Research Article

The Phenotypic Markers of CD4+CD25+ T Regulatory Lymphocytes

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Academic Editor: Jolanta Myśliwska

Abstract

The article gives the overview of both surface and intracellular markers present on T regulatory lymphocytes. Regulatory T cells (Tregs) play a principal role in immune reactions including autoimmunity, transplantation tolerance, anti-infectious immunity and cancer. Today the most widely accepted phenotype for Tregs is the coexpression of CD4, CD25 and FoxP3. But FoxP3 is an intracellular molecule, detection of which requires fixation and permeabilization of cells and the fixed cells cannot be used in studies of Treg function. Moreover intracellular FoxP3 staining is hardly usable on a daily clinical routine basis. Therefore several attempts have been made to characterize Tregs according their surface molecules. The particular molecules like CD25, CD127, CD26, CD39, CD45RA , CD49d, CD101, CD194 are described and their significance for distinguishing T regulatory lymphocytes from other T cells is discussed. Recently described surface molecule CD26 is another negative marker which seems to improve our possibilities in distinguishing this important population from effector T cells.

Keywords: T regulatory lymphocytes, Treg, surface markers, FoxP3

Introduction

Regulatory T cells (Tregs) play a principal role in immune reactions including autoimmunity, transplantation tolerance, anti-infectious immunity and cancer. These cells represent about <5% of the peripheral CD4+ T cell population and play a crucial role in the maintenance of immune homeostasis against self- antigens [1, 2]. Numerical or functional deficit of Treg cells is linked to many autoimmune diseases such as rheumatoid arthritis [3], multiple sclerosis or type 1 diabetes. These Treg features result from their potent capacity to reduce the activation and expansion of conventional T cells [4] by suppressing their biological activities like proliferation and blocking the production of proinflammatory cytokines TNF alpha and IFNgamma, among others [5].

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The two main groups of Tregs represent natural Tregs (nTregs) and induced or adaptive Tregs (iTregs) [6]. nTregs originate in thymus and express high levels of the IL-2 receptor α-chain (CD25) [7] and the forkhead-winged helix transcription factor FoxP3, which is inevitable for their development and function [8, 9]. FoxP3+ iTregs arise in periphery from conventional naive CD4+FoxP3− T cells. Under the influence of suppressive cytokines and antigen-specific activation they develop into FoxP3+ Tregs.[6].

FoxP3+ Tregs can be divided into two subpopulations based on the expression of ICOS (inducible T cell co-stimulator) [10]. These subpopulations are both anergic and suppressive, but exert different molecular mechanisms for suppression. While ICOS−FoxP3+ Tregs mediate their suppressive activity via TGF-β, ICOS+FoxP3+ Tregs moreover secrete IL-10.

Today the most widely accepted phenotype for Tregs is the coexpression of CD4, CD25 (α-chain of the IL-2 receptor), and of FoxP3 [10].

Mechanisms of Treg Function

Tregs can suppress a whole range of immune cells including B cells, NK cells, NKT cells, CD4+ or CD8+ T cells, and both monocytes and dendritic cells. The most important and extensively studied is the suppression of CD4+CD25- conventional T cells (T_{CONV}). Main function of Tregs is to suppress the activation of naive T_{CONV}, but they can also inhibit activated effector T cells and memory CD4+ [11] and CD8+ T cells [12]. Tregs can suppress their proliferation directly [13] without the presence of antigen presenting cells (APCs). There are evidences for either contact-dependent suppression or suppression via immunosuppressive cytokines or other factors. One efficient way to supppress immune responses is direct killing of CD4+ effector cells, which was described by Grossman et al.,[14]. They have shown that upon CD3/CD46 activation

human nTregs express the serine protease granzyme A and kill T_{CONV} in a perforin-dependent manner without CD95-CD95L interaction.

