inflammation was much higher as identified in histopathological examinations. Adoptive transfer of the purified CD4+CD25+ Tregs from wild-type mice to B7-2 knockout mice led to suppression of the inflammation in the stomach, but also allowed for an increase in pathogen load. In another study, TLR-2neg/neg mice have been shown to be less susceptible to *C. albicans* disseminated infection, have a decreased level of IL-10 production, and a 50% decrease in CD4+CD25+ Tregs [142]. In addition, the TLR-2neg/neg mice showed an increase in IFN-γ and effective antifungal macrophages, suggesting that *C. albicans* may incorporate the TLR-2 signalling pathway into its strategy for evading the host immune system.

As discussed above, several bacteria are capable of preventing their demise in the host by hijacking the immune system functions such as those involving the Tregs. Some benefit to the host can be seen in *H. pylori* infections where the re-infection rates are kept low due to the induction of CD4+CD25+ Tregs. However, other bacteria, such as *M. tuberculosis* have found mechanisms that allow them to be unaffected by CD4+CD25+ Tregs. It is clear by the described work that we are only on the cusp of understanding what
the role is that CD4+CD25+ Tregs play in the delicate balance between detrimental and supportive functions against bacterial and fungal infections.

**Parasitic infections**

Over 2 million deaths are caused every year by the malaria parasites. Recently, several research groups have showed that suppression of the immune system by CD4+CD25+ Tregs may help the malaria infection similar to bacterial infections (Table 2) [189–191]. In addition, many malaria patients show increased susceptibility to other diseases, such as human immunodeficiency virus (HIV), tuberculosis and parasitic worms, perhaps due to the suppression of the immune system [192–196]. One of the first papers to address the role of CD4+CD25+ T cells on malaria infections reported that mice depleted of CD25 were able to completely clear the infection after only two brief waves of parasitaemia while the wild-type mice died of malaria symptoms [189]. In DBA/2 mice, survival after malaria infection was attributed to the slow induction of CD4+CD25+ Tregs post-infection (5 days); conversely, in BALB/c mice that are unable to survive infection, with death occurring in all infected animals within 7 days post-infection, there is a significantly more rapid increase in CD4+CD25+ Tregs (3 days). In human volunteers infected with *Plasmodium falciparum*, rapid CD4+CD25+FoxP3+ Treg and TGF-β up-regulation occurs, which associates with immune suppression evidenced by decreased pro-inflammatory cytokine production and decrease of antigen-specific immune responses [190, 191]. Therefore, the induction of Tregs was considered a parasitic-specific virulence factor.

However, for other parasitic infections a positive role of Tregs was reported. Studies have shown that CD4+CD25+ Tregs are directly responsible for controlling *L. major* re-infection in the dermis by IL-10-dependent and independent mechanisms (Table 2) [29]. Thus, during chronic infection the number of parasites are maintained at a low level allowing retransmission to the natural vector, meanwhile preventing disease reactivation. Using mice deficient in IL-10 production, the protection against secondary infection is lost, which suggests that IL-10 producing CD4+CD25+ Tregs are involved in this delicate balance between infection and protection against *L. major* infection.

CD4+CD25+ T cells have been suggested in causing a Th1 to Th2 shift in mice infected with *S. mansoni* by preventing IL-12 secretion via IL-10 production (Table 2) [159]. Thus, using schistosome-infected IL-10neg/neg mice it was shown that activated CD4+CD25+ T cells were unable to control DC activation by IL-10 secretion. This inability to control DC activation caused Th1 responses to develop and the severity of the disease to increase in the IL-10neg/neg mice. In addition, elevated levels of IFN-γ were observed, which may be responsible for the decreased CD4+CD25+ Treg function and the shift to a Th1 immune response. Thus, elevated IFN-γ levels may be the root cause of other disease manifestations discussed in this section.

