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Phenotypic and Functional Differences Between Human $CD4^+CD25^+$ and Type 1 Regulatory T Cells

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Abstract T regulatory (Tr) cells have an essential role in the induction and maintenance of tolerance to both and foreign self-antigens. Many types of T cells with regulatory activity have been described in mice and humans, and those within the $CD4^+$ subset have been extensively characterized. $CD4^+$ Type-1 regulatory T (Tr1) cells produce high levels of IL-10 and mediate IL-10-dependent suppression, whereas the effects of naturally occurring $CD4^+CD25^+$ Tr cells appear to be cell-contact-dependent. Tr1 cells arise in the periphery upon encountering antigen in a tolerogenic environment. In contrast, it appears that $CD4^+CD25^+$ Tr cells can either arise directly in the thymus or be induced by antigen in the periphery. We have been interested in defining the phenotype

and function of different subsets of $CD4^+$ Tr cells present in human peripheral blood, with the ultimate aim of designing therapeutic strategies to harness their immunoregulatory effects. This review will discuss the similarities and differences between human Tr1 and naturally occurring $CD4^+CD25^+$ Tr cells, as well as evidence that indicates that they have nonoverlapping, but synergistic roles in immune homeostasis.

Abbreviations

Ag	Antigen
APCs	Antigen-presenting cells
DCs	Dendritic cells
GITR	Glucocorticoid-induced TNFR superfamily member 18
GVHD	Graft versus host disease
SIT	Specific immunotherapy
Tr	T regulatory
Tr1	Type-1 T regulatory

1 Introduction

T regulatory (Tr) cells have an essential role in the induction and maintenance of tolerance to both and foreign self-antigens (Ags). Many types of T cells with regulatory activity have been described in mice and humans [83, 85, 90, 107], and those within the $CD4^+$ subset have been extensively characterized. $CD4^+$ Type-1 T regulatory (Tr1) cells arise in the periphery upon encountering Ag in a tolerogenic environment via a process that requires IL-10. Tr1 cells produce high levels of IL-10 themselves, and mediate IL-10-dependent suppression of T cell responses. In contrast, $CD4^+CD25^+$ Tr cells can either arise directly in the thymus (the so-called naturally occurring subset) or be induced by Ag in the periphery. Naturally occurring $CD4^+CD25^+$ Tr cells do not produce IL-10, and mediate cell-contact-dependent suppression. We have been interested in better defining the phenotype and function of these different subsets of $CD4^+$ Tr cells present in human peripheral blood, with the ultimate aim of designing therapeutic strategies that harness their immunoregulatory effects. In this review, we will discuss the similarities and differences between human Tr1 and $CD4^+CD25^+$ Tr cells, and evidence that indicates that they have nonoverlapping, but nevertheless synergistic roles in immune homeostasis.

2 Tr1 Cells

Tr1 cells were first defined in in vitro differentiation systems that involved priming $CD4^+$ T cells in the presence of exogenous IL-10 [40]. These IL-

10-anergized T cells appear to undergo two stages of differentiation. First, they become nonresponsive and fail to proliferate or produce cytokines in response to Ag-specific or polyclonal activation [24, 40]. In this intermediate stage, although the T cells have already acquired the capacity to suppress naive T-cell responses, this function is not dependent on production of immunosuppressive cytokines, but is cell-contact-dependent [24]. A second stage of differentiation occurs following forced proliferation in vitro [42], and likely following repeated Ag exposure in vivo. The previously anergic cells regain some ability to proliferate, and acquire a unique profile of cytokine production (IL-$2^{-/low}$, IL-4^-, IL-5^+, IL-10^+, TGF-β^+), which is distinct from those of classical Th0, Th1, or Th2 cells [42]. In addition to IL-10 and TGF-β, human Tr1 cells also produce IFN-γ, although at levels that are at least 1 log lower than those produced by Th1 cells [8, 62]. In contrast, murine Tr1 cells usually do not produce IFN-γ [42]. The finding that these fully differentiated Tr1 cells mediate IL-10- and TGF-β-dependent suppression in vitro and in vivo, in both Th1 and Th2-mediated diseases [60, 82], sparked intensive interest in better defining their origins, phenotype, and potential clinical application.

2.1 Origins

In addition to T-cell priming in the presence of exogenous IL-10, many other methods can be used to promote the differentiation of Tr1 cells. Indeed, in the absence of antigen presenting cells (APCs), IL-10 alone is relatively inefficient at generating Tr1 cells, and addition of IFN-α can enhance its effects [62]. This was recently confirmed in vivo upon treatment with G-CSF, which appears to induce Tr1 cells via induction of IL-10 and IFN-α [84]. Stimulation of T cells in the presence of immunosuppressants such as vitamin D3 and dexamethasone has a similar Tr1-inducing effect, which depends on induction of autocrine IL-10 [12]. Interestingly, co-stimulation via CD2 [105] or with antibodies against CD46, a receptor that binds and inactivates complement C3b [52], also results in the generation of Tr1 cells. A general conclusion from these studies conducted in the absence of APCs, is that IL-10, be it from an autocrine, paracrine, or exogenous source, is necessary, but probably not sufficient, for the differentiation of Tr1 cells.

Many groups have investigated the capacity of different subsets of APCs to prime Tr1 cells. We recently studied the capacity of immature dendritic cells (DCs) to drive the differentiation of Tr1 cells upon repeated stimulation of naive peripheral blood $CD4^+$ T cells. Allogeneic immature DCs prime Tr1 cells via an IL-10-dependent mechanism, and the resulting cells suppress proliferation and cytokine production by an IL-10- and TGF-β-dependent

mechanism [61]. Induction of Tr1 cells by immature DCs does not require the presence of $CD4^+CD25^+$ Tr cells, and the resulting cells do not express high levels of CD25, providing further evidence that Tr1 and $CD4^+CD25^+$ Tr cells are distinct subsets (see also below). In contrast, when immature DCs are used to prime $CD4^+$ T cells isolated from cord blood, although the resulting T cells do produce IL-10, they do not mediate cytokine-dependent suppression [48]. The reason for this difference is not clear, but may be related to the fact that $CD4^+$ T cells from cord blood contain proportionally more $CD4^+CD25^+$ Tr cells and IL-10-producing cells than do adult peripheral blood T cells [62, 111], and therefore $CD4^+CD25^+$ Tr cells could have contaminated the cultures.