Nevertheless, the main mechanism of suppression of Tregs consist in influencing the activation status of APCs. In this regard the key molecule is CTLA4, which competitively inhibits the binding of CD28 to its ligands CD80 and CD86 and thus inhibits co-stimulation of effector T cells. CTLA4 together with the adhesion molecule LFA-1 also downregulates the expression of CD80 and CD86 on APCs [15, 16]. Capturing of CD80/86 ligands by CTLA4 is a process called trans-endocytosis [17]. Tregs do not only reduce antigen presenting activity of APCs, but also support an immunosuppressive cytokine milieu by reducing IL-6 while increasing IL-10 production by DCs [18].

Tregs can produce high amounts of membrane-bound and soluble TGF-ß, and blocking TGF-ß partially abrogated suppression of T cell proliferation in vitro using murine or human T cells [19, 20] by inhibiting the TCR/CD3 pathway [21]. An important role in Treg-mediated suppression plays cytokine IL-10, which exerts immunosuppressive effects on various cell types [22]. Another way of suppression offers IL-35, the cytokine involved in Treg-mediated suppression, which was shown to directly inhibit T_{CONV} proliferation [23].

Hydrolysis of extracellular ATP to ADP or AMP by the ectoenzyme CD39, expressed by all murine Tregs and by about 50% of human Tregs, represents another Treg-mediated anti-inflammatory mechanism [24].

Treg cells express high levels of CD25 and consecutively deprive the environment of IL-2, which can also affect the survival of effector T cells.

Human and Mouse Differences

To describe all differences between human and mouse systems is not the topic of this rewiev. Shortly speaking, in mice most of

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CD4^{+}CD25^{+} T cells are potently suppressive, whereas analogous population in humans, albeit larger, contains mainly activated effector cells and only a small percent comprising CD25^{high} cells represents Tregs [10, 25]. Particular differences are described if needed.

Intracellular Markers

FoxP3

The transcription factor FoxP3 is regarded to be a lineage molecule for Tregs. It is neccessary for their thymic development, function and phenotype and is responsible for controlling the expression of a number of genes including suppresive cytokines and Treg surface molecules [8, 10, 26, 27]. However, FoxP3+ T cells are phenotypically and functionally heterogeneous and involve both suppressive and nonsuppressive T cells. Moreover, FoxP3 is an intracellular molecule, detection of which requires fixation and permeabilization of cells and the fixed cells cannot be used in studies of Treg function. Intracellular FoxP3 staining is hardly usable on a daily clinical routine basis in large series of samples (several steps of incubation, washing, long incubation times, use of isotype control).

FoxP3 has been broadly used for characterization of thymus derived naturally occurring Tregs, although it is well known that is also expressed in peripheral induced Treg [28] and even in human activated CD4^{+}CD25^{−} T effector cells without suppressive activity [29-33]. 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. Despite these facts CD4^{+}CD25^{+}FoxP3^{+} is still generally accepted as the most reliable phenotype of Treg cells [8, 9, 34].

Because of the absence of a specific Treg cell marker, it has not been possible to distinguish between Treg cells and conventional activated T cells [35]. Later, epigenetic differences in DNA methylation within FoxP3 have been detected between Treg cells and non-regulatory T cells [36], which offered a promising option for the exact quantification of natural Treg cells. Tatura et al., [37] described the application of QAMA assay (quantitative analysis of methylated alleles), that is based on epigenetic differences within the FoxP3 Treg-specific demethylated region (TSDR) between Treg cells and all other major blood cells.

HELIOS

Helios is an Ikaros family transcription factor which was shown to play an important role in Treg function and which was at first believed to differentiate induced Treg cells from natural Treg cells [38, 39]. The aforesaid studies demonstrated the existence of two subsets of FoxP3^{+} Tregs which express Helios and which can discern nTregs (FoxP3^{+}Helios[+]) from peripherally-induced (FoxP3^{+}Helios[−]) iTregs [39]. But recently Himmel et al., found, that a lack of Helios expression does not exclusively identify human iTregs, and, their data provide the first evidence for the coexistence of Helios[+] and Helios[−] nTregs in human peripheral blood [40]. However, it has been reported very recently that Neuropilin 1 (Nrp1, CD304), highly expressed by most of nTregs, may be used to distinguish these two subsets [41, 42].