**Viral infections**

CD4+CD25+ Tregs have been studied in several viral infections, the best studied being herpes simplex virus (HSV), HIV, SIV, Friend virus, hepatitis B virus (HBV) and hepatitis C virus (HCV) (Table 2) [197–205]. Some studies have focused on acute infection (SIV, HSV), others on the chronic phase of infection (HCV, HIV, Friend virus, feline immunodeficiency virus [FIV], cytomegalovirus [CMV], Epstein-Barr virus [EBV]). From these studies several theories have evolved in which CD4+CD25+ Tregs can be positively induced by the virus either non-specifically by TLRs or by antigen specificity. These theories have helped partially answer the variability in disease outcomes seen between patients.

The first studies to show a role for Tregs during retroviral infections came from research with the murine Friend virus [205, 206]. The initial research, conducted without CD25 separation, characterized a role for CD4+ T cells during chronic viral infection in which elevated levels of CD4+ T cells were capable of suppressing the function of CD8+ T cells in vitro [205]. In addition, it was shown that tumour regression occurred in infected mice that were depleted of CD4+ T cells further suggesting an immunosuppressive role for CD4+ T cells [205]. Robertson *et al.* expanded upon this research by showing in vitro that CD4+CD25+ Tregs isolated from Friend-infected mice...
were the CD4+ T cell subset suppressing the function of activated CD8+ T cells [206].

HCV, which is transmitted primarily by blood, infects over 170 million people worldwide. In chronically infected HCV patients, the levels of IL-10 were found to be significantly higher than patients that were not displaying classic HCV symptoms [199]. IL-10- secreting Tregs were responsible for the patient’s chronic outcome due to their ability to modulate the protection conferred by IFN-γ. Two other recent studies reported additional results suggesting IL-10 may not be required for CD4+CD25+ Treg function during HCV infection. Boettler et al. demonstrated that CD4+CD25+ Tregs are ultimately responsible for the suppression of virus-specific CD8+ T cells [197]. In vitro depletion studies showed that CD4+CD25+ Tregs were capable in a dose-dependent, IL-10 independent, cell-to-cell contact manner of suppressing HCV-specific CD8+ T cells. A second study by Rushbrook et al. also reported that CD4+CD25+ Tregs were capable of suppressing virus-specific CD8+ T cells [200]. These studies showed that CD4+CD25+ Tregs taken from patients that fully recovered from HCV exerted less suppression of CD8+ T cells than the Tregs from chronically infected patients. While the IL-10 data contradicts the first study, the idea that HCV is hijacking the immune system remains clear. This finding corroborates the first study and suggests again that HCV is using CD4+CD25+ Tregs to induce persistent infection. Also, one must remember that, in other disease models, differences in CD4+CD25+ Treg suppression abilities and cytokine expression levels have been observed in vivo or in vitro, which could contribute to the conflicting results.

In HIV-infected individuals, progression to AIDS is directly associated with the level of immune activation, and only indirectly with viral load (VL) [207]. The first reports of CD4+CD25+ Tregs being associated with HIV infection came in 2004 [203, 208]. Recently, the idea that CD4+CD25+ Tregs may have a positive role in the prevention of HIV infection progression to AIDS has been studied. While it has been observed that HIV does not specifically target or replicate to higher VLs in CD4+CD25+ Tregs, Oswald-Richter et al. showed that naturally occurring and induced CD4+CD25+FoxP3+ Tregs were highly susceptible to HIV infection [209, 210]. Our group showed that the same is true for SIVmac, which is able to infect similar CD4+CD25+ Tregs (data not shown). This high virus susceptibility could ultimately diminish the suppressive abilities of CD4+CD25+FoxP3+ Tregs to the point that hyper-activation of the CD4+ T cell population occurs. Moreover, peripheral blood samples confirmed that patients with low levels of FoxP3 had higher levels of CD4+ T cell activation as compared to patients with high levels of FoxP3 [209]. Eggena et al. studied Tregs isolated from the blood of 81 HIV chronically infected Ugandan volunteers and showed that depletion of CD4+CD25+CD62L+ Tregs occurred throughout the course of the HIV infection as compared to non-infected controls [198]. This Treg depletion resulted in immune hyper-activation defined as co-expression of human leucocyte antigen (HLA)-DR and CD38 (a key indicator for progression to AIDS).

