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Resolving the identity myth: key markers of functional CD4⁺FoxP3⁺ regulatory T cells

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Abstract

Authenticating markers for the functional suppressive CD4⁺FoxP3⁺ regulatory T cells (Tregs) are important for the quantitative identification and enrichment of viable Tregs for possible therapeutic use. CD25 as a surrogate marker of Tregs has some limitations, which prompted investigators to identify more specific marker(s) of Tregs. The search for a firm molecular definition of Tregs resulted in the identification of FoxP3 as a better marker of this subset of CD4 cells. Nevertheless, FoxP3⁺ Tregs are phenotypically and functionally heterogeneous. Even in normal mice, only a minority of FoxP3⁺ T cells are potent suppressor cells. Therefore, additional marker(s) is required for delineation of truly functional Tregs. In this review, the studies identifying markers of functional Tregs, both in mouse and in human, and their functional implications are discussed. Our finding that TNFR2, which mediates the effect of TNF on the activation of Tregs, is a superb marker of the most suppressive subset of mouse Tregs and its application in the identification of functional human Tregs will also be reviewed.

Introduction

There is compelling evidence that CD4⁺FoxP3⁺ regulatory T cells (Tregs) play an indispensable role in the maintenance of immune homeostasis and prevention of autoimmunity, however, they also dampen host immune responses against pathogens and represent a major cellular mechanism by which tumors evade immunosurveillance [1–2]. As central regulator of immune responses, Tregs are considered to be important therapeutic target. Pharmacological manipulation of number and/or immunosuppressive function of Tregs has potential to be useful in the treatment of a number of major diseases, such as autoimmune disorders, allograft rejection, graft-versus-host disease and cancer [1–2].

Reliable surface markers are a prerequisite for the quantitative identification and enrichment of viable Tregs. Utilization of CD25 to define Treg, especially in human, is problematic, because this receptor is also expressed by activated effector T cells (Teffs). In addition,

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Tregs in both humans and mice are phenotypically and functionally heterogeneous. This prompted the investigators to search for more specific Treg markers, as well as markers which can identify functional subset of Tregs. It is clear now that Tregs express a panel of co-stimulatory/co-inhibitory molecules (CD28, CTLA-4, ICOS and PD-1 etc.), TNF receptor super family (TNFRSF) members (TNFR2, GITR, OX40, 4-1BB and FAS, etc.), chemokine receptors (CCR2, -4, -5, -6, -7 and -8, CXCR3 and -4, etc.) and TLRs (such as TLR1, -2, -4, -5, -6, -7, -8 and -9), as previously reviewed [3–6]. We will therefore focus on a number of molecules crucial for the delineation of functionality of Tregs. In an effort to unveil the mechanism underlying the activation of Tregs in the inflammatory environment, our laboratory unexpectedly found that TNF preferentially activates Tregs through TNFR2 [7]. Furthermore, the expression of TNFR2 actually identifies the maximally suppressive subset of mouse and human Tregs [8–9], including CD4⁺ as well as CD8⁺ Tregs [10–11].

Besides FoxP3⁺ Tregs, there are other types of Tregs that can be induced from naïve CD4 cells in the periphery during immune responses, such as IL-10-producing Tr1 cells [12] and TGFβ-producing Th3 cells [13]. In this review, our discussion will focus on the subset of naturally occurring, thymic-derived FoxP3⁺ Tregs.

CD25

Sakaguchi initially reported that transfer of purified CD4⁺CD25⁺ cells inhibited naïve CD4 cell-mediated autoimmunity in lymphopenic mice, and the lack of CD4⁺CD25⁺ cells was also responsible for the autoimmunity developed in neonatally thymectomized mice [14]. Although CD45RB^{low} was also identified as a marker of CD4 Tregs [15], only the CD25-expressing CD45RB^{low} cells were suppressive [16]. The in vitro model system that mimics the function of Tregs in vivo, established by Thornton/Shevach [17], greatly facilitated the recognition of CD25 as a relevant surface marker of Tregs. Up-regulation of CD25 expression is associated with enhanced function of Tregs upon TNF stimulation [7], suggesting high levels of CD25 expression may be an indicator of more suppressive Tregs in mice. Mice lacking both CD25 (and CD122) and IL-2 are defective in Tregs [18], suggestive of a functional role for CD25 and requirement for IL-2 by Tregs. Intriguingly, Tregs have no capacity to make IL-2 and they rely on IL-2-secreting T effector cells [19]. Thus, by expressing high levels of CD25, Tregs can deprive T effector cells of IL-2 and impair the proliferation and survival of T effector cells [20]. However, effector and memory T cells, when activated, also express CD25 [17]. Furthermore, a regulatory function has been demonstrated in CD4 subsets negative for CD25 [21–23]. Even in unmanipulated normal mice, as revealed by our study, a subset of highly suppressive TNFR2⁺FoxP3⁺ Tregs lacks CD25 expression [8]. Such CD25[−]FoxP3⁺ Tregs are increased in tumors [24], inflamed intestine [25] and aged mice [26]. Study of FoxP3 Tg mice and Scurfy mice also showed dissociation of CD25 and FoxP3 expression [27]. Thus, even in mice, the use of CD25 as a surrogate marker of functional Tregs yields false positives and false negatives.

