Review

The Phenotypic Characterization of Naturally Occurring Regulatory $CD4^+CD25^+$ T Cells

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The homeostasis of the immune system depends on the balance between the immune response to an invaded pathogen and the immune tolerance to self antigens. Both central and peripheral tolerances are important mechanisms for the induction and maintenance of T cell tolerance. Recently, much attention has been paid to regulatory T cells (Treg), which play a significant role in maintaining peripheral immune tolerance. So far, there has been no satisfactory advance regarding the surface markers of Treg cells, as none is unique for Treg cells. In this review, we summarize some important molecules expressed in naturally occurring $CD4^+CD25^+$ Treg cells (nTreg), including forkhead/winged-helix family transcriptional repressor p3 (Foxp3), the tumor necrosis factor receptor (TNFR) family, CD28/CTLA4 molecules, chemokine receptors, Toll-like receptors (TLRs), membrane-bound TGF-β and other molecules, such as neuropilin-1, lymphocyte activation gene-3 (LAG)-3 and granzyme. This review provides a collective view on current studies of nTreg cell activation and development related to the expression of molecules and cell phenotype markers, which is important for elucidation of nTreg cell origin, development and function. *Cellular & Molecular Immunology*. 2006;3(3):189-195.

Key Words: regulatory T cell, phenotype, immune tolerance

Introduction

The immune system can distinguish self from non-self structure and protects the host from pathogens. There are multiple mechanisms contributing to the maintenance of self-tolerance. The primary mechanism is thymic clonal deletion of potential autoreactive T cells, which is called central tolerance. However, some autoreactive T cells normally present in peripheral lymph organs. Therefore, peripheral tolerance approaches become quite essential to inactivate these potentially pathogenic T cells. There is ample evidence showing that suppressor T cells, now called regulatory T cells (Treg cells), suppress autoreactive T cells as an active mechanism for peripheral immune tolerance (1-3).

It is, thus far, firmly established that Treg cells can be divided into two different subtypes, namely natural (or constitutive) and inducible (or adaptive) populations according to their origins (4). In addition, a variety of Treg cell subsets have been identified according to their surface markers or cytokine products, such as $CD4^+$ Treg cells (including natural $CD4^+CD25^+$ Treg cells, IL-10-producting Tr1 cells, and TGF-β-producing Th3 cells), $CD8^+$ Treg cells, Veto $CD8^+$ cells, γδT cells, NKT ($NK1.1^+CD4^-CD8^-$) cells, $NK1.1^-CD4^-CD8^-$ cells, etc (4). Accumulating evidence has strongly shown that naturally occurring $CD4^+CD25^+$ Treg cells (called nTreg cells in later descriptions unless specially noted) play an active role in down-regulating pathogenic autoimmune responses and in maintaining immune homeostasis (5, 6). Because of the lack

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Abbreviations: APC, antigen presenting cell; CTLA-4, cytotoxic T lymphocyte-associated protein-4; DCs, dendritic cells; ELC, Epstein-Barr virus-induced molecule-1 ligand chemokine; Foxp3, forkhead/winged-helix family transcriptional repressor p3; GITR, glucocorticoid-induced tumor necrosis factor receptor; GVHD, graft versus host disease; IPEX, immune dysregulation, polyendocrinopathy, and enteropathy X-linked syndrome; LAG-3, lymphocyte activation gene-3; Nrp1, neuropilin-1; SLC, secondary lymphoid organ chemokine; TGF-β, transforming growth factor β; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor associated factor; Treg, regulatory T cells.

of specific reliable markers for identifying nTreg cells from other T subsets, the contribution of nTreg cells to immunologic self-tolerance or even their existence has been controversial until now. The lack of an unambiguous molecular definition of nTreg cells has severely hampered efforts to experimentally address the developmental process of these cells and their biological role in the immune system. Since the evolution of nTreg cells has been extensively covered in other articles (7-9), in this manuscript, we will mainly focus on the phenotypic characterization of nTreg cells.