Other studies have suggested that CD4+CD25+ Tregs may ultimately be involved in the progression of HIV to AIDS. Tsunemi et al. showed that patients with detectable levels of HIV-1 RNA (greater than 50 copies of HIV RNA/ml) had significantly higher levels of CD4+CD25+ Tregs as compared to infected patients with undetectable HIV-1 VLs as well as uninfected control patients [202]. In addition, patients with elevated levels of CD4+CD25+ Tregs were found to have inversely proportional levels of CD4+ T cells, suggesting that proliferation of CD4+CD25+ Tregs (and not VL) was directly associated with the observed lymphocytopenia. The levels of CD4+CD25+ Tregs may be related to the disease status of the patients tested [202]. Studies performed by Kinter et al. showed that CD4+CD25+ Tregs from healthy HIV-infected patients were capable of suppressing CD4+ and CD8+ T cell proliferation and cytokine production in response to HIV antigens [208]. In addition, patients with strong CD4+CD25+ Treg function in vitro had significantly lower levels of plasma viraemia and higher CD4+/CD8+ T cell ratios than patients with no detectable CD4+CD25+ Tregs. This data suggests that CD4+CD25+ Tregs may contribute to the diminution of HIV-specific T cell immune responses in HIV-infected patients [208]. Weiss et al. reported that CD4+CD25+ Tregs, expanded in peripheral blood of HIV-infected patients receiving highly active antiretroviral therapy (HAART), exhibit phenotypic, molecular and functional characteristics of regulatory T cells. CD4+CD25+ Tregs from HIV-infected patients did not proliferate in response to recall antigens and to p24 protein. The proliferative capacity of CD4+ T cells to tuberculin, CMV and p24 significantly increased following depletion of CD4+CD25+...
Tregs. Furthermore, addition of increasing numbers of CD4+CD25+ Tregs resulted in a dose-dependent inhibition of CD4+CD25+ T cells [203]. CD4+CD25+ Tregs responded specifically to p24 antigen stimulation by expressing TGF-β and IL-10, thus indicating the presence of p24-specific CD4+ T cells among the CD4+CD25+ Tregs [203]. Andersson et al. demonstrated for the first time in chronic HIV patient that Treg distribution changed in patients not receiving HAART treatment as compared to HAART-treated HIV-positive patients [37]. In untreated HIV patients, higher levels of Tregs were observed in the tonsils as compared to patients receiving HAART treatment, suggesting that Treg immunosuppression may be involved in preventing immune response to the virus [37]. Increases in Tregs in the gut and other LNs of HIV-infected patients have also been reported [37, 211, 212]. In support, Epple et al. showed that Tregs were increased in the gastrointestinal mucosa of non-treated HIV patients as compared to anti-retroviral treated patients [211]. In contrast, no difference in peripheral blood Treg levels was observed between these two patient groups. In both studies, Treg levels were found to be higher in non-treated patients suggesting that Tregs may be playing a vital role in harbouring the virus from immune control.

Recent work on the role of CD4+CD25+ Tregs in NHPs has yielded interesting results. SIV and NHPs are used as a parallel model to HIV in human beings due to the capability of expanding research that is not feasible or ethical in human beings. African green monkeys (AGMs) are used as a non-pathogenic model of HIV due to the animals retaining a persistent SIV infection, but rarely progressing to AIDS. In contrast, the RM is used as the pathogenic NHP model of HIV due to its eventual loss of CD4 T cells during infection which ultimately leads to AIDS, thus mimicking the disease progression seen in human HIV patients. Our group demonstrated that within 24 hrs of infection, the AGMs showed a strong increase in TGF-β1 and foxP3 expression [213]. This increase was correlated to enhanced levels of CD4+CD25+ and CD8+CD25+ T cells in blood shown to be at its maximum within the first few days. In addition, IL-10 levels were also found to increase in infected AGMs during primary infection in blood and tissues, whereas pro-inflammatory cytokines were rarely induced [212, 213]. Thus, an immediate anti-inflammatory immune response and increase in CD4+CD25+ Tregs may be involved in the prevention of the chronic T cell hyper-activation in non-pathogenic SIV infection. Our results also suggest that the very early immune balance following inoculation of the animal is important for the outcome of the disease and is demonstrated for other viral infections in the mouse model, providing another strong rationale for NHP research. We also showed that infected RM had increases in CD4+CD25+FoxP3+ Tregs, TGF-β1 and IL-10 starting from 7 days after infection suggesting that a parallel, but delayed induction of CD4+CD25+FoxP3+ Tregs and an immune response against the virus occurs [214]. In the LNs of RM, TGF-β levels also increased starting from day 7 p.i. [215]. In this model, the increase in suppressive CD4+CD25+FoxP3+ Tregs prevents the immune system response to the early stages of the virus infection when they would be needed the most for virus clearance. Thus, the timing of the Treg cell response in relation to immune activation and the adaptive immune response might be a critical determinant of outcome. The Treg cell response in SIV-infected RMs could be too late to limit the massive hyper-activation in the lymphatic compartments, but too early with respect to immune control. In AGMs, the Treg cell response is also too early to prevent an efficient control of viral replication, but is sufficiently early to prevent sustained immune activation.

