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REVIEW

Human T Regulatory Cells: On the Way to Cognition

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Abstract Forkhead box P3 (Foxp3)$^+$ T regulatory (Treg) cells are powerful controllers of the immune response and their role in the human immune system is indispensable. Since a number of revolutionary and very convincing results were brought to light, Foxp3 has unquestionably been thought to be the "master regulator" of Treg lineage commitment. Herein, we depict the revised view on the role of Foxp3 transcription factor, challenging this theory, as well as the growing significance of Runt-related transcription factor (RUNX) family proteins for Treg lineage. The review presents the current notion of Treg cell heterogeneity, molecular characteristics and their mechanisms of action.

Keywords T regulatory cells · Foxp3 · Treg immunophenotype · RUNX

Introduction

The subset of suppressor T cells loomed from the landmark work of Gershon and Kondo in the early 1970s (Gershon and Kondo 1970, 1971). Although not that widely noted, the existence of T cells with suppressive function was also suggested by Nishizuka and Sakakura (1969). Further investigations were difficult, however, due to the problems with delineating and purifying of this cell population from the pool of other T cells. The subject was revisited after Sakaguchi et al. (1995) applied the adoptive transfer model using CD4$^+$ T cells, previously depleted of CD4$^+$CD25$^+$ subpopulation, in *nu/nu* mice to study autoimmune reactions (Shevach 2011).

Since then, a great number of studies pertaining to the cells with regulatory characteristics have been published. However, these studies often show conflicting evidence which may be caused by the problems in isolating T regulatory (Treg) cells with full precision. The reason for that could be the lack of one ideal marker of Treg cell population. However, Treg population is heterogeneous and markers for specific subtypes are now the major objective.

Apart from CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, some Foxp3$^-$ T helper cells secrete interleukin (IL)-10 and/or transcription growth factor (TGF)-β (Jutel and Akdis 2011a; Sakaguchi et al. 2010). In addition CD8$^+$CD28$^-$ cells with suppressive capacity have also been described (Sakaguchi et al. 2010). This article will mainly focus on the naturally occurring Foxp3$^+$ Tregs (nTregs) because of their evident and indispensable role in maintaining the balance between immunological tolerance and response.

The General Classification of CD4$^+$Foxp3$^+$ T Cells

It has long been known that human T cells consist of cells with naïve and memory phenotype and can be identified in accordance to their expression of CD45RA or CD45RO, respectively (Michie et al. 1992). Consistent with these findings, CD4$^+$Foxp3$^+$ T cell subset was found to be

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diverse with regard to CD45 isoform expression and therefore subgrouped on this basis (Seddiki et al. 2006; Valmori et al. 2005). Subsequently, several studies concerning the characteristics of RA/RO subgroups have been published. Miyara et al. (2009) present a comprehensive picture of these observations. Three populations of Foxp3 expressing cells were suggested: $CD25^{+}CD45RA^{+}Foxp3^{lo}$ resting Treg cells (rTregs), $CD25^{hi}CD45RA^{-}Foxp3^{hi}$ activated Treg cells (aTregs), both potently suppressive in vitro, and $CD25^{+}CD45RA^{-}Foxp3^{lo}$ non-Treg nonsuppressive, cytokine secreting cells (Miyara et al. 2009). Importantly, resting and activated Tregs represent two developmental stages of the same cells (Miyara et al. 2009). Resting Tregs express CD45RA, low level of Foxp3 transcription factor, no Ki67 and most of them present the expression of CD31 which indicates recent thymic emigration (Marson et al. 2007). With nearly all $CD45RO^{+}$ Tregs being $CD31^{-}$ one can assume that Tregs migrate from the thymus in a resting state and become activated in the periphery. In vitro assays show that resting Treg cells are highly proliferative after T cell receptor (TCR) stimulation and are apoptosis-resistant (Miyara et al. 2009).