In addition to immature DCs, DCs that have developed and/or been activated in the presence of a variety of tolerogenic stimuli, including IL-10 itself [106], vitamin D3 [77], cholera toxin [58], or *Bordetella pertussis* toxin [71], all promote the differentiation of Tr1 cells. Although these agents seem to have little in common, they all in fact lead to suppression or inhibition of NFκB activation. In the absence of functional NFκB activity, IL-12 secretion would be inhibited, resulting in DCs that could predominantly secrete IL-10. In support of this concept, DCs that are genetically deficient for RelB (an NFκB family member) and lack expression of CD40, efficiently drive Tr1 differentiation [70]. Moreover, T cells primed with DCs previously treated with a proteasome inhibitor, which inhibits the degradation of IkB (and thus activation of NFkB), become Tr-like cells [113]. Thus, in the absence of a "normal" inflammatory response, Ag-loaded DCs might by default generate regulatory rather than effector T cells. Key open questions in this scenario include: where would priming of Tr1 cells take place in vivo? and would the resulting Tr1 cells always be phenotypically and functionally identical regardless of the tolerizing stimuli? Careful studies on the trafficking of tolerogenic DCs, the sites of DC–T cell interaction, and definition of molecular markers for Tr1 cells will be required to address these questions.

2.2 Phenotype

Currently, the defining phenotype of Tr1 cells is solely based on cytokine production, with the most consistent finding being T cells that secrete IL-10, but not IL-4, and very little, if any, IL-2. An unresolved question is whether co-secretion of TGF-β should also be included as part of the definition of Tr1 cells. Many reports describe a role for both IL-10 and TGF-β in their suppressive effects [26, 42, 116], whereas others describe an exclusive role for IL-10 [30]. Suppressive phenomena that are entirely dependent on T-cell-derived TGF-β have been attributed to either Th3 [23] or $CD4^+CD25^+$ Tr cells [74]. Until more

reliable molecular markers of these different subsets of Tr cells are found, we would argue that suppressive effects mediated by T-cell-derived IL-10 should be attributed to Tr1 cells, regardless of the presence or absence of TGF-β.

Like most Tr cells, Tr1 cells proliferate poorly following polyclonal or Ag-specific activation, but their proliferation can be significantly enhanced by exogenous IL-2 and/or IL-15 [8]. Despite this low proliferative capacity, Tr1 cells express normal levels of T-cell activation markers such as CD25, CD40L, CD69, HLA-DR, and CTLA-4 [8]. It should be noted that since Tr1 cells upregulate CD25 normally when activated, they could potentially fall into the $CD25^+$ pool. An important distinction from bona fide $CD4^+CD25^+$ Tr cells is that they do not continue to express high levels of CD25 in the resting phase. Thus, cells that are found to be $IL\text{-}10^+$ and $CD25^+$ should be re-analyzed for expression levels of CD25 after in vitro culture and entry into resting phase.

The difficulties associated with defining Tr1 cells solely on the basis of cytokine production have led to many studies designed to identify specific cell-surface markers. In the resting state, Tr1-cell clones constitutively express high levels of the IL-2/-15Rβ and γ common chains [8], and a vast repertoire of chemokine receptors, including some previously associated with the Th1 or Th2 phenotypes [89]. Notably, expression levels of FoxP3 (a transcription factor associated with $CD4^+CD25^+$ Tr cells, see below) in Tr1 cells do not differ from those in normal activated $CD4^+$ T cells ([102, 107] and our unpublished data). The group of Waldmann et al. has performed extensive serial analysis of gene expression (SAGE) experiments on murine Tr1 clones, and reported that prepro-enkephalin, GM2 ganglioside activator protein, glucocorticoid-induced TNFR superfamily member 18 (GITR), and integrin $\alpha E\beta 7$ (CD103) are all potential markers of Tr1 cells [114]. Further work to validate the specificity of these molecules, and to identify more Tr1-specific genes is required.

2.3 Mechanisms of Action and Functions

Tr1 cells regulate the responses of naive and memory T cells in vitro and in vivo and can suppress both Th1 and Th2 cell-mediated pathologies [39, 82]. Via production of suppressive cytokines, Tr1 cells exert suppressive effects on a variety of cell types in addition to T cells. For example, supernatants from activated Tr1 cells strongly reduce the capacity of DCs to induce alloAg-specific proliferation [22, 59]. Tr1-cell clones also suppress the production of immunoglobulins by B cells [54]. Furthermore, via local secretion of IL-10, it is likely that Tr1 cells will also educate naive $CD4^+$ T cells to become Tr1 cells. Data indicating that TGF-β is a differentiation/growth factor for $CD4^+CD25^+$

Tr cells [112], suggest that Tr1 cells may also be able to promote different Tr-cell subsets.

Evidence from both murine and human studies indicates that the major function of Tr1 cells is to control homeostasis of the response to foreign Ags in the periphery. Although Tr1 cells can also recognize self-Ags [43, 54] and tumor Ags [109], a majority of studies have reported IL-10-dependent regulation of responses to allergens, pathogens and alloAgs. Tr1 cells seem to be of particular importance in mucosal tissues, where foreign Ags are first encountered. Indeed, mucosal tissues may contain specialized subsets of dendritic cells that are dedicated to priming Tr1 cells [3, 4]. Also of particular interest is the remarkable ability of many bacteria [57, 71], parasites [88], and viruses [69, 66] to actively promote the generation of Tr1 cells. Study of the mechanisms that pathogens have evolved over millions of years to promote the generation of Tr1 cells will undoubtedly lead to new therapeutic strategies to induce their generation in clinically relevant situations.

2.4
In Vivo Evidence for Tr1 Cells in Humans

Following widespread revival of the concept of active suppression and armed with a phenotype (i.e., IL-10 production in the absence of IL-2 or IL-4) and the in vitro suppression assay, many groups have designed studies aimed at assessing the quantitative and qualitative presence of Tr1 cells in a variety of disease settings. Unfortunately, due to the ease of using CD25 as a marker to track and isolate Tr cells ex vivo, it is sometimes hard to dissect whether an effect attributed to IL-10$^+$CD25$^+$ Tr cells involves classical Tr1 cells, naturally occurring CD4$^+$CD25$^+$ Tr cells, Ag-induced CD4$^+$CD25$^+$ Tr cells, or some combination of these three cell types. Recent data that indicate CD4$^+$CD25$^+$ Tr cells may have a role in inducing both IL-10 and/or TGF-β-producing cells [30, 92] strongly suggest that the latter case may often be true. Table 1 highlights some recent reports that have found IL-10-producing Tr1 cells specific for a variety of Ags in humans. There is also an impressive number of studies that have found evidence of functional Tr1 cells in a variety of murine models (reviewed in [39, 41, 60, 82]). A general conclusion from these studies is that Tr1 cells are undoubtedly present naturally in vivo in both mice and humans, and regulate responses to a wide variety of Ags. However, thus far, it has been difficult to conduct quantitative studies that correlate relative numbers of Tr1 cells with clinical status.