The cytokine IL-2, which is involved in T cell growth and expansion, has been tested as an immune therapy alone or in a drug cocktail against HIV/SIV in attempt to boost virus-specific CD4+ and CD8+ T cell responses [213–214]. In HIV-infected human beings, intravenous administration of IL-2 at higher doses have not been tolerated well due to extensive inflammatory responses; however, an overall increase is observed in CD4+ and CD8+ T cell counts during and after termination of therapy [216]. Recently, a study investigated the effect of administering low doses of IL-2 as a component of a vaccine therapy over time to SIVmac251-chronically infected RM [217]. The RM were subjected to a low-dose IL-2 regimen that induced a marked increase in Gag-specific CD8+ T cells, reduction in Gag-specific CD4+ T cells, and a higher ratio of CD4+CD25+ Tregs. In contrast, in vitro studies demonstrated that the increases in CD4+CD25+ Tregs did not correlate with higher suppression of the CD4+ T cells in the IL-2 treated RM. Therefore, decreases in Gag-specific CD4+ T cells may be caused by an IL-2-specific mechanism and not suppression of the cells by CD4+CD25+ Tregs.
Other interesting work has been conducted on the role of CD4⁺CD25⁺ Tregs in other viral infections, such as those produced by HBV and HSV-2. HBV is considered to be a non-cytopathic virus that replicates within hepatocytes with liver damage primarily caused by the HBV-specific T cells in persistently infected individuals [218]. HBV-specific T cells from persistently infected individuals are unable to clear the virus which leads to extensive liver damage [218]. In addition, NK cells activated by IFN-α and IL-8 are thought to contribute to liver damage in chronic HBV cases [219, 220]. In chimpanzees, HBV clearance is also thought to be due to the non-cytolytic and cytolytic functions of CD8⁺ T cells [201]. Recently, CD4⁺CD25⁺ Tregs have been implicated in the progression of liver damage in chronic HBV-infected individuals [204]. CD4⁺CD25⁺ Tregs were found to be in higher frequency in the liver in patients with severe chronic HBV. In patients with acute and chronic forms of the disease, CD4⁺CD25⁺ Tregs were not only found to be positively correlated with the serum HBV VL, but also capable of suppressing HBV-specific immune responses. Therefore, CD4⁺CD25⁺ Tregs may be directly linked to the progression of HBV infection and represent an important cell type to possibly target and control during infection [201]. However, the dynamics of the HBV-specific immune responses still needs to be further investigated to determine the role each cell type plays in liver damage of chronically infected individuals.