Forkhead/winged-helix family transcriptional repressor p3 (Foxp3) expression in nTreg cells

Foxp3, encoding Scurfin, is a member of the forkhead/ winged-helix family of transcriptional repressors. At present, Foxp3 is still the most specific marker for distinguishing between Treg and non-Treg cells. Foxp3 mRNA and Scurfin are not only expressed specifically in peripheral nTreg cells and thymus counterparts in mice (10-12), but also in human $CD4^+CD25^+$ thymocytes (13-15). In addition, lower to intermediate level of Foxp3 can be detected in $CD4^+CD25^-$ T cells with regulatory activity in mice and humans (10, 14), Foxp3-expressing $CD25^-$ cells may constitute a peripheral reservoir of differentiated nTreg cells, which could recruit to the $CD25^+$ pool upon homeostatic expansion and/or activation (16). In addition, human $CD8^+CD25^+$ expressing Foxp3 possess a similar suppressive function with $CD4^+$ $CD25^+$ counterpart. As in a Foxp3 transgenic murine model, small populations of $Foxp3^+$ cells express CD4/CD8 double positive or CD4/CD8 double negative, this may be an artifact resulted from the insertion of Foxp3 gene, because few transcripts, if any, are detected in other lymphocyte subsets and tissues.

Foxp3 is considered as a key player in $CD4^+CD25^+$ Treg cell biology. First, Foxp3 is a key factor in controlling the development of nTreg (10, 11). The Scurfy mutant mice and the Foxp3-null mice have diminished $CD4^+CD25^+$ Treg cells in thymus and peripheral lymph organs, and develop an autoimmune lymphoproliferative disease (17). Similar to scurfy mice, IPEX (immune dysregulation, polyendocrinopathy, and enteropathy X-linked syndrome) patients have mutations in foxhead domain of Foxp3 gene, which lead to nTreg decrease. Meanwhile, decreased Foxp3 mRNA level is found in patients with virus infections or autoimmune diseases (18, 19). In addition, the transgenic mice over-expressing Foxp3 have a noticeably elevated number of nTreg cells (12).

Second, accumulating evidence suggests a vital role for Foxp3-expressing nTreg cells in the maintenance of dominant tolerance to self. Gain-of-function assay also provides direct evidence showing that mouse $CD4^+CD25^-$ cells-overexpressing Foxp3 acquire nTreg cell phenotype and function (10). $CD4^+CD25^-$ T cells could be converted into $CD25^+$ Treg in the presence of TGF-β (20), and estrogen could up-regulate Foxp3 gene, in turn, the suppressive activity of nTreg cells is strikingly enhanced (21). These results collectively indicate that Foxp3 is associated with the function of nTregs.

Surprisingly, when Foxp3 and/or Foxp3δ2, an isoform of Foxp3, are ectopically overexpressed in human $CD4^+$ T cells, it does not lead to the acquisition of significant suppressor activity *in vitro* (22). There are several possibilities: 1) Some factors other than Foxp3 are required during the process of activation and/or differentiation for the development of nTreg cells in human. 2) There are some diversity between the functions of Foxp3 in mice and humans.

Third, Foxp3 expression correlates to T cell activation. The activation of human $CD4^+CD25^-$ T cells through TCR leads to an increased expression of Foxp3 mRNA and CD25 (23). Gavin and his colleagues found that Foxp3 expression could be induced transiently in some human non-Treg $CD4^+$ and $CD8^+$ T cells upon activation. Contrasted to human, murine counterparts do not express Foxp3 mRNA, even though CD25 is markedly up-regulated. As above referred, this raises the possibility that human and murine Foxp3 possess some differences.

However, some problems should be noticed about Foxp3 as a specific marker to distinguish nTreg cells from non-regulatory cells. First, Foxp3 is a nuclear protein, which limits its application as a tool for isolating nTreg cells *ex vivo*. Second, its role in mediating Treg cells development has been controversial especially in humans.