Table 1 Summary of some recent studies that show quantitative and/or functional changes in Tr1 or $CD4^+CD25^+$ Tr cells in a variety of human diseases (*continued on next page*)

	Tr1/CD25	Ag specificity	Comments	Reference(s)
Autoimmunity				
Hemolytic anemia	Tr1	RhD	Mapped Tr1-specific epitopes	[43]
Multiple sclerosis	CD25	Unknown	Numbers not affected by treatment with Copaxone or IFN-α	[80]
	CD25	Unknown	Although numbers are normal, suppressive function abnormal	[103]
Systemic lupus erythematosus	CD25	Unknown	Decreased numbers in patients' PBMCs; no functional data	[65]
Rheumatoid arthritis	CD25	Unknown	Synovial fluid from patients contains functional Tr cells	[20,29]
Autoimmune polyglandular syndrome type II	CD25	Unknown	Although numbers are normal, suppressive function abnormal	[56]
Pemphigus Vulgaris	Tr1	Desmoglein 3	Classical Ag-specific Tr1 clones found in healthy donors	[100]
Myasthenia Gravis	CD25	Unknown	Normal numbers in thymus, but severely compromised function	[11]
Cancer				
Hodgkin's lymphoma	Tr1 and CD25	Unknown	Suppression in vitro largely IL-10-dependent	[68]
Gastrointestinal and esophageal malignancies	CD25, possibly Tr1	Unknown	Higher proportion in PBMCs and ascites correlates with poor prognosis	[45,87]
Melanoma	CD25 and Tr1	LAGE1	Ag-specific clones produce high levels of IL-10, but mediate contact-dependent suppression	[109]
	CD25, possibly Tr1	Unknown	Twofold increased frequency in metastatic lymph nodes	[104]
Ovarian carcinoma	CD25	Her2	Tr cells traffic to the tumor via CCR4-CCL22 interactions; their presence predicts poor survival	[28]

Table 1 (*continued*)

	Tr1/CD25	Ag specificity	Comments	Reference(s)
Infectious diseases				
EBV	Tr1	LMP1	IL-10-dependent suppression	[69]
Onchocerciasis (river blindness)	Tr1	Onchocerca Ags	Classical Ag-specific Tr1-cell clones found in chronically infected patients	[88]
Hepatitis C	Tr1	HCV core protein	HCV core-specific Tr1 cell clones isolated from chronically infected patients	[66]
HIV and CMV	CD25	viral Ags	Depletion of $CD25^+$ cells enhances in vitro responses to viral Ags	[1]
Allergy				
Cat allergy	Tr1	FelD1	Tr1 cells present in normal and allergic subjects, but increase following SIT	[81]
Grass pollen allergy	Tr1 and CD25	Phleum pratense	Increase in numbers of $CD25^+IL\text{-}10^+$ T cells following SIT	[36]
House dust and birch pollen allergies	Tr1 and CD25	Der p1 and Bet v1	Tr1 cells present in normal donors and increase following SIT in allergic patients	[50]
Allergic rhinitis	CD25	Grass or birch pollen extracts	No difference in numbers between normal donors and allergic patients	[15]
Nickel allergy	CD25	Nickel	Present in normal PBMCs, allergic patients not investigated	[21]
	Tr1	Nickel	Nickel-specific Tr1 clones	[22]
Celiac disease	Tr1	Gliadin	Tr1 clones isolated from mucosa of patients in remission	[37]
Transplantation				
Allogeneic hematopoietic stem cell transplantation	CD25	Unknown	Patients with chronic GVHD had elevated numbers of functional $CD4^+CD25^+$ T cells; did not assess alloAg-specific suppression	[25]
	CD25	Unknown	Patients with GVHD received grafts containing significantly higher frequencies of $CD4^+CD25^+$ T cells	[91]
	Tr1	Host alloAg	Donor-derived Tr1 cell clones specific for host alloAgs isolated ex vivo	[7]

3
Naturally Occurring Human $CD4^+CD25^+$ Tr Cells

Like their murine counterparts, $CD4^+CD25^+$ Tr cells isolated from human peripheral blood constitutively express the IL-2Rα chain [10, 78, 86]. However, in contrast to mice, where a distinct population of $CD25^+CD4^+$ cells can be identified by flow cytometry, human peripheral blood mononuclear cells (PBMCs) reveal a continuum of CD25 expression, with up to 20% of cells being positive if gates are set based on control antibodies. However, only the brightest $CD25^+$ cells (~3% of $CD4^+$ cells) are highly enriched for Tr activity, and the intermediate $CD25^+$ population contains a highly variable mixture of Tr cells and activated effector cells [9, 10]. Thus, in the human system, FACS sorting must be employed if long-term Tr cell lines and/or clones are to be generated. In fact, even the $CD4^+CD25^{bright}$ population does not contain Tr cells exclusively [64]. To accurately asses the purity of Tr cells within a population of $CD25^+$ cells, they must be allowed to rest in vitro; only true $CD4^+CD25^+$ Tr cells will maintain very high levels of CD25 expression in this phase [64].

3.1
Origins

Although it is clear that naturally occurring $CD4^+CD25^+$ Tr cells arise in the thymus, the cells, signals, and Ags that stimulate their development are poorly characterized. In humans, $CD4^+CD25^+$ Tr cells are present in the thymus, particularly in the perivascular areas of fibrous septa [5], and these cells share many phenotypic and functional similarities with their peripheral counterparts. Moreover, patients with thymic hypoplasia (DiGeorge syndrome) have low numbers of peripheral $CD4^+CD25^+$ Tr cells, supporting the concept that they are thymically derived [94]. Whether or not $CD4^+CD25^+$ Tr cells exclusively recognize self-Ags, or posses a repertoire broad enough to include foreign Ags, remains unclear. Analysis of V gene region diversity in human cell populations does not reveal any significant differences between that $CD4^+CD25^+$ Tr cells and nonsuppressive controls [51, 96], suggesting the Tr cells do not recognize a specialized subset of Ags. A definitive answer to this question is crucial to the therapeutic approach in settings such as allergy, since it determines whether it would be feasible simply to expand a pre-existing pool of Ag-specific $CD4^+CD25^+$ Tr cells, or whether, as for Tr1 cells, de novo differentiation would be required.