HSV-1 and HSV-2 infections are responsible for causing oral, ocular and genital lesions in human beings and several species of animals worldwide; in addition, HSV may contribute to HIV replication [13, 221, 222]. Immune responses to both viruses are regulated by feedback systems of the host and pathogen [222]. Recently, research to determine what role CD4⁺CD25⁺ Tregs may play during HSV infection has been studied. In HSV-1-infected rabbits, CD4⁺CD25⁺ Tregs isolated from the conjunctiva were found to have higher suppressive capacity against HSV-specific CD4⁺ and CD8⁺ T cells when compared to Tregs isolated from normal conjunctiva [223]. This increase in CD4⁺CD25⁺ Tregs was isolated to the conjunctiva, with no cell increase observed in the PBMCs or spleen [223]. CD4⁺CD25⁺ Tregs from human patients infected with HSV-2 were capable of suppressing CD4⁺ cells activated by HSV-2 antigen [221]. These observations may be important for potential development of a vaccine against HSV. Since CD4⁺CD25⁺ Tregs may ultimately control the suppression of human CD4⁺ and CD8⁺ T cell memory responses to HSV, it would be ideal to limit the suppressive activities of the CD4⁺CD25⁺ Tregs in order to expedite clearance of the virus. However, caution would need to be taken in vaccine development that involves depleting CD4⁺CD25⁺ Tregs due to observations in the murine model of HSV-1. Using the mouse model of the HSV-1, lesions were shown to be more severe in animals depleted of CD4⁺CD25⁺ Tregs [13]. Depletion of CD4⁺CD25⁺ Tregs in the mouse model resulted in a significant increase in immune response and viral clearance; however, Treg depletion also triggered corneal damage in the mice [13]. Therefore, the development of immunotherapy needs to consider the role that CD4⁺CD25⁺ Tregs are playing in order to prevent significant side effects.

To conclude, as we have seen with bacteria and fungal infections, the role of CD4⁺CD25⁺ Tregs in viral infections has been previously underestimated. The most likely reason for the various roles CD4⁺CD25⁺ Tregs in viral infections is the mechanism of action of each individual virus.

Autoimmunity and cancer

Since their discovery, CD4⁺CD25⁺ Tregs have been found to play an important role in preventing natural and transplant-derived autoimmune diseases. CD4⁺CD25⁺ Tregs are responsible for the suppression of effector T cells, the majority of which are specific for self-antigens; without suppression these cells would possibly cause unnecessary damage to the host. Recent research has been carried out to learn how to control the suppressive functions of CD4⁺CD25⁺ Tregs for many different autoimmune diseases, transplantation therapies and cancer treatments.

CD4⁺CD25⁺ Tregs have been studied in several different autoimmune diseases and animal models: type 1 diabetes, MS, Behcet's disease, idiopathic thrombocytopenic purpura (ITP), IPEX and systemic lupus erythematosus [224–230]. ITP, a heterogeneous autoimmune disease due to the appearance of anti-platelet autoantibodies, has very few therapy options. Recently it was found that individuals with platelet counts below 100 x10⁹/L had unchanged numbers of CD4⁺CD25⁺ Tregs compared to healthy volunteers, whereas patients that had recently undergone a splenectomy (an effective treatment) to
increase platelet counts had significantly higher Tregs and foxp3 mRNA [229]. These results suggest that the increased numbers of CD4^+CD25^+ Tregs are associated with improved platelet counts, but the actual role of Tregs in the pathogenesis of ITP remains to be deciphered. Similar results were found in individuals inflicted with Behcet’s disease, an autoimmune disease associated with recurrent outbreaks of inflammation usually resulting in ulcers and arthritis [226]. Patients with active cases of Behcet’s disease were found to have higher expression of foxp3 and CTLA-4 mRNA as compared to remission and normal volunteers. Moreover, CD4^+CD25^+ Tregs taken from patients with active disease had no diminished abilities in suppressing CD4^+CD25^{neg} T cells. This suggests that the higher levels of CD4^+CD25^+ Tregs found in patients with active Behcet’s disease may be accountable for the recurrent inflammation that is characteristic of this disease [226].

The use of non-obese diabetes (NOD) mouse models in research has led to major developments in our understanding of type 1 diabetes. CD4^+CD25^+ Tregs have been reduced in numbers in NOD mice and diabetes can be controlled when these cells are expanded in vitro and transferred back to the mice [231]. In a recent study it was determined that CD4^+CD25^+ Tregs from NOD mice are not necessarily impaired in their ability to suppress cells, but rather it is the inability of the APCs to activate the regulatory cells that causes the defect [224]. Therefore, targeting APC populations could provide a better route in the development of therapies for patients with type 1 diabetes. The idea of activating CD4^+CD25^+ Tregs in NOD mice is also supported by data obtained with a DNA vaccine which when administered early, induced an increase in the number of Tregs in the mice and improved the outcome of the disease [232].