Tumor necrosis factor receptor (TNFR) family expressed on nTreg cells

During the past several years, some research groups performed a series of gene expression array analyses to identify a specific gene expression pattern of nTreg cells, which might be used as satisfactory marker of nTreg cells. TNFR superfamily is a related family, which includes Glucocorticoid-induced tumor necrosis factor receptor (GITR), 4-1BB, OX40 (CD134), CD95, etc. Some of them play an important role in the functions of nTreg cells.

GITR, a 70 kD homodimeric type I transmembrane glycoprotein, is found by Nocentini et al (24). GITR lacks the intracellular death domain, which is required for the induction of apoptosis and mediate intracellular signaling by recruiting TNF receptor associated factor (TRAF) proteins to their cytoplasmic tails. It is expressed on various lymphocytes at different levels. High surface expression of GITR is only confined to resting nTreg cells in the periphery and thymus. GITR may affect nTreg cells in several aspects as follows: First, because GITR expression can make T cells (both thymocytes and peripheral T cells) resistant to TCR-induced apoptosis (24), high GITR expression may render $CD4^+CD25^+$ thymocytes resistant to thymic negative selection and promote the development of nTreg cells. Second, GITR abrogates the suppressive activity of nTreg cells when ligated with its ligand or agonistic antibodies (25), which is consistent with co-stimulatory function of GITR (26). However, Stephens considers that GITR ligand

provides an important signal for $CD25^-$ T cells, rendering them resistant to nTreg cell-mediated regulation at the initiation of the immune response, since the susceptibility of effector T cells to suppressor activity is enhanced when GITR ligand is down-regulated by inflammatory stimuli (27). In addition, *in vivo* administration of anti-GITR can enhance the activity of $CD4^+CD25^-$ autoreactive cells, which further suggests that GITR-GITR ligand signaling does not appears to directly interfere with tolerance, but enables autoreactive T cells to circumvent suppression by nTreg cells (28).

OX40 (CD134) is transiently expressed on T cells after TCR ligation. Both naïve and activated nTreg cells express OX40 (29, 30). The number of nTreg cells decreases in the spleens of OX40-deficient mice, while increases in the spleens of mice overexpressing OX40 ligand (OX40L). These data suggest that OX40 participates in the development and homeostasis of nTreg cells. Suppression of T cell responses by nTreg cells is significantly impaired in the absence of OX40 (31), with indicateds that, in addition to its homeostatic functions, OX40 contributes to efficient nTreg cell-mediated suppression.

4-1BB (CD137) plays a co-stimulatory role in the activation and the maintenance of survival of CD4, CD8 and NK cells. 4-1BB is also expressed on nTreg cells (32). 4-1BB could induce the proliferation of nTreg cells both *in vitro* and *in vivo*, the 4-1BB-expanded nTreg cells are functional, as they remain suppressive to other T cells in co-culture systems (33). Collectively, 4-1BB may be related to nTreg cell expansion.

Nevertheless, like CD25 and other TNFR superfamily members, three molecules above are up-regulated on $CD25^-$ T cells following activation, rendering them not as a specific marker for nTreg cells.

The expression of CD28/cytotoxic T lymphocyte-associated protein-4 (CTLA-4) on nTreg cells

The CD28/CTLA-4 family interacts with their ligands, B7 family, to provide a second signal for T cell activation/anergy. These interactions also seem to play an important, albeit not well understood, role in the development of nTreg cells.

CD28 co-stimulation is a very important "second signal" in classical T cells activation. Similar levels of CD28 were expressed on both $CD25^+$ and $CD25^-$ T cell populations. CD28-deficient NOD mice present a profound decrease of the nTreg cells in thymus and peripheral, which suggests that CD28 plays a critical role in the development and homeostasis of nTreg cells (34). The suppressive activity of nTreg cells from CD28-deficient and CD7/CD28-double-deficient mice is less potent than that of wild-type nTreg cells (35). Moreover, CD28 engagement promotes the survival of nTreg cells *via* regulating IL-2 production by conventional T cells and CD25 expression on nTreg cells themselves (36). CD28 superagonist administration *in vivo* leads to the preferential expansion and strong activation of $Foxp3^+$ nTreg cells (37).