In human, co-expression of CD4 and CD25 is reportedly also able to define functional Tregs present in the PBMCs [28]. However, up to 30% of PB CD4⁺ T cells express CD25 [28], which likely include activated T effector cells. Actually, only 1–2% of cells with highest CD25 expression are enriched in human Tregs with consistent suppressive function [29–30] and high levels of FoxP3 expression [31–32], akin to the mouse CD4⁺CD25⁺ Tregs [29]. However, at least 50% of total FoxP3⁺ human Tregs reside in CD25^{low} and even some of the CD25[−] population is FoxP3-expressing. Thus, identification of Tregs based on CD25^{high} will miss majority of human CD4⁺FoxP3⁺ Tregs [9]. In addition, there is no firm criterion to define CD25^{high}. It is also problematic that activated human CD4⁺ T effector cells can express high levels of CD25 [33]. Combination of CD25^{+/high} with other markers therefore has been proposed as a means of identifying functional human Tregs.

FoxP3

The search for a firm molecular definition of Treg lineage resulted in the identification of Foxp3 [27, 34–35]. Foxp3 is generally considered as a master regulator of biological processes of Tregs, including lineage commitment and developmental differentiation. This conclusion is favored by the study in Scurfy mice, a strain with X-linked autoimmune disease and dysregulated T cell function caused by disabling mutation of FoxP3 [36]. However, instead of being a monomorphic lineage rigorously controlled by FoxP3, even in unmanipulated normal mice, FoxP3⁺ cells are phenotypically and functionally heterogeneous, and more than half of FoxP3⁺ cells have minimal or no suppressive ability [8, 24].

In human, the crucial role of FoxP3 in Tregs is demonstrated by cells from patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome due to mutations in the foxp3 gene [37]. However, FoxP3 expression can also be induced by TCR stimulation of human Teffs which do not have suppressive activity [38]. In addition, proinflammatory cytokine producing, non-regulatory FoxP3^{low}CD4⁺ Teffs are present in the PB of normal humans [31]. Thus, expression of FoxP3 is less reliable in defining human Tregs than mouse Tregs. Suppressive Tregs display a demethylated Foxp3 promoter, which is only partially demethylated in non-suppressive FoxP3⁺ T cells [39]. Sustained high FoxP3 expression by activated T cells can confer regulatory capacity [40–41], while loss of FoxP3 expression over time in long-term culture decreases their suppressive ability [42]. Thus, FoxP3 expression is necessary, but not sufficient, to identify functional Tregs.

CTLA-4

CTLA-4 (CD152) down-regulates T cell activation by competing with CD28 for B7 binding. CTLA-4 is constitutively expressed by approximately ~40% of mouse Tregs [16, 43], and also rapidly induced in Teffs upon activation [43]. This molecule is reported to be critically required for the function of Tregs in vivo [44]. However, Tregs from CTLA-4-deficient mice are still functional [45]. The expression levels of CTLA-4 should be able to indicate functional status of Tregs. Indeed, mouse Tregs with maximally suppressive capacity express markedly higher levels of CTLA-4, as compared with inactive CTLA-4^{low} Tregs [8]. Human Tregs also express high levels of CTLA-4 [46] and human PB CD4⁺CD25⁺ Tregs that exhibit consistent suppressive capacity harbor the highest CTLA-4-expressing cells, as compared with other subsets [9]. However, FACS detection of CTLA-4 requires intracellular staining, which precludes its use as a surface marker of Tregs.