$CD45RO^{+}$ regulatory T cells are a population of activated or "effector" Tregs, considered to develop from $CD45RA^{+}$ resting Tregs. Apart from the certain CD45 isoform expression, they are $Ki67^{+}$, maintain high levels of CD25 and Foxp3 and also upregulate CTLA-4, CD95 and glucocorticoid-induced TNF-receptor-related protein (GITR) (Sakaguchi et al. 2010). CTLA-4, high expression of which is characteristic only to the $CD45RO^{+}Foxp3^{hi}$ subset of Tregs (Miyara et al. 2009) is a molecule being crucial for Treg function (Shevach 2009; Wing et al. 2008). Contrary to $CD45RA^{+}$, $CD45RO^{+}$ regulatory cells are prone to apoptosis (Fritzsching et al. 2006) and, consequently, they are hyporesponsive in vitro (Miyara et al. 2009).

CD45RA delineates naïve population of T cells, however, this name is not exactly proper for Treg cells. At least in mice, constant stimulation by their specific antigen is needed for the maintenance of Tregs in the periphery (Fisson et al. 2003). Therefore they are not strictly naïve and the proposed term "resting" better describes their real nature. Similarly, although CD45RO marks a memory phenotype of conventional T cells, $CD45RO^{+}$ Treg cells are not literally memory cells. Even if the population of long-surviving Tregs exists, currently specified $CD45RO^{+}$ subset is characterized by the rapid turnover (Vukmanovic-Stejic et al. 2006).

$Foxp3^{+}$ cells may arise in vitro from both human and murine naïve $Foxp3^{-}CD4^{+}$ cells in response to stimulation in specific cytokine milieu containing IL-2 and TGF-β (Chen et al. 2003; Fantini et al. 2004). The induction of these cells is further promoted by the addition of retinoic acid (Lu et al. 2010; Xiao et al. 2008). There is a dispute over the functional characteristics of such induced Treg cells (iTregs) in human beings. Also the stability, fate and prevalence of this population among other $Foxp3^{+}$ cells in the periphery is controversial. While induction of Foxp3 in murine naïve $CD4^{+}$ cells undoubtedly confers them natural Treg-like phenotype and regulatory capacity (Fantini et al. 2006; Huter et al. 2008), it is unclear whether human induced $Foxp3^{+}CD4^{+}$ cells are suppressive (Tran et al. 2007; Walker et al. 2003). It seems possible that the population of induced, nonsuppressive in vitro $Foxp3^{+}$ cells (Tran et al. 2007) matches the subgroup of human peripheral blood $CD25^{+}CD45RA^{-}Foxp3^{lo}$ cells presented by Miyara et al. (2009). *Foxp3* gene methylation studies showed high level of methylation in both populations while naturally occurring Tregs (both resting and activated) display full *Foxp3* demethylation (Baron et al. 2007; Floess et al. 2007).

Despite initial findings suggesting the possibility of differentiation of thymus- and periphery-derived $Foxp3^{+}$ cells on the basis of Helios (the member of Icaros family of transcription factors) expression (Thornton et al. 2010), subsequent studies proved that Helios expression is not exclusive to nTregs as under certain conditions the protein is upregulated in the iTregs both in vitro (Akimova et al. 2011; Gottschalk et al. 2012) and in vivo (Gottschalk et al. 2012). It was also shown that Helios expression is independent of Foxp3 induction and is rather associated to the recent T cell activation (Akimova et al. 2011). However, it has been reported very recently that Neuropilin 1 (Nrp1), highly expressed by most of nTregs, may be used to distinguish these two subsets (Weiss et al. 2012; Yadav et al. 2012). Such distinction eventually becoming possible would mean a great step forward on the way to understand Treg biology.

Key messages:

- There are three main populations of $Foxp3^{+}$ T cells: $CD25^{+}CD45RA^{+}Foxp3^{lo}$ rTregs, $CD25^{hi}CD45RA^{-}Foxp3^{hi}$ aTregs and $CD25^{+}CD45RA^{-}Foxp3^{lo}$ nonsuppressive non-Tregs;
- aTregs emerge from rTregs and express high levels of activation markers: CTLA-4, CD95 and GITR
- Foxp3 can be induced in $Foxp3^{-}CD4^{+}$ cells under certain conditions, however, the role of such cells in humans is not clear;
- Nrp1 is a newly discovered marker for distinguishing nTreg and iTreg groups.