CTLA-4 is the homologue of CD28, having 70% sequence homology with CD28, and is an important negative regulator of T cell activation to attenuate T cell response. CTLA-4 in reticulum is intracellularly localized, and relocates to the cell surface when activated. nTreg cells are constitutively expressed at a high level of intracellular CTLA-4 and low level of surface CTLA-4, and surface CTLA-4 expression is elevated after nTreg cells activation (38). The interaction between CTLA-4 and B7 molecules offers a co-stimulation signal and results in nTreg cells activation (39). These data suggest that CTLA-4 is involved in nTreg cell regulatory activity. *In vivo* administrating non-activating anti-CTLA-4 mAb abolishes the protective activity of nTreg cells in IBD models, but does not reduce the number of nTreg cells in normal mice. *In vitro* blockade of CTLA-4 neutralizes the suppression function of nTreg cells, which suggests that CTLA-4 expression is not required for the development of nTreg cells, but is necessary for their function (40, 41).

The expressions of chemokine receptors on nTreg cells

In immune response, leukocyte recruitment can be induced through the actions of complement components, leukotrienes, platelet activating factor or other chemotactic agents, but the most important factor is chemotactic cytokines, termed chemokines. In order to turn off adaptive immune responses, nTreg cells may need to migrate to a specific location.

Analysis by confocal microscopy shows that the most of human blood-borne nTreg cells co-express CCR4 and CCR8, whereas more $CD4^+CD25^-$ T cells stain positive for CCR4 expression, and few cells expressed CCR8, so CCR8 is more restricted to the nTreg cell populations (42, 43). Almost 90% of human $CD4^+CD25^+$ thymocytes express CCR8 and 14% of these cells express CCR4, while only 8% and 10% of $CD4^+$ $CD25^-$ T cells express CCR8 and CCR4, respectively (43). Chemotaxis assays show that nTreg cells respond more potently and efficaciously to CCL17 and CCL22, and nTreg cells also respond to CXCL11 and CCL19.

nTreg cells express high levels of CCR5 (44, 45). After giving adoptively transferred $CCR5^{-/-}$ nTreg cells to irradiated allogeneic recipients, the survival of animals is significantly decreased, and GVHD scores are enhanced compared with animals receiving wild-type nTreg cells, even though $CCR5^{-/-}$ nTreg cells can suppress naïve T cell proliferation functions. The lack of CCR5 results in impaired accumulation of nTreg cells in the liver, lung, spleen, and mesenteric LNs more than one week after transplantation, which definitively demonstrates a requirement for CCR5 in nTreg cells function (45).

In Szanya's experiment, miurine $CD62L^+$ nTreg cells can express CCR7 at a high level, and respond to Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC) and secondary lymphoid organ chemokine (SLC) (46), as well as human nTreg cells (47). Because the combined expression of CD62L, CCR7 and CD27 is described as a characteristic of central memory T cells that continuously screen lymphoid organs for recall antigens, this phenotype of nTreg cells suggests a similarity to activated/memory T cells.

Gene array analysis of mouse nTreg cells indicates that

CCR6 mRNA is significantly more abundant in nTreg cells than $CD25^-$ cells. $CCR6^+$ nTreg cells exhibit a phenotype of activated effector-memory cells with a rapid turnover rate. Moreover, $CCR6^+$ nTreg cells can also be detected in human blood (48). In $CCR6^{-/-}$ mice, the generation of nTreg cells residing inside intestinal Peyer's patches is significantly reduced (49). Furthermore, CCR2, CXCR3 and CXCR4 also are found in αE^+CD25^+ T cells at intermediate levels (50).

Toll-like receptors (TLRs) expressed on nTreg cells

TLRs are primary sensors of both innate and adaptive immune systems and play a pivotal role in the immune response directed against structurally conserved components of pathogens.