HLA-DR

HLA-DR is expressed by approximately one third of effector Tregs in adult human PB. HLA-DR⁺ Tregs express higher levels of FoxP3 and are responsible for contact-dependent in vitro suppression, while the suppression by HLA-DR⁻ Tregs is initially mediated by IL-4 and IL-10 and later by a contact-dependent mechanism [47]. Activation and expansion of HLA-DR⁻ Treg cells in vitro leads to the generation of HLA-DR⁺ Treg cells [47–48]. Both ex vivo-isolated and in vitro-generated HLA-DR⁺ Treg cells suppress more effectively than HLA-DR⁻ Treg cells [47]. Thus, HLA-DR defines a terminally differentiated subset of functional Tregs. HLA-DR is expressed only on human Tregs, not on mouse Tregs [49].

CD103

10–30% of mouse peripheral Tregs express CD103 [50–52]. In contrast to CD25, the expression of CD103 is not induced upon activation, and therefore is not an indicator of activation status of Tregs [51]. However, the in vivo inflammatory environment appears to

up-regulate CD103 expression on Tregs [50, 53]. Although exhibiting an activated phenotype [52, 54], in vitro suppressive capacity of CD103⁺ Tregs is marginally higher than that of CD103⁻ Tregs [52, 54]. CD103⁺ Tregs express higher levels of IL-10 than CD103⁻ Tregs, and induce IL-10 production from co-cultured T_H17 cells [51–52]. However, their suppressive function can not be abrogated by neutralization of IL-10 [51], and CD103⁺ Tregs from IL-10 KO mice are also suppressive [52]. Therefore, CD103⁺ Tregs are not Tr1 cells. The role of CD103 is the retention of T cells within epithelial compartments [55]. Thus, the ability of CD103⁺ Tregs to traffic to certain inflamed sites, especially in the skin and mucosal membrane, may explain their superb in vivo anti-inflammatory activity at these sites [56].

Human CD25^{high}FoxP3⁺ Tregs are undetectable or express extremely low levels of CD103 (<5%) in various tissues, including blood [57–60]. Nevertheless, human CD4 T cells can be induced by different stimuli in vitro to express CD103 [61–62] and the percentage of CD103⁺ cells among CD4⁺ CD25^{high} T cells is significantly higher than among CD4⁺CD25⁻ T cells in patients with multiple sclerosis [60]. Further study is needed to determine whether CD103 is up-regulated on human Tregs in other inflammatory conditions.

CCR6

In the periphery, CCR6 is preferentially expressed by 15~32% of mouse Tregs. CCR6⁺ Tregs are generated in vivo from CCR6⁻ Tregs after encounter with antigen, and CCR6⁺ Tregs exhibit an effector-memory phenotype, although they possess equal suppressive ability as CCR6⁻ Tregs [63]. They respond rapidly to re-stimulation in vitro with up-regulation of IL-10. CCL20, the chemokine ligand for CCR6, can induce robust chemotactic response by CCR6⁺ Tregs [63]. In the mouse experimental autoimmune encephalomyelitis (EAE) model, CCR6⁺ Treg cells are enriched in the PB and accumulate in the central nervous system [63]. Lack of CCR6 reduces the recruitment of Tregs, as well as of pathogenic Th17 cells which also express CCR6, to the inflamed tissues in EAE model [64]. Thus, CCR6⁺ effector/memory-like Tregs appear to function as a natural counterbalance to effector-memory T_H17 cells, destined to control potential destructive immune responses directly at inflamed sites [63]. This notion is supported by the observation that CCR6 expression on Treg cells was required for the fully effective Treg-mediated suppression in the T cell-transfer model of colitis [65].

In normal human PBMCs, 30~80% of the CD25^{high} T cells and 20~70% of the CD25^{-/low} population express CCR6 [63]. CCR6 expression is strictly correlated with CD45RO. Thus, CCR6 expression defines a subset of memory Tregs. Purified human CCR6⁺ Tregs are actively suppressive [63]. A subset of CCR6-expressing memory CD4⁺FoxP3⁺ Tregs express ROR γ t and produce IL-17 upon activation or in the presence of inflammatory stimulation, while preserving suppressive capacity [66–68], suggesting they may play additional proinflammatory functions.