RUNX

The role of Runt-related transcription factor (RUNX) family proteins in relation to Treg development and function has been explored during last few years. It was shown that Foxp3 expression in nTregs is dependent on RUNX proteins and correlates with the binding of RUNX to Core-binding factor β (CBF-β). Heterodimers of RUNX and CBF-β have been shown to play an important role in maintenance and development of Tregs in both mice and humans, since they controll Foxp3 expression and also the expression of target genes [43]. Complex of RUNX1 and

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CBF-β seems to be indispensable for the suppressive function of human nTregs [44]. Similar importance has the complex of CBF-β with RUNX1 and RUNX3 for the TGF-β mediated iTreg development and function [45]. RUNX deficient mice produce autoimmune disorders resembling those occurring in Foxp3-mutants, although the symptoms are less severe [44].

Surface Markers

CD25

Lot of studies indicate that CD25 (IL-2R –α) is a principal cell surface marker of Tregs [25, 46], but some of them have showed that only the CD4+ T cell subset expressing the high levels of CD25 (termed CD25high) exerts in vitro suppressive activity and has the greatest regulatory potential [25, 47]. However, conventional/effector T cells [10, 25, 48] and a portion of CCR7+ central memory T lymphocytes start expressing CD25 upon TCR-mediated activation [49]. Therefore, even highly pure CD4+CD25high Treg populations may contain a significant fraction of proinflammatory T effector cells [13].

CD26

CD26 is an extracellular serine protease with dipeptidyl peptidase IV (DPPIV) activity [50]. High expression of CD26 has been traditionally used as an indicator of immune activation and effector functions in T cells. Activated and memory T cells display a CD26high phenotype, and T cytokines like IL-12 raise the number of CD26 molecules on T lymphocytes. High surface levels of this protease indicate T_{H1} effector responses [50, 51].

Recently interesting data has been published by Salgado et al., [52] Their flow cytometry outcomes demonstrated high percentage of CD26 within CD4+CD25⁻ or CD4+FoxP3⁻/low effector T lymphocytes, but negative or low levels (CD26⁻/low) in Treg cells, which were gated as the CD4+CD25high or the CD4+FoxP3high phenotype. While the negative marker CD127 is down modulated in CD4+ T effector lymphocytes after their activation, CD26 molecule within these activated cells is upregulated and becomes a CD4+CD25+/high CD26+ phenotype. The upregulation of CD26 within Treg cells (CD4+CD25high CD26⁻/low) is only slight. The differences between Treg and activated effector T cells regarding CD26 levels seem to be stable, therefore assessment of CD26 molecule, in combination with currently used markers as CD25, FoxP3, CD194 and others may be useful in distinguishing Treg cells from activated T effector cells. This approach could help to quantify or isolate Tregs from samples of patients with autoimmune or inflammatory diseases.

CD39

Two studies highlighted the importance of CD39/NTPDase1 (ecto-nucleoside triphosphate diphosphohydrolase 1) as a useful sign for the identification of Tregs [24, 53]. Mandapathil et al., [54] have further focused on CD39 as a positive selection marker of Tregs. Their paper pointed out that CD39 molecule can be successfully used for routine characterization and isolation of functionally unharmed human T regulatory cells from the peripheral blood of patients or healthy donors. The ectoenzyme NTPDase1 catalyzes the generation of AMP from ATP, which is necessary to produce adenosine, an important mediator of active suppression [24, 55]. Some papers have also been published about the frequencies of CD39+CD4+ T cells of patients infected with HTLV-1 [56] or in HIV patients [57], regarding their correlation to Tregs.