The Further Heterogeneity Inside $CD4^{+}Foxp3^{+}$ Subset

Evidence has been presented implying the existence of another, terminally differentiated Treg population expressing HLA-DR and constituting a considerable portion of human effector Tregs in the peripheral blood (Baecher-Allan et al. 2001). These cells express higher

levels of Foxp3 and have greater suppressive capacity as compared to HLA-DR$^-$ Tregs (Baecher-Allan et al. 2006). Interestingly, HLA-DR$^+$ and HLA-DR$^-$ Tregs demonstrate different mechanisms of action in vitro: the former subset suppresses in an early, contact-dependent manner while the latter secretes IL-10 and the contact-dependent component emerges in the late phase (Baecher-Allan et al. 2006). Moreover, CD25^{hi} HLA-DR$^-$ Tregs become CD25^{hi} HLA-DR$^+$ when cultured in activating conditions (Baecher-Allan et al. 2006).

Recent findings showed that the pool of effector Tregs in the periphery can be divided into ICOS$^+$ and ICOS$^-$ subsets. Ito et al. (2008) demonstrated that although all ICOS$^+$Foxp3$^+$ Tregs were CD45RO expressing cells, those ICOS$^-$ were either CD45RA$^+$ or CD45RO$^+$ and the phenotype ICOS$^-$ or ICOS$^+$ correlated to the preferential production of TGF-β and IL-10, respectively. Based on the fact that myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) prime T cells preferentially using different pathways (ICOS/ICOS-L and CD28 pathways, respectively) (Janke et al. 2006), the experiment proved that pDCs selectively induce the proliferation of ICOS$^+$ Treg cells and mDCs tend to regulate the proliferation of ICOS$^-$ Tregs (Janke et al. 2006). The connection between ICOS and HLA-DR expression remains to be explored.

The above featured model of classification by Miyara et al. (2009) is elegant and consistent, nevertheless it is not contradictory to the findings of other teams. Indeed, other groups also postulated similar diversification of CD4$^+$Foxp3$^+$ cells: the types of nTregs (naïve-like and memory-like phenotypes), characterized by various suppression capabilities, expression levels of molecules such as CD25, Foxp3 and different apoptosis and stimulation susceptibility (Baecher-Allan et al. 2001; Banham et al. 2006; Fritzsching et al. 2006), and on the other hand, the group of non-suppressive cells, still, expressing certain amounts of CD25 and Foxp3 (Allan et al. 2007; Gavin et al. 2006; Morgan et al. 2005).

Apart from this most basic and primary classification, various experiments describe more subtle subgroups of Treg cells (Table 1), which are typically found in certain immunological environments and sites of the body either under normal conditions or in pathology. Hence, a subset of potent regulatory cells, very efficient in migration to acute inflammatory sites, was found to be expressing the integrin α_E (Huehn et al. 2004). Another interesting experiment described extrathymical production of Foxp3$^+$ Tregs in the gut. The process is mediated by the specialized CD103$^+$ DC population and involves retinoic acid and TGF-β (Coombes et al. 2007; Sun et al. 2007). Treg cells from the colonic lamina propria are abundant producers of IL-10 (Kamanaka et al. 2006), which cytokine is highly important in protection against *Helicobacter hepaticus*-induced colitis (Kullberg et al. 2002). Recent studies bring us another unique population: Treg cells from murine adipose tissue impact metabolism and have their own phenotypic signature. Imbalance of this subset seems to be an important feature in a model of type-2 diabetes (Cipolletta et al. 2011).

These examples show the direct clinical relevance of certain Treg populations. Reciprocal relations and the position of such individual subgroups in collective Treg-genealogy need to be further investigated.