Caramalho reports that TLR-4 expression in mouse $CD4^+$ T cell subsets is restricted to subpopulations with regulatory functions and might represent a specific marker for nTreg cell differentiation. Three additional TLRs (TLR-5, -7, and -8) are found to be preferentially expressed by $CD4^+CD45RB^{low}$ cells. TLR-5 expression is similar to TLR-4 (markedly increased in the $CD25^+$ subset) when compared with $CD45RB^{low}CD25^-$ cells. TLR-4 expression appears to be the most selective for nTreg cells. Stimulation of nTreg cells *via* TLR4 with LPS *in vitro* is able to elicit their proliferation, to prolong their survival and to augment their suppressive activity, even in the absence of APCs. This suggests that LPS may directly act on TLR4 expressed by nTreg cells (51, 52). Crellin's study reveals that TLR5, expressed on human nTreg cells at levels comparable to those on monocytes and DCs, interacts with its ligand (flagellin) to potently increase their suppressive capacity and enhance the expression of Foxp3 (53).

TLR-8 appears specifically expressed in the $CD4^+$ $CD45RB^{low}$ compartment independent of its CD25 phenotype (51). Peng and his colleagues also reveal TLR8 as a receptor is highly expressed by human nTreg cells, but with little or no TLR7 or TLR9 expression (54). Furthermore, siRNA assays suggest that TLR8-MyD88-IRAK4 signaling pathways may be necessary and sufficient for directing the reversal of the suppressive function of nTreg cells, which indicates that a shift for the functional balance between nTreg cells and effector T cells occurs through TLR8 signaling (54). In addition, more results demonstrate that Toll pathway-dependent blockades could alter nTreg cell-mediated suppression (55, 56).

Membrane-bound transforming growth factor-β (TGF-β)

It has been shown repeatedly that nTreg cells can suppress the proliferation of $CD4^+CD25^-$ T cells *via* cell-cell contact-dependent fashion. Study shows that this suppression may attribute to TGF-β1 expression on the surface of nTreg cells, and stimulated nTreg cells express high levels of TGF-β in both surface-bound and secreted form, anti-TGF-β abolishes nTregs cell-mediated immunosuppression on T cell proliferation (39). Furthermore, Green confirms that nTreg cells *in vivo* also express membrane TGF-β1 and TGF-β1 signalings correlate with delay in diabetes progression (57). A decline in the expression of TGF-β is responsible for gradual loss of function by nTreg cells with aging. Moreover, $CD4^+CD25^-$ T cells can be induced to $CD4^+CD25^+Foxp3^+$ Treg cells with TGF-β *in vitro* (20). However, there are some controversial results about the role of TGF-β in nTreg cells. Piccirillo finds that anti-TGFβ mAb is not able to neutralize the suppression of resting or activated nTreg cells, and nTreg cells from $TGF\beta^{-/-}$ mice are similar with wild-type counterparts in suppression properties (58). In addition, nTreg cells from dnTGF-βRII mice remain to inhibit colitis, which suggests that TGF-β signaling is not required for the development or peripheral function of nTreg cells (59). In short, TGF-β is not a suitable marker for distinguishing between nTreg cells, its role in the development or function of nTreg cells still need to be investigated.

Some new molecules expressed on nTreg cells

Neuropilin-1(Nrp1) is a co-receptor to a tyrosine kinase receptor for both the vascular endothelial growth factor family and semaphorin (Sema) family members. Nrp1 plays critical roles in neuronal guidance, cardiovascular development, angiogenesis and tumor progression. Nrp1 expression is significantly down-regulated after T cell activation (60). On the basis of global gene expression profiling, Bruder finds that Nrp1 is constitutively expressed on the surface of nTreg cells independent of their activation status, being co-regulated with Foxp3. $CD4^+Nrp^{high}$ cells exhibit high levels of Foxp3 mRNA and only $CD4^+Nrp1^{high}$ T cells suppress proliferation of naïve $CD4^+CD25^-$ T cells (61). So Nrp1 may be considered as a candidate marker of nTreg cells.