3.2 Phenotype

In addition to CD25, $CD4^+CD25^+$ Tr cells isolated from peripheral blood constitutively express high levels of CTLA4, GITR, CD71, HLA-DR, CD45RO, IL-2Rβ (CD122), IL-2Rγ (CD132), PD-L1, and ICOS [10]. In contrast to murine cells, human $CD4^+CD25^+$ Tr cells do not express high levels of the integrin CD103 ([92] and our unpublished data), but high expression of the chemokine receptors CCR4 and CCR8 may be functionally relevant [46]. Unfortunately, none of these markers have proven to be truly specific for $CD4^+CD25^+$ Tr cells and their expression is merely indicative of an apparently constitutive state of T cell activation. Indeed, $CD4^+CD25^+$ Tr cells have short telomeres, suggesting that these cells have experienced repeated episodes of Ag-specific stimulation in vivo [96].

The cytokine production phenotype of human $CD4^+CD25^+$ Tr cells has been intensively studied. A majority of studies have failed to detect significant production of IL-10 in vitro [31, 49, 63]. In contrast, human $CD4^+CD25^+$ Tr cells can secrete TGF-β, although at levels that are not significantly different from nonsuppressive cells [64]. We failed to detect membrane bound TGF-β on suppressive $CD4^+CD25^+$ Tr cell clones, and instead found evidence that positive staining of freshly isolated cells was due to an artifact of purification by magnetic beads [64]. The phenotype of human $CD4^+CD25^+$ Tr cells in terms of other Th cytokines is quite remarkable: they fail to produce detectable levels of IL-2, IL-4, IL-5, IL-10 or IFN-γ [9, 64]. In fact, they are the only human T cell clones that we have found not to produce IFN-γ. Thus, a crucial difference between Tr1 and $CD4^+CD25^+$ Tr cells lies in their cytokine production profiles.

Despite their inability to produce IL-2, this cytokine is a key growth factor for $CD4^+CD25^+$ Tr cells both in vitro and in vivo. As for Tr1 cells, IL-15 can completely replace IL-2 as a growth and survival factor for $CD4^+CD25^+$ Tr cells in vitro [31]; however, data from IL-$2^{-/-}$ mice indicate that in vivo this is not the case [79]. In human cell cultures, IL-4 cannot replace IL-2 as a growth factor (our unpublished data), whereas in mouse cultures it can [98]. This may be due to differential receptor expression and/or signaling. IL-2 or IL-15 can also rescue $CD4^+CD25^+$ Tr cells from apoptosis, likely via induction of Bcl-2 expression [95] and allowing exit from cell cycle arrest in the G1/G0 phase [49]. Interestingly, IL-2-induced activation of the PI3'kinase/Akt pathway appears to be defective in $CD4^+CD25^+$ Tr cells [16], which may provide an explanation for their poor proliferative capacity, at least in vitro. Evidence that $CD4^+CD25^+$ Tr cells are not anergic in vivo [33] suggests that current in vitro culture conditions may still be lacking essential growth factors(s).

A major advance in the study of $CD4^+CD25^+$ Tr cells came with the finding that a transcription factor known as FoxP3 may not only be a novel Tr-

cell marker, but it may also be necessary for their development [34, 44, 53]. Thus, when mouse $CD4^+$ T cells are forced to overexpress Foxp3, they adopt a phenotype virtually identical to naturally occurring $CD25^+CD4^+$ Tr cells [34, 44]. These "artificial" Tr cells are even able to suppress autoimmune bowel disease in vivo. Notably, both mice and humans deficient for FoxP3 rapidly develop systemic autoimmunity, which correlates with the notion that FoxP3 is required for the development and/or function of $CD4^+CD25^+$ Tr cells [17, 110]. This finding also supports the hypothesis that the primary role of $CD4^+CD25^+$ Tr cells is controlling responses to self-Ags.

Like murine cells, human $CD4^+CD25^+$ Treg cells express significantly more FoxP3 mRNA and protein than do $CD4^+CD25^-$ T cells [108]. However, expression of FoxP3 can also be induced upon TCR-mediated activation of normal human $CD4^+CD25^-$ Tr cells. Indeed, induction of FoxP3 in effector $CD4^+$ T cells may be a natural mechanism that allows the peripheral induction of Tr cells [108].

3.3 Functions

All published reports agree that human $CD4^+CD25^+$ Tr cells potently suppress the proliferation and effector functions of both $CD4^+$ and $CD8^+$ T cells. Interestingly, there may be an age-dependent loss of this suppressive activity, possibly correlated with decreased thymic function [99]. The effectiveness of $CD4^+CD25^+$ Tr cells in vivo is likely related to the state of activation of their targets, since when strongly stimulated by polyclonal [10] or Ag-specific activation [76], their targets become transiently resistant to suppression. $CD4^+CD25^+$ Tr cells that are fully functional in vitro, are found in inflamed tissues (see Table 1), but whether they would be functional in vivo in an environment full of pro-inflammatory cytokines and T cell stimuli is an important, and as yet unanswered, question.

Originally, it was hypothesized that the major role of naturally occurring $CD4^+CD25^+$ Tr cells was to regulate tolerance to self-Ags. In fact, a recent study suggests they may recognize epitopes within the TCR itself [18]. However, a growing body of literature suggests that, like Tr1 cells, they may also be key regulators of tolerance to foreign Ags [79]. This is illustrated in Table 1, where several recent reports of isolation of $CD4^+CD25^+$ Tr cells specific for foreign Ags (e.g., allergens and viruses) are summarized. However, since any activated Tr cell could be in the $CD25^+$ pool in vivo, experiments that truly define the nature of the TCR repertoire of these naturally occurring Tr cells are required before conclusions about Ag specificity can be drawn.

Human $CD4^+CD25^+$ Tr cells potently suppress IL-2, IFN-γ, and IL-13 production by $CD4^+$ T cells [9], although some evidence indicates that Th2 cells may be less susceptible than Th1 cells to their suppressive effects [27]. Human $CD4^+CD25^+$ Tr cells can also potently inhibit the cytotoxic activity of $CD8^+$ T cells by down-regulating perforin and granzyme B [19], and $Va24^+$NKT cell proliferation and cytokine production [6]. Presumably, these actions allow $CD4^+CD25^+$ Tr cells to control adaptive immune responses by multiple mechanisms.

3.4 Mechanisms of Action

Almost 10 years after their initial description, the mechanism(s) by which $CD4^+CD25^+$ Tr cells achieve these remarkable effects remain unclear. Some reports indicate that they may act by down-regulating the function of APCs [72], whereas others have not seen a similar effect [75, 79]. Although suppression in vitro is undoubtedly dependent upon direct cell-to-cell contact, the majority of reports have not found a role for the highly expressed cell-surface molecules CTLA-4, ICOS, PDL1, or GITR [9, 63, 64].