It was found, that 50% to 90% of CD4+CD39+ T lymphocytes are FoxP3+ and express low levels of CD127 [54]. But further studies have shown that CD39 expression could serve as a marker to identify not only suppressive CD4+T cells, but also a CD4+ T-cell subpopulation with immunostimulatory properties [58]. Ndhlovu et al., [59] reported that the CD39 could identify new subset of "inducer" CD4+ T cells that significantly

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increases the proliferation and cytokine production of responder T cells. This unique cell subset produces a distinct repertoire of cytokines in comparison to the other CD4+ T cell subsets. It was proposed that this novel CD4+ T cell population counterbalances the suppressive activity of Tregs in periphery and serves as a calibrator of immunoregulation.

So the CD39 molecule expressed on CD4+ T cells together with CD25 co-expression, is the marker of T cells with distinct effector (CD39+CD25−) or regulatory (CD39+CD25+) function.

Moreover, further studies have shown, that activated T cells upregulate CD39 [60], and a new subset of human CD4+CD39+FoxP3− T cells that produce IFN-gamma and IL-17 has been found [61].

CD45RA

The naïve T cell marker CD45RA and FoxP3 have been identified on the population of CD4+CD25+ T cells from peripheral blood and these cells showed potent suppressive functions [62]. Miyara et al., [27] described CD45RA+FoxP3low T cells as a resting Tregs and Tregs with a CD45RA−FoxP3high phenotype as activated Treg cells. While activated Tregs are terminally differentiated and rapidly die, resting Tregs show a high proliferative capacity and convert into their activated descendants. The subpopulation of CD45RA+ Tregs can be used for cellular therapies, because it retains its suppressive activity also after in vitro expansion [63].

CD49d

Markus Kleinewietfeld et al., [64] have reported that the application of depleting antibodies for CD49d (alpha chain of VLA-4 integrin), a marker of effector T cells producing proinflammatory cytokines, will eliminate conventional T cells from Tregs and in combination with the CD127 antibody can give highly pure population of FoxP3+ Tregs.

CD49d molecule is expressed on most of IFN-gamma or IL-17-producing proinflammatory T cells and is reduced on Tregs, even though later data from the same group [24] as well as other researchers [65] admitted, that some degree of CD49d is expressed also in some subsets of Treg cells.

CD101

CD101 molecule, previously described as V7, is a type I transmembrane glycoprotein, which is expressed on monocytes, granulocytes, dendritic cells and activated T cells [66]. Binding of anti-CD101 monoclonal antibody on CD101 molecule on T cells blocks TCR/CD3 –induced proliferation by inhibiting calcium flux and activation of tyrosine kinase, leading to the suppression of IL-2 transcription.
In the study of Fernandez et al., cell surface expression of CD101 well correlated with functional suppressor activity of CD4+ CD25+ Treg cells in mice [67]. Tregs with high expression of CD101 are supposed to be activated Tregs.

CD127

In 2006 Liu et al., [47] discovered important hallmark for human Treg delineation: the surface molecule CD127, interleukin 7 receptor- α (IL7R- α) chain. They showed that the expression of CD127 inversely correlates with FoxP3 expression and suppressive activity of Tregs. The combination of CD127 with CD25 then allowed the isolation of highly purified Treg population [68].

The comparisons of intracellular FoxP3 staining with CD4+CD25+CD127− phenotype initially reported good correlation. The experiments were made in healthy subjects [68], in septic patients [69], in viremic HIV patients [57, 70] and in patients with systemic lupus erythematosus [71]. Isolated CD4+CD25highCD127− were used several time for clinical applications [72]. CD4+CD25hiCD127− isolated Tregs showed the best reached Treg population regarding

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purity, function, stability and in vitro expansion capacity [73], promising isolation of pure Treg populations with high suppressive functionality [68, 74]. Later on, most studies of Tregs started to use CD127 marker not only to isolate Tregs, but also to directly characterize them in human peripheral blood [75]. Yu et al., [71] found that $CD4^+CD25^+CD127^{low/-}$ T cells expressed the highest level of FoxP3 and had the strongest correlation with $CD4^+CD25^+FoxP3^+$ T cells, the accepted identifying characteristics for "real" nTreg cells. Moreover, functional data showed that $CD4^+CD25^+CD127^{low/-}$ T cells could effectively suppress the proliferation of $CD4^+CD25^-$ T cells, suggesting that these T cells best fit the definition of naturally occurring regulatory T cells in human peripheral blood.