MS is an autoimmune disease characterized by a chronic inflammation of the central nervous system where T cells attack the myelin sheath eventually causing permanent paralysis and death of the patient. The idea that CD4^+CD25^+ Tregs could be used to suppress these damaging T cells has been studied mainly in the EAE mouse model, which shares many of the disease characteristics of MS. Zhang et al., using SJL mice that are highly susceptible to proteolipid protein (PLP) 139-151-induced EAE, found that CD4^+CD25^+ Tregs are increased in mice that recovered from EAE [233]. In addition, depleting CD4^+CD25^+ Tregs during the recovery phase aggravated the clinical signs of the disease in mice and also caused an increased number of PLP 139–151 positive cells. TGF-β was also shown to be important in the EAE recovery due to the increased numbers of CD4^+ T cells in the spleen and blood that also expressed TGF-β. When TGF-β was neutralized in vivo, mice were unable to recover from EAE. This indicates not only an importance for Tregs in general in the pathogenesis of MS, but more specifically the role that TGF-β has in recovery from induced EAE. Chen et al. recently showed that a combination of two currently used therapies for MS could be used to enhance the population of CD4^+CD25^+FoxP3^+ Tregs, but not T effector cells [234]. Thus, following administration of IL-2 and Dexamethasone, CD4^+CD25^+FoxP3^+ Tregs increased in the lymphoid tissue of EAE animals. More importantly, this treatment allowed for a partial restoration of the suppressive activity of splenic CD4^+CD25^+ Tregs and inhibited EAE disease advancement. This work provides support for the use of combination therapies like Dexamethasone and IL-2 that could provide a novel approach against natural and induced autoimmune diseases [234].

Three research groups recently investigated the functionality and quantity of CD4^+CD25^+ Tregs in human beings derived from the peripheral blood of MS patients with either relapse-remitting (RR-MS) or secondary progressive (SP-MS) forms of the disease. Huan et al. demonstrated that foxp3 mRNA and protein levels were reduced in RR-MS patients and that these data correlated with the suppression abilities of the CD4^+CD25^+ Tregs [235]. Unlike IPEX patients that have no FoxP3 expression due to genetic defects, MS patients may have reduced levels of FoxP3 due to an unknown defect in a regulatory pathway. Venken et al. observed no quantitative or phenotypic differences in CD4^+CD25^+ Tregs in patients with either RR-MS or SP-MS [236]. However, as seen in the previous study, foxp3 mRNA levels were reduced in patients with RR-MS, but not in SP-MS individuals. These data also correlated with a reduced suppressor function of CD4^+CD25^+ Tregs from RR-MS patients that was also not seen with cells from SP-MS individuals, suggesting that Treg function is altered early on in disease. Finally, Kumar et al. published contrasting...
results that showed significantly higher levels of CD4+CD25+ Tregs in MS patients, and that these CD4+CD25+ Tregs isolated from MS patients were unable to suppress CD4+CD25- T cells stimulated by the myelin-based protein antigen in vitro at the same level as CD4+CD25+ Tregs taken from healthy patients [237]. The discrepancy could be related to the heterogeneous nature of MS disease and/or the definition of Tregs.

The history of transplantation has been plagued with a high level of graft rejection by the recipient patient. While suppressive drugs have been relatively successful, the need to continue the medication for the life of the patient is both costly and cumbersome. Recent transplantation research has focused on developing therapies involving CD4+CD25+ Tregs that may make suppressive drugs more efficient or ultimately unnecessary. Using Tregs generated by the retroviral transfer of the foxp3 gene into CD4+CD25neg T cells in vitro, Chai et al. demonstrated that allogenic skin graft rejections were minimal in animals that received these in vitro generated cells [238]. Moreover, the patients that developed graft versus host disease after transplantation had a decreased level of CD4+CD25+ Tregs as compared to healthy individuals [239]. After steroid treatment, the levels of CD4+CD25+ Tregs increased in these individuals concomitant with a decrease in inflammation. This study was the first tightly time-lined one that gives us a strong indication that the levels of CD4+CD25+ Tregs are somehow correlated with the onset of graft versus host disease, suggesting that CD4+CD25+ Tregs should be closely monitored and enhanced as necessary after transplantation in order to prevent graft rejection.