CD127

Mouse Treg are generally characterized by low CD127 (IL-7R α) expression when compared with T_H17 cells [69]. However, the expression levels of CD127 on mouse Tregs vary, depending on their localization and activation status. ICOS⁺ and CD103-expressing Tregs express higher levels of CD127. Mouse Tregs significantly up-regulate their CD127 expression during in vitro and in vivo activation, and Tregs in bone marrow and skin also express high levels of CD127 [70].

Absence of CD127 together with CD25^{+/high} has been widely used for the past 5 years to define functional human Tregs [32–33]. It has been shown that CD4⁺CD25⁺CD127[−] identify human FoxP3⁺ Tregs even in patients with ongoing inflammatory diseases [32, 71]. However, only about 40% of the CD127^{low} population is Foxp3⁺ [72] and non-suppressive FoxP3^{low}CD45RO⁺ T cells present in the normal humans also express low levels of CD127 [31]. Expression of CD127 is also downregulated early in the course of activation of Tregs [73]. Thus, additional marker(s) have to be used together with CD127^{−/low} and CD25^{high} to define human Tregs, such as CD62L⁺ which may differentiate Tregs from recently activated Tregs [74]. Furthermore, low expression of CD127 is not an intrinsic attribute of Tregs, since it is known that Treg are able to respond to IL-7 [75].

CD45RO/CD45RA

CD45RO and CD45RA are exclusively expressed by distinct subsets of human CD4 cells, and can be used to divide human CD4⁺FoxP3⁺ T cells into three phenotypically and functionally distinct subpopulations: CD45RA⁺CD25⁺FoxP3^{low} resting Treg cells and CD45RO⁺CD25^{high}FoxP3^{high} activated Treg cells, both of which were suppressive in vitro, and proinflammatory cytokine-producing CD45RO⁺CD25⁺FoxP3^{low} nonsuppressive Tregs [31]. Human CD4⁺ FoxP3⁺ T cells with a naive phenotype (expressing CD45RA) nevertheless also possess moderate suppressive function [31], and are present in peripheral blood and prevalent in cord blood [76–78]. The CD45RO⁺CD25^{high}FOXP3^{high} cells are activated and functionally differentiated subset of Tregs. They are highly proliferative in vitro and in vivo, have more potent suppressive activity and are derived mainly from CD45RA⁺CD25⁺FOXP3^{low} naive Tregs [31].

ICOS

Co-stimulatory receptor ICOS is expressed by the majority of mouse Tregs, and FoxP3^{high}CD44^{high} Tregs express higher level of ICOS [79]. In a model of contact hypersensitivity, a subset of FoxP3⁺ Tregs expressing high levels of ICOS possesses superior suppressive activity. Distinct from other subset FoxP3⁺ Tregs, ICOS⁺ Tregs in this model also produce IL-10, IL-17 and IFN γ [80]. In other models, Tregs highly expressing ICOS have the capacity to express IL-10 [81–82], therefore can be considered as Tr1-like cells.

Human FoxP3⁺ Tregs present in the thymus and periphery can be divided into two functionally distinct subsets: ICOS⁺ Tregs and ICOS[−] Tregs. Whereas the ICOS⁺ Tregs use IL-10 to suppress DC function and TGF β to suppress T cell function, the ICOS[−] Tregs used TGF β only. Almost all ICOS⁺ Tregs exhibit CD45RO⁺ memory phenotype, while ICOS[−] Tregs contained both CD45RO⁺ and CD45RO[−] cells, suggesting they were more related to naive Tregs in phenotype [83].

LAG-3

LAG-3 (lymphocyte-activation gene 3), a ligand of MHC II, can be induced on activated T cells and negatively regulates T cell homeostasis by Treg-dependent and independent mechanisms [84]. Mouse Tregs selectively express high levels of the LAG-3 gene, however, surface expression of LAG-3 protein on Tregs can only be detected on ex vivo TCR-activated Tregs, not on freshly isolated Tregs [85]. Interestingly, LAG-3 is constitutively expressed on IL-10-producing CD4⁺CD25[−]FoxP3[−] cells [86] and may therefore be used as a surface marker of mouse Tr1 cells.