Much effort has gone into investigating the potential role of IL-10 and/or TGF-β in suppression. Classical in vitro suppression assays are not reversed by neutralizing anti-IL10 antibodies [31, 49, 63]. In addition, even at the clonal level, suppressive $CD4^+CD25^+$ Tr-cell clones do not produce detectable amounts of IL-10 [64]. These data, combined with those that demonstrate that IL-10-induced anergy, and differentiation of Tr1 cells by IL-10 and IFN-α [64] or by immature DCs [61] can all occur in the absence of $CD4^+CD25^+$ Tr cells, indicate that $CD4^+CD25^+$ Tr cells are distinct from Tr1 cells and do not need IL-10 for their induction. Thus, in vivo studies that found a role for IL-10 may be attributed to either an Ag-induced subset of $CD4^+CD25^+$ Tr cells that has a distinct mechanism of action from the naturally occurring subset [14, 57] and/or de novo differentiation of Tr1 cells (possibly induced by the naturally occurring $CD4^+CD25^+$ Tr cells themselves, see below [30, 47]).

In contrast, the role of TGF-β in suppression mediated by $CD4^+CD25^+$ Tr cells is much less clear. Like all human T cells, $CD4^+CD25^+$ Tr cells produce low levels of TGF-β [64]. Although neutralizing antibodies can have a small effect at high concentrations, they are never able to completely reverse suppression in vitro [9, 31, 49, 63, 64, 75]. Furthermore, addition of recombinant TGF-β cannot suppresses $CD4^+$ T cell proliferation to the same degree as $CD4^+CD25^+$ Tr cells (unpublished data). Nevertheless, much in vivo data suggests that TGF-β, like IL-10, does have a role in $CD4^+CD25^+$ Tr cell-mediated suppression [67, 73]. Again, these findings may be due to the induction of new Tr cell subsets.

Improved molecular markers for the different Tr cell subsets (i.e., $CD25^+$ vs Tr1 vs Th3) will undoubtedly shed more light on this issue.

Remarkably, human $CD4^+CD25^+$ Tr cells can be split into two subsets with distinct functions based on differential expression of integrins. Those expressing $\alpha 4\beta 7$, which binds to vascular adressins expressed by venules in mucosal tissues, have the capacity to induce de novo differentiation of IL-10-producing Tr1 cells [92]. In contrast, $CD4^+CD25^+$ Tr cells expressing $\alpha 4\beta 1$, which binds to VCAM1 on the endothelium of inflamed tissues, induce the differentiation of TGF-β-producing Th3 cells [30, 47, 92, 93]. It will be important to define the mechanisms involved in this induction of Tr1 vs Th3 cells, and their respective target Ags. If these in vitro findings are confirmed, this phenomenon would offer an attractive explanation for the studies discussed above that found a role for IL-10 and/or TGF-β in naturally occurring Tr-mediated suppression.

3.5
In Vivo Evidence for $CD4^+CD25^+$ Tr Cells in Humans

Given the ease of monitoring and isolating $CD25^+$ cells, and their undeniable importance in immune homeostasis, many groups have sought alterations in their number and/or function in patients with a variety of diseases. Table 1 summarizes some of these recent reports. A key question these studies raise is: in the absence of an Ag-specific assay, how meaningful are changes in the numbers and/or function of the total population of $CD4^+CD25^+$ Tr cells? For example, a recent study reported that the in vitro function of $CD4^+CD25^+$ Tr cells from patients with MS is dramatically impaired upon polyclonal activation [103], findings difficult to reconcile with the fact that these patients do not suffer from systemic autoimmunity. Moreover, similar to Tr1 cells, in many cases apparently normal $CD4^+CD25^+$ Tr cells can be isolated from patients with disease, perhaps highlighting the inadequacy of our current in vitro assays as a surrogate marker of in vivo functionality.

4
Peripheral Generation of $CD4^+CD25^+$ Tr Cells

In addition to thymus-derived $CD4^+CD25^+$ Tr cells, cells with a similar phenotype can also be generated from naive peripheral T cells. For example, activation in the presence of TGF-β induces naive $CD45RA^+$ T cells to up-regulate FoxP3 and develop contact-dependent, cytokine-independent suppressive activity [32, 115]. Remarkably, these induced $CD4^+CD25^+$ Tr cells also seem to have the capacity to induce differentiation of Tr1 cells [115]. Similarly, costimulation blockade [101] and expansion with IL-15 induces contact-dependent

$CD4^+CD25^+$ Tr cells [55]. In addition, cells from the $CD25^-$ pool that fail to down-regulate CD25 after activation acquire a phenotype and function that appear indistinguishable from those of naturally occurring $CD4^+CD25^+$ Tr cells [108]. Analysis at the single cell level will be required to determine the proportion of Tr cells in these induced populations, and whether they are truly phenotypically and functionally equivalent to the naturally occurring subset.

5 Networks of Tr1 and $CD4^+CD25^+$ Tr Cells

This review has attempted to highlight the similarities and differences between human Tr1 and naturally occurring $CD4^+CD25^+$ Tr cells, summarized in Table 2. It can be concluded that although these two subsets have nonredundant roles in tolerance induction and maintenance, they probably achieve their effects in synergy. At birth, thymic-derived natural Tr cells would be immediately ready to protect us against autoreactive T cells. Subsequent exposure to foreign Ags would then stimulate the development of interdependent networks of cytokine-dependent (i.e., Tr1) and -independent (i.e., $CD4^+CD25^+$ Tr) regulation. The concept that naturally occurring $CD4^+CD25^+$ Tr, Ag-

Table 2 Comparison of some of the salient features of human Tr1 and naturally occurring $CD4^+CD25^+$ Tr cells

	Tr1 cells	$CD4^+CD25^+$ Tr cells
Origins	Peripheral	Thymic
	Primed by immature or tolerogenic DCs	Subset(s) of DCs required unknown
	Require exogenous/ autocrine IL-10	Require exogenous IL-2
Ag specificity	Primarily foreign	Primarily self, but possibly also foreign
Mechanism of action	Secreted factors (IL-10 ± TGF-β)	Cell-contact-dependent in vitro
Growth and survival factors	IL-2 and/or IL-15	IL-2 and/or IL-15
Expression of CD25	Inducible	Constitutive
Expression of FoxP3	Low levels	High constitutive levels
Cytokine production	IL-$2^{+/-}$, IL-4^-, IL-10^+, IFN-γ^+, TGF-β^+	IL-2^-, IL-4^-, IL-10^-, IFN-γ^-, TGF-$\beta^{+/-}$

induced $CD4^+CD25^+$ Tr and Tr1 cells could all be present simultaneously offers an explanation for conflicting results regarding cytokine-dependent and -independent regulation. The relative importance of Tr1 vs $CD4^+CD25^+$ Tr cells, in any given instance, is likely dictated by the nature of the Ag, the context of Ag presentation, and the biology of specific tissues. One further level of control could be trafficking, since Tr1 and $CD4^+CD25^+$ Tr cells appear to have distinct migratory behaviors [35]. A major area to be investigated is the Ag specificity of these networks: do different Tr cell subsets recognize distinct or overlapping subsets of Ags?