But later outcomes implicated, that the negative correlation with $FoxP3^+$ T cells is not absolute.

$CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{high}FoxP3^+$ were used at once to determine the frequency of Tregs in two different groups of human immunodeficiency virus (HIV) infected persons, one viremic and the other aviremic. As expected, a strong correlation between both Treg phenotypes was observed in the aviremic group, but surprisingly, in the viremic group this correlation was completely absent. These findings on T cell activation levels suggested that the $CD4^+CD25^{high}CD127^{low}$ population corresponds with the elevated numbers of activated non-regulatory T cells in the viremic HIV group, suggesting that CD127 molecule is greatly influenced by mere T cell activation [76]. Very similar outcomes reported Rios et al., [77]. In HIV patients detection of Tregs as $CD4^+CD25^+CD127^{Low/-}$ cells resulted in a significantly lower percentage of cells in comparison with $FoxP3^+$ population.

These results were confirmed by measurement of DNA-methylation (QAMA) in samples of $CD4^+$ T cells isolated from blood of septic patients and healthy donors. Data were compared with measurement of $CD4^+CD25^{high}CD127^{low}$ population by flow cytometry.

In healthy subjects, the results obtained by both methods were clearly positively correlated, while the correlation between both methods in septic patients was only poor [37]. This finding revealed that quantification of Treg cells by QAMA detects $CD4^+$ T cells with unmethylated FoxP3–TSDR, hidden in the $CD25^{med/low}$ fraction of flow cytometry.

So that although CD127 was for a long period an efficient tool to determine the phenotype and functional activity of Treg [47, 78], there were increasing controversy in comparisons with $CD4^+CD25^+FoxP3^+$ cells, particularly in the context of chronic infections [77]. It became obvious, that the mere existence of an underlying disease (e.g., HIV infection) [76] or the in vitro activation [79] cause an intense CD127 down modulation on formerly $CD127^+$ T effector cells.

FR4

The folate receptor (FR), is also known as the folic acid binding protein. There are four isoforms of the human FR: FR-α,-ß,-γ and–δ. They are expressed on epithelial cells, macrophages and malignant cells. Since FRs are overexpressed on approximately 40% of human cancers, folate conjugates have been used to deliver attached imaging and therapeutic agents selectively to malignant cells. Recently, FR- δ has been described also on human Tregs [80].
In mice has been described highly homologous molecule to human FR-δ, called FR4 [81].
Studies in mice determined that FR4 is expressed at particularly high amounts in nTregs and plays an important role in the maintenance of the Treg phenotype [82].

Other Surface Molecules

Besides CD25, FoxP3 and above mentioned molecules, Treg cells constitutively express

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surface proteins like CD45RO, cytotoxic T-lymphocyte-associated protein (CTLA-4) [83, 84], activation marker HLA-DR, or glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR) [10, 25], CD45RB, CD62L [85], neuropilin 1 (NRP1, CD304), CD103, CD195 (CCR5), ICOS [57] or lymphocyte activation gene LAG-3, that show enriched expression in Treg cell populations [86]. Unfortunately, all these markers exhibit lack of specifity, because they are neither present in 100% of Tregs or exclusive of this cell lineage and this phenotype is also shared by memory T_H cells or activated Th cells. Tregs also epress high amount of chemokine receptor CCR4 (CD194), which is the reason why they can migrate to several different types of chemotactic ligand CCL17- and CCL22-secreting tumors to facilitate tumor cell evasion from immune surveillance [87].