In normal human PBMCs, about 3% of CD4⁺CD25^{high} or CD4⁺FoxP3⁺ Tregs are LAG-3⁺ cells. The proportion of LAG-3⁺ Tregs expand to 8~10% in PBMC, LNs and TILs (tumor

infiltrating lymphocytes) from cancer patients. LAG-3⁺ Tregs exhibit an effector-memory phenotype and a more potent inhibitory potential. Although LAG-3⁺ Tregs present in tumor patients express IL-10 and TGF β , they differ from classic Tr1 or Th3 cells, because their inhibitory activity is cell contact-dependent and IL-10- and TGF β -independent [87]. Thus, LAG-3 can identify a subset of activated and terminally differentiated, tumor-associated human Tregs. However, in tumor patients, LAG-3 is also up-regulated on FoxP3⁻ cells [88] and presumably these LAG-3⁺FoxP3⁻ cells exert their suppressive function by a different mechanism(s) than classic FoxP3⁺ Tregs.

GARP

The gene encoding GARP (glycoprotein A repetitions predominant, also known as leucine-rich-repeat-containing protein 32 or LRRC32), a cell surface molecule, is expressed 100 fold more by activated human Tregs as compared with Teffs [89–90]. Upon activation, GARP is expressed on a subset of CD25⁺ (or FoxP3⁺) Tregs which have more potent suppressive capacity as compared with GARP⁻CD25⁺ cells which contain IL-17-producing cells. Expression of GARP is associated with FoxP3 expression and suppressive function of Tregs [89, 91]. Remarkably, GARP is selectively up-regulated on activated Tregs, but is not induced on activated Teffs or TGF β -induced FoxP3⁺ cells without suppressive function [91]. In HIV patients, although the percentage of FoxP3⁺ cells was increased, the proportion of TCR activation-induced GARP⁺ cells in FoxP3⁺ subset is the same as in healthy donors (~20%) [91], suggesting this molecule is useful as a functional Treg marker even in an ongoing inflammatory response. GARP is critical for tethering TGF β to the cell surface and thus the activated Tregs co-express latent TGF β and GARP on their cell membranes [92–93]. GARP is not detectable on freshly isolated Tregs and the expression of GARP on TCR-activated Tregs is not stable [91, 93], more information is needed to be able to exploit this molecule as marker for functional human Tregs.

LAP/CD121a/CD121b

Latency-associated peptide (LAP) and IL-1 receptor type I (CD121a) and II (CD121b) are not expressed constitutively on resting or expanded FoxP3⁺ Tregs, but are rapidly induced and expressed only on FoxP3⁺ Tregs for a short period after TCR-mediated activation. These three molecules are selectively expressed on activated Tregs with fully suppressive capacity, but not expressed on either activated CD4⁺FoxP3⁻ Teffs, or by proinflammatory cytokine-producing FoxP3⁺ non-suppressive cells and TGF β -induced FoxP3⁺ non-suppressive cells [94]. Furthermore, TCR stimulation-induced expression of LAP, CD121a and CD121b also define potent suppressive Tregs from patients with inflammatory responses [94]. CD121a is a decoy receptor for IL-1 and LAP expression on the activated Tregs represents the latent TGF β complex [95]. Thus, these molecules may also contribute to Treg functions. The interaction of IL-1 with CD121a is critical for the early programming of Th17 cells [96]. Whether CD121a⁺ Tregs actually can be reprogrammed into Th17 cells by IL-1 needs further study.

CD39

In PBMCs from normal humans, CD39 is expressed almost exclusively by ~60% of Foxp3⁺ cells. There is a linear correlation of expression of CD39 and FoxP3 [97]. The mechanism of suppression by CD39 (nucleoside triphosphate diphosphohydrolase-1 [NTPDase 1]) is based on inactivation of extracellular proinflammatory ATP. CD39⁺ Tregs co-express CD45RO and CCR6, thus they represent a subset of activated effector/memory-like Tregs [97]. Although both CD4⁺CD25^{high}CD39⁺ and CD4⁺CD25^{high}CD39⁻ T cells suppress proliferation and IFN γ production by responder Teffs, only CD4⁺CD25^{high}CD39⁺ cells suppress IL-17 production [98]. Another study shows that CD4⁺CD25⁺CD39⁺ T cells

expressed an activated Treg molecular signature, while lack of CD39 on CD4⁺CD25⁺ T cells identifies cells with Th17 characteristics [99]. Patients with relapsing-remitting multiple sclerosis (MS) or with renal allograft rejection have strikingly reduced numbers and suppressive activity of CD39⁺ Tregs in the blood, but no differences in CD39⁻ Treg levels [97–99]. Thus, CD39 expression may also be used as a marker of functional Tregs in patients with inflammatory responses.