6 Therapeutic Opportunities

The idea that manipulation of the frequency and/or function of Tr1 or $CD4^+CD25^+$ Tr could be used therapeutically has generated much excitement. Indeed, significant progress toward proof of this principle has already been made in animal models. We are currently investigating whether, as in murine models [24], alloAg-specific T cells that have been anergized in vitro upon addition of IL-10 are suppressed in their capacity to mediate graft versus host disease (GVHD), and whether they represent the precursors of Tr1 cells.

However, in general it has been difficult to establish rapid and efficient methods to expand homogenous populations of Ag specific Tr1 cells in vitro that could be used as a cellular therapy in vivo. Thus, it is currently more realistic to contemplate clinical protocols that involve boosting the numbers of Tr1 cells directly in vivo, via administration of some combination of tolerogenic agents [2, 82, 107]. Of particular interest is the remarkable efficiency of in vivo administration of the combination of rapamycin and IL-10, in a murine model of pancreatic islet allograft rejection, in inducing tolerogenic Tr1 cells [13]. Moreover, in vivo induction of Tr1 cells in humans seems to have already been achieved by specific immunotherapy (SIT) for allergens [36, 50, 81], although the precise mechanisms involved in this system remain unknown. Studying the mechanisms that pathogens have evolved over millions of years to induce Tr1 cells in vivo may reveal new strategies to achieve this goal.

With respect to $CD4^+CD25^+$ Tr cells, caution should be adopted when considering cellular therapy with non-Ag-specific populations that have been expanded in vitro. In murine systems, this approach has already proven successful at establishing long-term tolerance in the setting of bone marrow transplantation [97]. However, human $CD4^+CD25^+$ Tr cells are a much more heterogeneous mixture of activated effector and Tr cells. Although short-term expansion ex vivo preserves the potent suppressive effects of bulk populations

[38, 63], in the long-term, contaminating nonsuppressive cells overtake the cultures. What would happen to such a heterogeneous population in vivo could be a dangerous question to ask, especially if in vitro Ag-specific priming is involved. On the other hand, it is also possible that mechanisms of infectious tolerance will dominate, and that the non-Tr cells would eventually join the Tr network. Judging by the number of citations dealing with Tr cells in recent years, a similar mechanism already seems to have resulted in an increased number of Tr immunologists!

References

1. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF (2004) Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. J Virol 78:2454–2459
2. Adorini L, Giarratana N, Penna G (2004) Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. Semin Immunol 16:127–134
3. Akbari O, DeKruyff RH, Umetsu DT (2001) Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2:725–731
4. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, Umetsu DT (2002) Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat Med 8:1024–1032
5. Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, Romagnani P, Maggi E, Romagnani S (2002) Phenotype, localization, mechanism of suppression of CD4(+)CD25(+) human thymocytes. J Exp Med 196:379–387
6. Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H (2003) Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. Cancer Res 63:4516–4520
7. Bacchetta R, Bigler M, Touraine JL, Parkman R, Tovo PA, Abrams J, de Waal Malefyt R, de Vries JE, Roncarolo MG (1994) High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. J Exp Med 179:493–502
8. Bacchetta R, Sartirana C, Levings MK, Bordignon C, Narula S, Roncarolo MG (2002) Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. Eur J Immunol 32:2237–2245
9. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA (2001) CD4+CD25high regulatory cells in human peripheral blood. J Immunol 167:1245–1253
10. Baecher-Allan C, Viglietta V, Hafler DA (2004) Human CD4+CD25+ regulatory T cells. Semin Immunol 16:89–98
11. Balandina A, Lecart S, Dartevelle P, Saoudi A, Berrih-Aknin S (2004) Functional defect of regulatory CD4+CD25+ T cells in the thymus of patients with autoimmune Myasthenia Gravis. Blood 105:735–741; e-pub Sept. 28 DOI 15454488
12. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A (2002) In vitro generation of

interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 195:603–616
13. Battaglia M, Stabilini A, Draghici E, Gregori S, Bonifacio E, Roncarolo MG (2004) Rapamycin and IL-10 treatment induces antigen specific T regulatory type 1 (Tr1) cells that mediate transplantation tolerance (submitted)
14. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL (2002) CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 420:502–507
15. Bellinghausen I, Klostermann B, Knop J, Saloga J (2003) Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. J Allergy Clin Immunol 111:862–868
16. Bensinger SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC, Thompson CB, Burchill MA, Farrar MA, Turka LA (2004) Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. J Immunol 172:5287–5296
17. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 27:68–73
18. Buenafe AC, Tsaknaridis L, Spencer L, Hicks KS, McMahan RH, Watson L, Culbertson NE, Latocha D, Wegmann K, Finn T et al (2004) Specificity of regulatory CD4+CD25+ T cells for self-T cell receptor determinants. J Neurosci Res 76:129–140
19. Camara NO, Sebille F, Lechler RI (2003) Human CD4+CD25+ regulatory cells have marked and sustained effects on CD8+ T cell activation. Eur J Immunol 33:3473–3483
20. Cao D, Malmstrom V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C (2003) Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. Eur J Immunol 33:215–223
21. Cavani A, Nasorri F, Ottaviani C, Sebastiani S, De Pita O, Girolomoni G (2003) Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. J Immunol 171:5760–5768
22. Cavani A, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G (2000) Human CD4+ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. J Invest Dermatol 114:295–302
23. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237–1240
24. Chen ZM, O'Shaughnessy MJ, Gramaglia I, Panoskaltsis-Mortari A, Murphy WJ, Narula S, Roncarolo MG, Blazar BR (2003) IL-10 and TGF-beta induce alloreactive CD4+CD25– T cells to acquire regulatory cell function. Blood 101:5076–5083
25. Clark FJ, Gregg R, Piper K, Dunnion D, Freeman L, Griffiths M, Begum G, Mahendra P, Craddock C, Moss P, Chakraverty R (2004) Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. Blood 103:2410–2416
26. Cong Y, Weaver CT, Lazenby A, Elson CO (2002) Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. J Immunol 169:6112–6119