Using a murine bone marrow transplantation model, Zeiser et al. showed that CD30 expression on CD4+CD25+ Tregs was a critical player in the prevention of aGVHD [240]. It has also been suggested that Tregs are important for the induction of graft tolerance [241]. Mice that were deficient in CD30 production died via aGVHD by day 35. Furthermore, by employing CD30 blocking experiments at different time points after transplantation, it was shown that expression of CD30 early after bone marrow transplantation is critical for the induction of donor cell apoptosis by Tregs. In human transplantation experiment, Kreijveld et al. showed that renal transplant patients receiving a single dose of the anti-CD25 drug daclizumab did not suffer graft rejection and were capable of recovering fully functional CD4+CD25+ Tregs after clearance of the drug [175]. Another drug used in transplant therapy, Rapamycin, was recently shown to expand murine CD4+CD25FoxP3+ Tregs in vitro. Furthermore, these expanded Tregs were fully functional in vitro and prevented allograft rejection in vivo [129]. Furthermore, the effects of rapamycin were not reversed by a combinational therapy of rapamycin and daclizumab [175].

CD4+CD25+ Tregs have also been implicated in several forms of cancer. Since the cancer field is incredibly large and diverse, we will only explore the role of CD4+CD25+ Tregs in ovarian, prostate and breast cancer research (Table 2). We saw earlier that the suppressive function of Tregs could be used against the host in individuals with certain diseases, and the same holds true in cancer patients. It is thought that the presence of Tregs prevents the effector cells of the immune system from attacking and ultimately destroying the tumour. The key in recent cancer research has been the development of therapies or vaccines that target the Tregs in order to knock down their suppressive function.

Ovarian cancer is the leading cause of gynaecological cancer deaths. There is a high level of interest for an improved therapy due to the high mortality rate (30% mortality within 5 years). Elevated CD4+CD25+ Tregs' percentages in ovarian tumour-associated lymphocytes were first reported in late-stage patients in 2001 [242]. This increase in CD4+CD25+ Tregs was further confirmed in PBMCs and tumour infiltrating lymphocytes isolated from patients with ovarian carcinoma [243]. CD4+CD25+ Tregs isolated from ovarian cancer patients were shown to secrete TGF-β. Furthermore, T cells isolated from the tumour-associated lymphocytes and stimulated with anti-CD3 and anti-CD28 were shown to have an increase in IL-2, TNF-α, IFN-γ secretion as compared to normal T cells [242]. Li et al. recently demonstrated in vitro that TGF-β could convert a percentage of ovarian carcinoma CD4+CD25neg cells to CD4+CD25+ Tregs, which suggests that targeting TGF-β in ovarian cancer patients could have positive results [243]. CD4+CD25+ Tregs accumulation at the sight of the tumour was associated with poor prognosis. Curiel et al. recently demonstrated that CD4+CD25+ Tregs suppress tumour-specific T cell immunity which ultimately contributes to the progression of tumour
enlargement. In addition, CCL22 (binds CCR4 expressed on Tregs) is involved in vitro in CD4⁺CD25⁺ Treg migration, and this same pathway may also be involved in trafficking CD4⁺CD25⁺ Tregs to the tumour site in vivo [244]. Therefore, a possible immunotherapy against ovarian cancer could involve prevention of Treg migration.