Perforin/granzyme (granule exocytosis) pathway is the major mechanism by which cytotoxic lymphocytes (NK and cytotoxic T lymphocytes) kill intracellular pathogens and tumor cells (62). Activated human nTreg cells express GZ-A and very little GZ-B, while inducible Treg cells express more GZ-B. Furthermore, both of Treg subtypes display perforin-dependent cytotoxicity against autologous target cells, including activated $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, and both immature and mature DCs. This cytotoxicity is dependent on CD18 adhesive interactions but is independent on Fas/FasL. This pathway may partly explain the contact-dependent suppression by nTreg cells (63, 64). However, a recent study in mice reveals that GZ-B is one of the key components of nTreg cell-mediated suppression, and the induction of regulatory activity is correlated with the up-regulation of GZ-B expression (65), which demonstrates that GZ-B is involved in the contact-mediated suppression by nTreg cells in mice. Intracelluar expression and different expression fashion of granzyme between humans and mice demonstrate that this molecule is not suitable as a marker for nTreg cells.

LAG-3 is an MHC class II-binding CD4 homolog.

Table 1. The phenotypes of human and mouse nTreg cells

	Human	Mouse
Cell surface receptors	$CD25^{high}$	$CD11b/CD18^{high}$
	CD27	$CD25^{intermediate\sim high}$
	$CD45RO^{high}$	CD30
	CD58	$CD45RB^{low}$
	CD83	$CD5^{high}$
	$CD95^{high}$	CD69
	$HLA\text{-}DR^{low}$	$CD150^{low}$
	a4β1	TNFRII
	a4β7	OX40
	CCR4	4-1BB
	CCR8	TRANCER
	Granzyme A	CD103(aE)
		CCR2
		CCR6
		CCR7
		CXCR3
		LAG-3
		Nrp1
		PD-1
		TLR4
		TLR7
		galectin-1
		$Ly6A/E^{high}$
		TGFβR1
		Granzyme B
	$CD122^{high}$, CD132, CD28, $CD38^{high}$, $CD44^{high}$, CD62L, CD54, CD71, CD127, GITR, TLR5, TLR8, TGF-β	
Intracellular molecules	$CTLA4^+$, $Foxp3^+$	

Previous studies suggest that LAG-3 may have a negative regulatory function (66). Comparative analysis of gene expression arrays reveals that LAG-3 mRNA is selectively expressed in nTreg cells, but no LAG-3 protein is detected on the cell surface of nTreg cells directly *ex vivo*. Furthermore, LAG-3 mRNA is up-regulated after $CD4^+CD25^-$ T cells activation in a dose-dependent manner. Anti-LAG-3 antibodies could inhibit the suppression mediated by purified nTreg cells *in vitro* (67), which demonstrates that LAG-3 involves in the regulatory function of nTreg cells.

Ectopic expression of LAG-3 with MSCV-based retrovirus potently suppresses the proliferation of naïve T cells in a contact-dependent fashion. The suppressive capacity of LAG-3 is comparable to that seen in parallel experiments with ectopically expressed Foxp3 (67). However, the lack of surface LAG-3 expression excludes the possibility of LAG-3 as a surface marker.

Using a gene microarray technique, many genes are found to be expressed in resting or activated nTreg cells, the expression patterns of these molecules on mouse and human nTreg cells are summarized in Table 1.

Concluding remarks

A number of candidate markers for distinguishing nTreg cells have been reported. Unfortunately, most of these cell surface molecules currently used to mark nTreg cells, including CD25, CD62L, CD44, CD45, TNFR family, CTLA-4, etc., are highly up-regulated on effector/memory cells upon antigen-driven activation or are also expressed by either naïve or other Treg cell populations. They are therefore not nTreg cell-specific. Although CD25 is primarily used as a marker to study the function of nTreg cells, there are numerous reports showing that $CD4^+CD25^-$ cell populations can also suppress certain immune functions (68, 69). Foxp3 is the most specific molecule for Treg cells at present, but its location limits its application as an effective isolating tool. So the lack of Treg cell-specific cell surface markers is still an impediment to the subsequent studies on nTreg cells. Solving this problem will significantly facilitate the elucidation of nTreg cell origin, development and function, as well as its clinical applications.

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