27. Cosmi L, Liotta F, Angeli R, Mazzinghi B, Santarlasci V, Manetti R, Lasagni L, Vanini V, Romagnani P, Maggi E et al (2004) Th2 cells are less susceptible than Th1 cells to the suppressive activity of CD25+ regulatory thymocytes because of their responsiveness to different cytokines. Blood 103:3117–3121
28. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942–949
29. De Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, De Jager W, Pugayung G, Giannoni F, Rijkers G et al (2004) CD4(+)CD25(bright) regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol 172:6435–6443
30. Dieckmann D, Bruett CH, Ploettner H, Lutz MB, Schuler G (2002) Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells. J Exp Med 196:247–253
31. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler S (2001) Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. J Exp Med 193:1303–1310
32. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF (2004) Cutting edge: TGF-beta Induces a regulatory phenotype in CD4(+)CD25(-) T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 172:5149–5153
33. Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL (2003) Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J Exp Med 198:737–746
34. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4:330–336
35. Foussat A, Cottrez F, Brun V, Fournier N, Breittmayer JP, Groux H (2003) A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation. J Immunol 171:5018–5026
36. Francis JN, Till SJ, Durham SR (2003) Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 111:1255–1261
37. Gianfrani C, Levings MK, Sartirana C, Mazzarella G, Zanzi D, Iaquinto G, Giardullo N, Auricchio S, Troncone R, Roncarolo MG (2004) Gliadin-specific type 1 T regulatory cells are present in the intestinal mucosa of treated coeliac disease patients. (submitted)
38. Godfrey WR, Ge YG, Spoden DJ, Levine BL, June CH, Blazar BR, Porter SB (2004) In vitro-expanded human CD4(+)CD25(+) T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. Blood 104:453–461
39. Groux H (2003) Type 1 T-regulatory cells: their role in the control of immune responses. Transplantation 75:8S–12S
40. Groux H, Bigler M, de Vries JE, Roncarolo MG (1996) Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med 184:19–29
41. Groux H, Cottrez F (2003) The complex role of interleukin-10 in autoimmunity. J Autoimmun 20:281–285

42. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737–742
43. Hall AM, Ward FJ, Vickers MA, Stott LM, Urbaniak SJ, Barker RN (2002) Interleukin-10-mediated regulatory T-cell responses to epitopes on a human red blood cell autoantigen. Blood 100:4529–4536
44. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061
45. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H (2003) Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 9:4404–4408
46. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D (2001) Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. J Exp Med 194:847–853
47. Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH (2002) Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. J Exp Med 196:255–260
48. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH (2000) Induction of interleukin-10-producing, nonproliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogenic immature human dendritic cells. J Exp Med 192:1213–1222
49. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH (2001) Identification and functional characterization of human CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. J Exp Med 193:1285–1294
50. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, Akdis CA (2003) IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 33:1205–1214
51. Kasow KA, Chen X, Knowles J, Wichlan D, Handgretinger R, Riberdy JM (2004) Human CD4(+)CD25(+) regulatory T cells share equally complex and comparable repertoires with CD4(+)CD25(–) counterparts. J Immunol 172:6123–6128
52. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature 421:388–392
53. Khattri R, Cox T, Yasayko SA, Ramsdell F (2003) An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 4:337–342
54. Kitani A, Chua K, Nakamura K, Strober W (2000) Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T regulatory cell 1 T cells. J Immunol 165:691–702
55. Koenen HJ, Fasse E, Joosten I (2003) IL-15 and cognate antigen successfully expand de novo-induced human antigen-specific regulatory CD4+ T cells that require antigen-specific activation for suppression. J Immunol 171:6431–6441
56. Kriegel MA, Lohmann T, Gabler C, Blank N, Kalden JR, Lorenz HM (2004) Defective Suppressor Function of Human CD4+ CD25+ Regulatory T Cells in Autoimmune Polyglandular Syndrome Type II. J Exp Med 199:1285–1291

57. Kullberg MC, Jankovic D, Gorelick PL, Caspar P, Letterio JJ, Cheever AW, Sher A (2002) Bacteria-triggered CD4(+) T regulatory cells suppress Helicobacter hepaticus-induced colitis. J Exp Med 196:505–515
58. Lavelle EC, Jarnicki A, McNeela E, Armstrong ME, Higgins SC, Leavy O, Mills KH (2004) Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. J Leukoc Biol 75:756–763
59. Lecart S, Boulay V, Raison-Peyron N, Bousquet J, Meunier L, Yssel H, Pene J (2001) Phenotypic characterization of human CD4+ regulatory T cells obtained from cutaneous dinitrochlorobenzene-induced delayed type hypersensitivity reactions. J Invest Dermatol 117:318–325
60. Levings MK, Bacchetta R, Schulz U, Roncarolo MG (2002) The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. Int Arch Allergy Immunol 129:263–276
61. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG (2004) Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Treg cells. Blood 105:1162–1169; e-pub Oct12 DOI 15479730
62. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG (2001) IFN-alpha and IL-10 Induce the differentiation of human type 1 T regulatory cells. J Immunol 166:5530–5539
63. Levings MK, Sangregorio R, Roncarolo MG (2001) Human CD25+CD4+ T regulatory cells suppress naive and memory T-cell proliferation and can be expanded in vitro without loss of function. J Exp Med 193:1295–1302
64. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC, Roncarolo MG (2002) Human CD25+CD4+ T suppressor cell clones produce TGF-β, but not IL-10 and are distinct from type 1 T regulatory cells. J Exp Med 196:1335–1346
65. Liu MF, Wang CR, Fung LL, Wu CR (2004) Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. Scand J Immunol 59:198–202
66. MacDonald AJ, Duffy M, Brady MT, McKiernan S, Hall W, Hegarty J, Curry M, Mills KH (2002) CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. J Infect Dis 185:720–727
67. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F (2003) CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 197:111–119
68. Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, Vickers MA (2004) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. Blood 103:1755–1762
69. Marshall NA, Vickers MA, Barker RN (2003) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. J Immunol 170:6183–6189
70. Martin E, O'Sullivan B, Low P, Thomas R (2003) Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. Immunity 18:155–167