Conclusion

Finding a specific cell surface marker of Treg cells which would allow their identification and isolation was the topic of a past decade. Presumably no other Treg-specific positive surface marker shall be identified in the future, but revealing the molecule CD26 as another negative marker in combination with other listed molecules could slowly amend our possibilities in distinguishing this important population from effector T cells and improve our diagnostic possibilities.

Table 1: The most important surface molecules on Tregs and their role.

Molecule	Alternative name	Function/expression on Tregs	References
CD25	IL-2 receptor	Constitutively expressed on all Tregs, but also on T_{CONV} upon activation	[44, 45]
CD26	DPP IV ectoenzyme	Activated and memory T cells display a CD26high phenotype. High surface levels of this protease indicate T_{H1} effector response, while Tregs display negative or low levels of CD26	[50, 51, 79]
CD31	PECAM-1, platelet endothelial cell adhesion molecule	CD31 positive Tregs are resting Tregs which recently migrates from thymus	[88]
CD39	Ecto-nucleoside triphosphate diphosphohydrolase 1	50% to 90% of CD4+CD39+ T lymphocytes are FoxP3+, but CD39 is also expressed on effector T cells upon activation. Also a new subset of human CD4+CD39+FoxP3– T cells that produce IFN-gamma and IL-17 has been found	[23, 49, 53, 59, 60]
CD45RA		Tregs expressing CD45RA are resting /naive Tregs, while CD45RA negative are called effector or activated Tregs	[10, 27]
CD45RO		CD45RO+ Tregs are so called memory Tregs, and exert enhanced suppressive effects on T cell proliferation and cytokine production	[89]
CD49d	VLA4α, Integrin α4	Marker of effector T cells producing proinflammatory cytokines, has been used for negative selection of Tregs, but in some degree expressed also on Tregs	[23, 63, 64]

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Molecule	Alternative name	Function/expression on Tregs	References
CD62L	L-selectin, LECAM1	Present on all T cells, B cells and NK cells, does not contribute to Treg selection	[84]
CD101	IGSF2, V7	Cell surface expression of CD101 correlates with functional suppressor activity of Tregs. Tregs with high expression of CD101 are supposed to be activated Tregs	[66, 67]
CD103	HML-1, integrin α E	Distinguishes subset of highly suppressive Tregs which migrates to the sites of acute inflammation	[90]
CD127	IL7 Rα	The expression of CD127 inversely correlates with FoxP3 expression and suppressive activity of Tregs and the combination of negative marker CD127 with CD25 allowed the isolation of highly purified Treg population. However, underlying disease or the in vitro activation cause an intense CD127 down modulation on formerly CD127^{+} T effector cells	[46, 50-52, 72, 83]
CD152	CTLA-4, Cytotoxic T-Lymphocyte Antigen 4	CTLA-4 is inhibitory molecule present on the surface of T_H cells and it is a ligand for CD28 molecule on APCs. CTLA4 on Tregs competitively inhibits the binding of CD28 to its ligands CD80 and CD86 and thus inhibits co-stimulation of effector T cells	[65, 68]
CD194	CCR4	Chemotactic ligand facilitating the migration to tumors, expressed on T cells and macrophages	[71]
CD195	CCR5	CCR5 is a chemotactic mediator for inflammatory cells as well as Tregs. It also tightly regulates the balance between Treg and Th17 cells in the site of imflammation	[91-93]
CD223	LAG-3, Lymphocyte activation gene 3	Lymphocyte activation gene-3 (LAG-3) is a CD4-related transmembrane protein expressed by Tregs that binds MHC II on APCs. It was shown that during Treg:DC interactions, LAG-3 engagement with MHC class II inhibits DC activation	[70, 83]
CD304	Neuropilin 1	Highly expressed by most of nTregs, but not on iTregs	[41, 42]
FR4	Folate receptor 4	Expressed at particularly high amounts in nTregs	[82]

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