CD49d

CD49d, the α -chain of the integrin VLA-4 ($\alpha 4\beta 1$), is expressed on the majority of proinflammatory PBMCs but is absent on Tregs [100]. Therefore, exclusion of CD49d⁺ cells can be used to remove the contaminating IFN γ and IL-17 producing CD25⁺ Teffs from CD25⁺ Tregs. The purity of Tregs can be further enriched by removal of CD49d⁺/CD127⁺ cells. The resultant CD4⁺CD25^{high}CD49d⁻CD127⁻ cells yield Foxp3⁺ Tregs virtually free of contaminating CD25⁺ Teffs [100]. Thus, the absence of CD49d may be used as an additional surface marker to identify functional human Foxp3⁺ Tregs.

TNFR2

Our studies show that the classical proinflammatory cytokine TNF exerts a negative feedback activity on inflammatory responses by activating and expanding Tregs through TNFR2 [7, 101]. TNFR2 is preferentially expressed by 30~40% of Tregs present in the peripheral lymphoid tissues of normal mice, while fewer than 10% of Teffs express TNFR2 at lower levels on a per cell basis [8, 24]. TNFR2-expressing CD25⁺ cells or FoxP3⁺ cells exhibited the most potent suppressive activity, while TNFR2⁻ Tregs even if they are CD25⁺ and FoxP3⁺ in normal C57BL/6 mice had only minimal or no suppressive ability [8, 24]. The majority of tumor infiltrating Tregs are highly suppressive TNFR2⁺ cells [8], and depletion of TNFR2⁺ Tregs results in tumor eradication after cyclophosphamide treatment [102]. TNFR2⁺ Tregs are highly proliferative in vivo upon inflammatory stimulation ([24] and our unpublished data) and Tregs deficient in TNFR2 are not able to control inflammatory responses in vivo [103]. Remarkably, TNFR2 identifies more mouse peripheral Tregs (30~40%) than CD103 (10~30%), and TNFR2⁺ Tregs are markedly more suppressive than CD103⁺ Tregs [8]. Further, TNFR2 can define highly suppressive FoxP3⁺ Tregs present in the CD25⁻ subset, consequently, flow-sorted CD4⁺TNFR2⁺ cells are up to ~4-fold more suppressive than flow-sorted CD4⁺CD25⁺ cells [8]. Thus, TNFR2 expression is a superb marker for defining highly functional mouse Tregs.

In human, TNFR2 is also constitutively expressed on thymic Tregs, but not on thymic Teffs [104]. Human circulating FoxP3⁺ cells present in CD25^{high}, CD25^{low} and even CD25⁻ subsets of CD4⁺ cells expressed markedly higher levels of TNFR2 (~70%), as compared with CD4⁺FoxP3⁻ Teffs (~20%) [9, 103, 105]. TNFR2 is also expressed on antigen-specific CD4 Tregs induced by tolerogenic DCs [11] and CD8⁺ Tregs generated by anti-CD3 treatment [10]. In combination with other markers, expression of TNFR2 identifies suppressive human Tregs even in the CD25^{low} or CD25⁻ sub-population. We have shown that TNFR2-expressing CD4⁺CD25⁺ T cells include all of the FoxP3⁺ cells present in the CD4⁺CD25^{high} subset as well as a substantial proportion of the FoxP3⁺ cells present in the CD4⁺CD25^{low} subset. Flow-sorted CD4⁺CD25⁺TNFR2⁺ Tregs express high levels of FoxP3 and have potent suppressive activity [9]. This method may be further improved by combination with other markers, such as CD127^{-/low} or CD62L⁺. Interestingly, even in the CD25⁻ fraction, TNFR2⁺CD127^{-/low} cells comprise some FoxP3⁺ cells (our unpublished data), which may be identifiable by additional marker(s). Naive FoxP3⁺ Tregs, as shown by CD45RA⁺ or CD45RO⁻, also expressed higher levels of TNFR2, as compared with naïve Teffs (our unpublished data). Thus, TNFR2 is not only helpful in the identification of

activated/memory subset of Tregs [8], it may also be used to identify naïve suppressive Tregs.