71. McGuirk P, McCann C, Mills KH (2002) Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. J Exp Med 195:221–231
72. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV (2004) Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. J Immunol 172:4676–4680
73. Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, Strober W (2004) TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J Immunol 172:834–842
74. Nakamura K, Kitani A, Strober W (2001) Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 194:629–644
75. Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI (2001) Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. Blood 98:2736–2744
76. Pasare C, Medzhitov R (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299:1033–1036
77. Penna G, Adorini L (2000) 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 164:2405–2411
78. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H, Shevach EM (2002) CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J Exp Med 196:237–246
79. Piccirillo CA, Shevach EM (2004) Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. Semin Immunol 16:81–88
80. Putheti P, Pettersson A, Soderstrom M, Link H, Huang YM (2004) Circulating CD4(+)CD25(+) T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs. J Clin Immunol 24:155–161
81. Reefer AJ, Carneiro RM, Custis NJ, Platts-Mills TA, Sung SS, Hammer J, Woodfolk JA (2004) A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. J Immunol 172:2763–2772
82. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK (2001) Type 1 T regulatory cells. Immunol Rev 182:68–79
83. Roncarolo MG, Levings MK (2000) The role of different subsets of T regulatory cells in controlling autoimmunity. Curr Opin Immunol 12:676–683
84. Rutella S, Bonanno G, Pierelli L, Mariotti A, Capoluongo E, Contemi AM, Ameglio F, Curti A, de Ritis DG, Voso MT et al (2004) Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha. Eur J Immunol 34:1291–1302
85. Sakaguchi S (2000) Regulatory T cells: key controllers of immunologic self-tolerance. Cell 101:455–458

86. Sakaguchi S (2004) Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 22: 531–562
87. Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A (2003) CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. Cancer 98:1089–1099
88. Satoguina J, Mempel M, Larbi J, Badusche M, Loliger C, Adjei O, Gachelin G, Fleischer B, Hoerauf A (2002) Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). Microbes Infect 4:1291–1300
89. Sebastiani S, Allavena P, Albanesi C, Nasorri F, Bianchi G, Traidl C, Sozzani S, Girolomoni G, Cavani A (2001) Chemokine receptor expression and function in CD4+ T lymphocytes with regulatory activity. J Immunol 166:996–1002
90. Shevach EM (2000) Regulatory T cells in autoimmmunity. Annu Rev Immunol 18:423–449
91. Stanzani M, Martins SL, Saliba RM, St John LS, Bryan S, Couriel D, McMannis J, Champlin RE, Molldrem JJ, Komanduri KV (2004) CD25 expression on donor CD4+ or CD8+ T cells is associated with an increased risk for graft-versus-host disease after HLA-identical stem cell transplantation in humans. Blood 103:1140–1146
92. Stassen M, Fondel S, Bopp T, Richter C, Müller C, Kubach J, Becker C, Knop J, Enk AH, Schmitt S et al (2004) Human CD25+ regulatory T cells: two subsets defined by the integrins alpha4beta7 or alpha4beta1 confer distinct suppressive properties upon CD4+ T helper cells. Eur J Immunol 34:1303–1311
93. Stassen M, Schmitt E, Jonuleit H (2004) Human CD(4+)CD(25+) regulatory T cells and infectious tolerance. Transplantation 77: S23–S25
94. Sullivan KE, McDonald-McGinn D, Zackai EH (2002) CD4(+) CD25(+) T-cell production in healthy humans and in patients with thymic hypoplasia. Clin Diagn Lab Immunol 9:1129–1131
95. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN (2001) Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol 31:1122–1131
96. Taams LS, Vukmanovic-Stejic M, Smith J, Dunne PJ, Fletcher JM, Plunkett FJ, Ebeling SB, Lombardi G, Rustin MH, Bijlsma JW et al (2002) Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. Eur J Immunol 32:1621–1630
97. Taylor P, Lees CJ, Blazar BR (2002) The infusion of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-versus-host disease lethality. Blood 99:3493–3499
98. Thornton AM, Piccirillo CA, Shevach EM (2004) Activation requirements for the induction of CD4+CD25+ T cell suppressor function. Eur J Immunol 34:366–376
99. Tsaknaridis L, Spencer L, Culbertson N, Hicks K, LaTocha D, Chou YK, Whitham RH, Bakke A, Jones RE, Offner H et al (2003) Functional assay for human CD4+CD25+ Treg cells reveals an age-dependent loss of suppressive activity. J Neurosci Res 74:296–308
100. Veldman C, Hohne A, Dieckmann D, Schuler G, Hertl M (2004) Type I regulatory T cells specific for desmoglein 3 are more frequently detected in healthy individuals than in patients with pemphigus vulgaris. J Immunol 172:6468–6475

101. Vermeiren J, Ceuppens JL, Van Ghelue M, Witters P, Bullens D, Mages HW, Kroczek RA, Van Gool SW (2004) Human T cell activation by costimulatory signal-deficient allogeneic cells induces inducible costimulator-expressing anergic T cells with regulatory cell activity. J Immunol 172:5371–5378
102. Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC, O'Garra A (2004) IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4(+)CD25(+) regulatory T cells. J Immunol 172:5986–5993
103. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA (2004) Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 199:971–979
104. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, Bachelez H, Kourilsky P, Ferradini L (2004) Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 173:1444–1453
105. Wakkach A, Cottrez F, Groux H (2001) Differentiation of regulatory T cells 1 is induced by CD2 costimulation. J Immunol 167:3107–3113
106. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H (2003) Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity 18:605–617
107. Waldmann H, Graca L, Cobbold S, Adams E, Tone M, Tone Y (2004) Regulatory T cells and organ transplantation. Semin Immunol 16:119–126
108. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. J Clin Invest 112:1437–1443
109. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF (2004) Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity 20:107–118
110. Wildin RS, Smyk-Pearson S, Filipovich AH (2002) Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 39:537–545
111. Wing K, Ekmark A, Karlsson H, Rudin A, Suri-Payer E (2002) Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood. Immunology 106:190–199
112. Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA (2001) A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. J Immunol 166:7282–7289
113. Yoshimura S, Bondeson J, Brennan FM, Foxwell BM, Feldmann M (2001) Role of NFkappaB in antigen presentation and development of regulatory T cells elucidated by treatment of dendritic cells with the proteasome inhibitor PSI. Eur J Immunol 31:1883–1893
114. Zelenika D, Adams E, Humm S, Graca L, Thompson S, Cobbold SP, Waldmann H (2002) Regulatory T cells overexpress a subset of Th2 gene transcripts. J Immunol 168:1069–1079
115. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA (2004) Natural and induced CD4(+)CD25(+) cells educate CD4(+)CD25(–) cells to develop suppressive activity: the role of IL-2, TGF-beta, IL-10. J Immunol 172:5213–5221

116. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, Bowen G, Rook G, Walker C (2002) Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 8:625–629