Table 1 The "classical" categorization of Foxp3-expressing Treg cells and examples of more specific subpopulations

Group	Brief description	References
CD4$^+$CD25$^+$CD45RA$^+$Foxp3^{lo}	The phenotype of classically distinguished population of resting/naïve-like Tregs which upregulates Foxp3 and CD25 upon activation	Miyara et al. (2009) and Sakaguchi et al. (2010)
CD4$^+$CD25^{hi}CD45RA$^-$Foxp3^{hi}	The second typically delineated group of Tregs, called effector, activated or memory-like Tregs, consists of activated and potently suppressive cells	Miyara et al. (2009) and Sakaguchi et al. (2010)
Nrp1$^+$ Tregs	High expression of Nrp1 in the population of thymus-derived Tregs enables purification of these "naturally occurring" cells from the pool containing also Foxp3$^+$ cells induced in the periphery	Weiss et al. (2012) and Yadav et al. (2012)
CD4$^+$CD25$^+$CD103$^+$ Foxp3$^+$ cells	CD103 (integrin α_E) delineates the subset of highly suppressive Tregs efficiently migrating to the sites of acute inflammation	Huehn et al. (2004)
IL-10-producing intestinal Treg cells	Very high secretion level of this cytokine is characteristic of the group of colonic Tregs and plays a significant role in the control of colitis	Izcue and Powrie (2008) and Kamanaka et al. (2006)
Visceral adipose tissue Tregs	This subset influencing organismal metabolism has characteristic expression profile of many Treg-typical molecules and unique TCR repertoire. Imbalance of this subset was observed in a model of type-2 diabetes	Cipolletta et al. (2011) and Feuerer et al. (2009)
CD8$^+$Foxp3$^+$ Tregs	Although little is known about this population, CD8$^+$ Tregs constitute an inherent part of both human and murine immune systems and are capable of preventing pathology in several disease models	Endharti et al. (2011) and Smith and Kumar (2008)

Key messages:

- $CD25^{hi}$ Tregs can be further activated which results in HLA-DR expression and a change in suppressive mechanisms;
- Effector Tregs can be grouped into $ICOS^{+}$ and $ICOS^{-}$ populations;
- There are specialized subsets of Tregs which can only be found in certain immunological environments;
- Many pathologies can be linked to the prevalence or dysfunction of certain types of Treg cells.

Cell Surface Immunophenotype

Relying on the discovery that Treg cells in mouse constitutively express CD25 and subsequent assessments for human beings, Tregs were initially described as $CD4^{+}CD25^{+}$ cells (Jonuleit et al. 2001; Levings et al. 2001; Ng et al. 2001). Later investigations revealed *Foxp3* gene expression as being key factor of Treg cell development and regulatory function in mice and humans (Fontenot et al. 2003; Yagi et al. 2004). Foxp3 expression still remains the most important marker of natural Tregs, however, it was revealed that both Foxp3 and CD25 are also expressed in the conventional effector T cells upon activation (Gavin et al. 2006; Morgan et al. 2005). Therefore, to delineate Treg subset with maximal precision, the use of quantitative analysis and a combination of other markers rather than just qualitative assessment of Foxp3 and CD25 expression is necessary.

Unlike in mice, where most of $CD4^{+}CD25^{+}$ T cells are potently suppressive, analogous population in humans, albeit larger, contains mainly activated effector cells and only a small percent comprising the cells with the highest levels of CD25 seem to represent Tregs (Baecher-Allan et al. 2001; Sakaguchi et al. 2010). Yet, the boundary between $CD25^{lo}$ and $CD25^{hi}$ subsets has not been strictly defined. Considering the role of Foxp3 in Tregs, the observations of the potent suppressive capacity of $CD25^{hi}$ population are in line with the fact that the levels of CD25 and Foxp3 expression are proportional in human Tregs (Miyara and Sakaguchi 2011).

One of the most useful cell surface markers in Treg analyses is CD127 (α-chain of IL-7 receptor): suppressive capacity and Foxp3 expression in human $CD4^{+}$ pool are linked to low levels of CD127 (Liu et al. 2006). Nevertheless, utilization of this marker is hindered, similarly to the case of CD25, because of the interference with the population of recently activated effector T cells: CD127 expression decreases to low levels after cell stimulation (Aerts et al. 2008). Nevertheless, the discrimination of recently activated normal T cells and Tregs can apparently be done with CD62L (L-selectin): the subsets are $CD62L^{lo}$ and $CD62L^{+}$, respectively (Sakaguchi et al. 2010). Another idea for making such distinction exploits the absence of CD49d protein on the Treg cells, in contrast to its presence on normal $CD4^{+}$ effector cells (Kleinewietfeld et al. 2009).