Only a minority of resting mouse peripheral Tregs (30~40%) express TNFR2 which can be used as marker of maximally suppressive Tregs [8], while the majority of normal human PB Tregs (~70%) express this molecule [9]. Nevertheless, in an ongoing inflammatory condition, up-regulation of TNFR2 expression on human Tregs also can serve as an indicator of more suppressive Tregs, as shown in malaria patients [106]. TNFR2 expression on human Tregs present in the synovial fluid of rheumatoid arthritis (RA) patients is also up-regulated [105], presumably reflecting their enhanced suppressive capacity [107]. TNFR2-expressing Tregs in mouse and human demonstrate memory/effector phenotype. Strikingly, they express highest levels of CTLA-4, a molecule reported to play a critical role in Treg activity [44], among CD4 subsets.

The functional implications of TNFR2 in human Treg activity also are becoming evident. For example, both activated human and mouse Tregs produce high levels of soluble TNFR2 which contribute to their immunosuppressive activity [103]. TNF-TNFR2 interaction promotes the survival of Tregs in cancer and at inflammatory sites by inducing thioredoxin-1, a major antioxidative molecule, on Tregs in a NF κ B-dependent manner [108]. Furthermore, as in mice, TNF-TNFR2 interaction increases FoxP3 expression on human Tregs (our unpublished data).

Several other TNFRSF members, such as GITR [54], 4-1BB and OX40 [4], are also preferentially expressed by resting or activated Tregs. Although GITR was initially recognized as a marker of Tregs [4, 54], Tregs also express this molecule even in the resting state and GITR expression on Treg subsets is not directly correlated with their suppressive function [8]. Expression of 4-1BB and OX40 is preferentially up-regulated on highly suppressive Tregs, such as IL-2/TNF-activated Tregs or Tregs present in the tumor environment [109]. Thus these two molecules may be used as markers of activated Tregs.

Closing remarks

A number of molecules or their combinations have been shown to be able to identify functional suppressive Tregs in mouse or humans, as summarized in Table 1 and 2. These molecules, although diverse in nature, collectively have provided useful tools in the delineation of functional Tregs, and advance our understanding of biological processes and suppressive mechanisms of this important subset of CD4 cells. Most of these “Treg markers” are also expressed by activated Tregs, nevertheless, in combination with other molecules, they can still serve as markers of activated Tregs with highly suppressive activity. Identification of a more specific Treg cell marker(s), especially if expressed on the surface of functional Tregs, is still needed.

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highlights

1. FoxP3⁺ Tregs are phenotypically and functionally heterogeneous
2. Additional markers are needed to identify highly potent functional Tregs
3. A number of molecules or their combinations have provided useful tools in the delineation of functional Tregs, and advance our understanding of biological processes and suppressive mechanisms of this important subset of CD4 cells
4. Identification of a more specific Treg cell marker(s), especially if expressed on the surface of functional Tregs, is still needed

Table 1

Markers of mouse functional Tregs

Marker	Function/comments	Ref.
CD25 ⁺	CD4 ⁺ CD25 ⁺ T cells in mouse are generally FoxP3 ⁺ Tregs which are heterogenous and contain both highly and minimally suppressive subsets. However, CD25 can be expressed on activated Teffs and memory T cells, and CD25 ⁻ T cells can be potent suppressive FoxP3 ⁺ Tregs	[14, 17]
FoxP3 ⁺	Master regulator and to date the best intracellular marker of Tregs. FoxP3 ⁺ Tregs are phenotypically and functionally heterogenous, including both highly suppressive and minimally suppressive Tregs	[27, 34–35]
CTLA4 ⁺	Constitutively and preferentially expressed intracellularly by Tregs. Expression levels may indicate the suppressive potency of Tregs	[16, 43–45]
CD103 ⁺	Memory/effector Tregs with potential to produce IL-10. Marker of skin- and mucosal-homing Tregs	[51–52]
CCR6 ⁺	Identifies effector-memory Tregs with potential to produce IL-10	[63–65]
CD127 ^{-/low}	Mouse Tregs express relative lower levels of CD127 (IL-7Ra) than Teffs. However, ICOS ⁺ and CD103 ⁺ Tregs express high level of CD127; its expression can be up-regulated on Tregs upon in vitro and in vivo activation	[69–70]
ICOS ⁺	Expressed on most of Tregs in normal mice. High expression identifies IL-10 producing Tregs or Ag-specific Tregs which share features of Tr1/Th1/Th17 cells	[79–82]
LAG-3 ⁺	Expressed on TCR-activated Tregs and contributes to Treg function. Also expressed on activated Teffs	[85]
CD39 ⁺	Constitutively expressed on majority of mouse Tregs. Moderately up-regulated upon in vitro TCR stimulation, but markedly enhance suppressive activity of Tregs on APT-mediated maturation of DCs.	[97]
TNFR2 ⁺	Predominantly expressed by 30~40% of highly suppressive peripheral Tregs and ~80% thymic Tregs. Transduce activating effect of TNF on Tregs and shed by Tregs as suppressive molecule	[7–8, 24] [103]