Prostate cancer is one of the leading causes of death in North American and European men. While early detection can lead to a successful recovery, currently no treatment exists for late-stage cancer. While the reasons for unsuccessful late-stage treatment are unknown, the thought is that the presence of CD4⁺CD25⁺ Tregs is somehow involved [245]. Using peripheral blood and malignant tissue obtained from patients with early stage prostate cancer, CD4⁺CD25⁺ Tregs were significantly increased in peripheral blood from prostate patients as compared to healthy donors [245]. In addition, malignant tissue harboured a significantly higher level of CD4⁺CD25⁺ Tregs as compared to benign tissue from the same prostate. This work is supported by two earlier studies that observed increased levels of Tregs in the mouse prostate cancer model. In the first study, CD4⁺CD25⁺ Tregs were found to be significantly higher in the draining LNs of the mouse model of prostate cancer as compared to normal controls [246]. In another study, it was observed that an increased frequency of CD4⁺ T cells within the tumour was stage-independent and could be used as an indicator of poor prognosis in prostate cancer patients [247]. Together, these studies suggest that elevated levels of CD4⁺CD25⁺ Tregs are linked to prostate cancer and therefore the need exists to develop a therapy that directly targets this CD4⁺CD25⁺ Tregs population.

Breast cancer is one of the leading causes of death in women worldwide. Similar to prostate cancer patients, breast cancer patients have levels of CD4⁺CD25⁺ Tregs in blood significantly higher than healthy volunteers [245, 248]. One study, however, evaluated peripheral blood from breast cancer patients and found no difference in CD4⁺CD25⁺ Tregs when compared to healthy individuals [249]. In another study, Leong et al. attempted to phenotype the tumour-infiltrating lymphocytes of patients with infiltrating ductal carcinoma and reported that 60% of the CD4⁺ T cells expressed CD25⁺ and of this population 44% co-expressed FoxP3 and CD152 [250]. In addition, a high number of CD8⁺CD28neg suppressor cells were also detected in the carcinomas. While a correlation in the number of CD4⁺CD25⁺ Tregs and the stage of the tumour could not be established, a correlation between the presence of CD4⁺CD25⁺ Tregs and the age of the individual was identified. Individuals older than 50 years of age were found to have significantly lower levels of CD4⁺CD25⁺ Tregs as compared to younger patients. However, these correlations need to be further tested and confirmed.

Therapies are being developed against breast cancers that are specifically focusing on lowering the level of circulating CD4⁺CD25⁺ Tregs. Herceptin is a monoclonal antibody prescribed for the treatment of metastatic breast cancer that overexpress the HER2 protein; however, its mode of function is still unknown (unpublished data). Recently, a study by Slavina et al. demonstrated that patients treated with Herceptin had decreased numbers of circulating CD4⁺CD25⁺ Tregs in the peripheral blood [251]. This study was supported by a group developing a vaccine against HER2 [252]. The HER2/neu peptide vaccine (E75) was recently used in a clinical trial to test its effectiveness. The breast cancer positive patients were found to have two-fold higher levels of CD4⁺CD25⁺ Tregs and three-fold higher levels of TGF-β in peripheral blood as compared to healthy individuals. However, after vaccination, levels of CD4⁺ CD25⁺ Tregs were found to be lower and, in addition, an increase in activated CD4⁺ CD69⁺ T cells was observed. Also, two-thirds of vaccinated individuals showed lower levels of TGF-β. A longer follow-up is needed in order to confirm the duration of the Tregs decrease in these patients and the impact of the vaccine on the patients’ prognosis and survival. Future studies should incorporate assays investigating CD4⁺CD25⁺ Tregs activity to determine if any increase or decrease is found in their suppressive abilities in the vaccinated patients.

**Conclusions**

Our knowledge of CD4⁺CD25⁺ Tregs has increased rapidly during the past decade. However, our level of understanding will need to continue to improve in order to truly allow medicine to use these cells to
their fullest. While in some cases CD4⁺CD25⁺ Tregs seem to protect the host from damage, in other cases they are directly associated with the intensification of the disease. The key probably ends up being somewhere in the middle in that CD4⁺CD25⁺ Tregs are capable of protecting the host from the effects of disease by regulating the effector cells’ functions, thus minimizing inflammation in the infected area. However, the difficulty in understanding the role these cells play in protecting individuals from disease and their eventual ability to be used as therapy treatments may ultimately come down to organ specificity. As mentioned here, CD4⁺CD25⁺ Tregs can employ different levels of suppression based on their location in the host. This aspect among many others brought up throughout this review presents new avenues of research.

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