Other molecules, inseparably connected to Treg functions, helpful for describing this subset include cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152), GITR, CD39 (nucleoside triphosphate diphosphohydrolase-1), CD95 (also known as FAS). Their role in Treg isolation is limited, however, because they are typically expressed in effector T cells upon activation (Shevach 2009).

Key messages:

- Conventional effector T cells express Foxp3 and CD25 upon activation;
- Treg cells express no or low levels of CD127;
- With the combination of surface markers, Tregs are usually characterized as $CD25^{hi}CD127^{lo}CD62L^{+}$ cells.

Foxp3 and RUNX Transcription Factors

The role of forkhead box P3 transcription factor for immune tolerance has been well described by observations in both mice and humans with mutations of *Foxp3* gene. Scrufy mice with a frameshift mutation of *Foxp3* gene are deficient of Tregs and develop multiorgan inflammatory disease (Brunkow et al. 2001). Foxp3 mutations in humans lead to immune dysregulation, polyendocrynopathy, enteropathy, X-linked (IPEX) syndrome, characterized by serious autoimmune, allergic and inflammatory disorders that affect the skin, endocrine organs, joints and the intestines (Bennett et al. 2001; Wildin et al. 2001).

Similar, IPEX-like symptoms are observed in patients with IL-2R α-chain (CD25) mutations (Caudy et al. 2007; Roifman 2000). This can be explained by impaired STAT5-dependent signaling in such individuals: under normal conditions IL-2 binding to its receptor activates STAT5, which in turn binds to *Foxp3* gene promoter and induces Foxp3 transcription (Burchill et al. 2007; Yao et al. 2007).

Genome analysis of Foxp3 target genes showed that there are hundreds of genes, the expression of which is modulated by Foxp3. Among those are the ones connected to Treg characteristics and functions e.g. *Il2ra* (*CD25*) *Tnfrsf18* (*GITR*) *Nrp1, Ccr4, Icos* (Marson et al. 2007; Zheng et al. 2007). Such experiments, arguing the evident connection between Foxp3 and Treg function and development, led to the conviction that Foxp3 is the "master regulator" of this subset, however, this opinion has recently been questioned (Curiel 2007). Irrespective of the extent to what Foxp3 is necessary in Treg development (see below),

it is certainly a critical factor for maintaining of Treg function (Wan and Flavell 2007).

Recently, the role of Runt-related transcription factor (RUNX) family proteins in the context of Treg development and function has been addressed. The results are encouraging. Heterodimers of RUNX and Core binding factor (CBF)-β have been shown to play an important role in both maintenance and development of Tregs in mice by promoting stability of Foxp3$^+$ phenotype (Rudra et al. 2009), controlling Foxp3 expression and also in the downstream expression of target genes (Bruno et al. 2009). Other experiments confirmed these findings in humans. RUNX1/CBF-β complex seems to be indispensable in the functioning of human nTregs (Kitoh et al. 2009) and the complexes of CBF-β with RUNX1 and RUNX3 were found to play a significant role in TGF-β mediated iTreg development and function (Klunker et al. 2009). RUNX-mutant mice produce illnesses resembling those occurring in Foxp3-mutants, although the symptoms are not that severe (Kitoh et al. 2009).

Key messages:

- Foxp3 mutations cause severe inflammatory disorders;
- The expression of many Treg key molecules (e.g. CD25, GITR, ICOS) is regulated by Foxp3 transcription factor;
- RUNX/CBF-β heterodimers are important factors for Treg development, stability and function.