Table 2**Markers of human functional Tregs**

Marker	Function/comments	Ref.
CD25 ^{high}	Top 1~2% of brightest CD25-expressing, FoxP3 ^{high} and CD45RO ⁺ CD4 cells are highly suppressive Tregs	[29–30]
FoxP3 ⁺	FoxP3 expression is necessary but not sufficient to identify human Tregs. Tregs can express FoxP3 upon activation, especially in the presence of TGFβ. Functional suppressive FoxP3 ⁺ Tregs can be identified by using other markers, e.g.: CD45RO ⁺ FoxP3 ^{high}	[37–38]
CTLA-4 ⁺	Constitutively and preferentially expressed intracellularly by Tregs. Expression levels correlate with the suppressive potency of Tregs	[46] and others
CD103 ⁺	Expressed very low levels on human Tregs, which may be up-regulated in inflammatory responses or by TGFβ	[59–62]
HLA-DR ⁺	Identify mature, terminally differentiated, high FoxP3-expressing Tregs responsible for contact dependent inhibition.	[30, 47]
CCR6 ⁺	Identifies memory Tregs and IL-17-producing suppressive Tregs	[63, 66–68]
CD127 ^{~low}	Human Tregs are characterized by low CD127 expression. CD4 ⁺ , CD25 ⁺ and CD127 [~] are generally used to define human Tregs. This combination may be further improved by additional marker, such as CD62L ⁺ . Tregs also down-regulate their CD127 expression upon activation	[32–33, 71]
CD45RO ⁺ (or CD45RA [~])	CD45RO ⁺ CD25 ^{high} FoxP3 ⁺ identify effector Tregs with highly potent suppressive capacity; CD45RA ⁺ CD25 ⁺ FoxP3 ^{low} identify resting Tregs with moderate suppressive function; CD45RO ⁺ CD25 ⁺ FoxP3 ^{low} identify non-suppressive FoxP3 ⁺ T cells which can produce proinflammatory cytokines	[31]
ICOS ⁺	ICOS identifies memory-like Tregs. ICOS ⁺ Tregs produce IL-10 and express membrane bound TGFβ	[83]
LAG-3 ⁺	Constitutively expressed by ~3% of PB Tregs and expanded by activation in tumor patients. Identifies an activated and terminally differentiated Tregs with superior suppressive potential	[87]
GARP ⁺	Upon activation, GARP is selectively expressed on highly potent suppressive Tregs, but not expressed on activation-induced FoxP3 ⁺ non-suppressive cells	[89–93]
LAP ⁺ /CD121a ⁺ /	After TCR stimulation, expression of LAP, CD121a and CD121b can identify functional suppressive FoxP3 ⁺ Tregs	[94]
CD49d [~]	Absence of CD49d excludes CD25 ⁺ Tregs from Tregs. CD4 ⁺ CD25 ^{high} CD49d [~] CD127 [~] identifies highly pure FoxP3 ⁺ functional Tregs	[100]
TNFR2 ⁺	Constitutively and preferentially expressed by ~70% of PB FoxP3 ⁺ Tregs and all of human thymic Tregs. Highly expressed by memory/effector Tregs, while naïve Tregs also express higher TNFR2 than naïve Tregs. Combination of CD25 ⁺ and TNFR2 ⁺ can identify more FoxP3 ⁺ Tregs in human PB than CD25 ^{high} . Combination with additional marker, such as CD127 ^{~low} , may yield the better identification of Tregs. Also expressed by tolerogenic DC-induced Ag-specific CD4 Tregs and anti-CD3 induced CD8 Tregs. Up-regulated on activated Tregs by inflammation and promotes survival of Tregs in inflammatory environment	[9–11, 103–106, 108]