Foxp3: Function Controller Rather than Lineage Development Decision-Maker

Recent assays challenge the initial view that Foxp3 is a "master regulator" of Treg lineage commitment (Fontenot et al. 2003; Hori et al. 2003). Several teams have shown that Foxp3 expression does not equal Treg characteristics and vice versa. Some of the Treg phenotype attributes are not necessarily associated with Foxp3 as they are sustained in Foxp3-deficient individuals (Bacchetta et al. 2006; Lin et al. 2007; Williams and Rudensky 2007). Conversely, Foxp3 is not sufficient to develop regulatory features as shown by activation-induced Foxp3 expression in human T effector cells without gaining suppressive function (Allan et al. 2007). A number of genes were found to be coregulated with Foxp3 when the connections between the expression of Foxp3 and other Treg hallmark genes were analyzed. However, no direct induction of these genes by Foxp3 was observed (Hill et al. 2007). Therefore, it may be assumed that there is some superior transcription regulator which administers the transcription of *Foxp3* in line with several other genes and Foxp3 itself plays role in gaining regulatory function only (Hill et al. 2007). Gavin et al. (2007) suggested that Foxp3 importance in Treg cells consists in multiplying and stabilizing regulatory features. Although without giving a factual mechanism, it was also suggested that Foxp3 positively regulates its own expression (Gavin et al. 2007). In the light of new experimental results, this observation becomes virtually certain: RUNX1 and Foxp3 are parts of a feed-forward mechanism that keeps *Foxp3* locus in an active state (Bruno et al. 2009; Zheng et al. 2010).

Key messages:

- Foxp3 seems to be the key factor for Treg function and not the "master regulator" of lineage commitment;
- Foxp3 in complex with RUNX1 positively regulates its own transcription therefore stabilizing Foxp3-dependent Treg regulatory features.

Suppressive Mechanisms of FoxP3$^+$ Treg Cells

It is already known thanks to the numerous suppression assays carried out in many laboratories that the suppressive function of FoxP3$^+$ Tregs is based on multiple mechanisms. These mechanisms can be primarily grouped into: impeding the function of antigen-presenting cells (APCs), secretion of suppressive cytokines like IL-10 and TGF-β and the contact-dependent effect. Importantly, one has to notice that the mechanisms shown in vitro do not necessarily parallel to in vivo behavior of Tregs. CTLA-4 appears to be the key molecule for CD25^{hi}Foxp3^{hi} aTreg function both in vitro and in vivo (Shevach 2009; Wing et al. 2008). It competitively inhibits the binding of co-stimulatory signal-providing CD28 to its ligands CD80 and CD86. CTLA-4 also negatively regulates the expression of CD80 and CD86 by APCs. Treg-specific knockout of CTLA-4 abolishes Treg function and promotes autoimmunity (Wing et al. 2008). Another effector mechanism of Tregs, also suppressing the CD28-dependent co-stimulatory pathway, is IL-10 and TGF-β production, with the latter cytokine inhibiting the TCR/CD3 pathway as well (Jutel et al. 2003; Jutel and Akdis 2011b). Treg cells express high levels of CD25 and consecutively deprive the environment of IL-2 thus affecting effector T cell survival. There are other molecules, the expression of which was experimentally proved to take part in Treg-mediated suppression. CD39 and CD73 proteins enable Tregs to inactivate proinflammatory pericellular ATP (Borsellino et al. 2007; Deaglio et al. 2007). Fibrinogen-like protein 2 is secreted by Treg cells and seems to negatively affect DC function (Shalev et al. 2008). The rest of known Treg suppression modes involve for

example lymphocyte-activation gene 3 (LAG3) protein, galectin 1, IL-35, granzymes and CD95–CD95L interaction (Sakaguchi et al. 2010; Shevach 2009).

Key messages:

- FoxP3^{+} Tregs present several ways of suppression, the main ones being direct inhibition of effector cells, reducing activating capability of APCs and modifying cytokine milieu;
- CTLA-4 is a crucial suppressive factor of Tregs which inhibits CD28 co-stimulatory pathway;
- CD25 (highly expressed by Tregs) consumption of IL-2 promote responder cell apoptosis;
- Other molecules such as CD95L, galectin 1, LAG3, granzymes are also engaged in Treg-mediated suppression.

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