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Induced regulatory T cells in inhibitory microenvironments created by cancer

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Abstract

Introduction—Regulatory T cells (Tregs) accumulating in the peripheral circulation and tumor sites of patients contribute to tumor escape from the host immune system. Tregs encompass subsets of immune cells with distinct phenotypic and functional properties. Whereas natural (n) or thymic-derived (t) Tregs regulate responses to self-antigens, inducible (i) or peripheral (p) Tregs generated and expanded in regulatory microenvironments control immune responses to a broad variety of antigens.

Areas covered—Tregs accumulating in the tumor microenvironment (TME) are contextually regulated. They acquire phenotypic and functional attributes imposed by the inhibitory molecular pathways operating *in situ.* Several molecular pathways active in human cancer are reviewed. The pathways may differ from one tumor to another, and environmentally induced Tregs may be functionally distinct. Potential therapeutic strategies for selective silencing of iTregs are considered in the light of the newly acquired understanding of their phenotypic and functional diversity.

Expert opinion—Human Tregs accumulating in cancer comprise ‘bad’ subsets, which inhibit antitumor immunity, and ‘good’ anti-inflammatory subsets, which maintain tolerance to self and benefit the host. Future therapeutic strategies targeting Tregs will need to discriminate between these Treg subsets and will need to consider reprogramming strategies instead of Treg elimination. Re-establishment of effective antitumor immune responses in cancer patients without disturbing a normal homeostatic T-cell balance will greatly benefit from insights into inhibitory pathways engaged by human tumors.

Keywords

cancer; immune suppression; inducible regulatory T cell; inhibitory pathways; regulatory T cell-directed therapy; tumor microenvironment

Declaration of interest

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1. Introduction

Regulatory T cells (Tregs) have been a subject of intense scientific and clinical interest in the past decade. One reason for this is the acceptance of the fact that Tregs are indeed responsible for maintaining immune responses in balance. The other reason is the realization that limited understanding of the Treg biology interferes with progress in achieving a complete grasp of the mechanisms regulating this balance, which is clearly necessary for health. In cancer, for example, Tregs have emerged both as contributors to cancer progression, because of their ability to block antitumor immune responses, and as inhibitors of cancer progression via their ability to suppress cancer-promoting inflammation [1,2]. The vigorous debate that has ensued in trying to understand and reconcile these two opposing effects mediated by Tregs has been fueled by additional unanswered questions about their heritage, development, differentiation and functions. Further, whereas experiments in murine models of cancer growth have provided many mechanistic insights into *in vivo* interactions of Treg, studies of human Tregs have uncovered some differences that interfere with translating behavior of mouse Tregs to human Tregs. For example, although FOXP3 transcription factor is a reliable marker of murine Tregs, its expression in human inducible (i) Treg may be downregulated, and it appears in activated T cells which do not mediate suppression. This and other differences in Treg phenotype between mouse and human were previously discussed by us and others [2,3].

One unifying, albeit still perplexing, characteristic which is equally applicable to murine and human Tregs concerns the remarkable phenotypic and functional diversity of these cells [4]. It is perhaps due to this diversity that we have had difficulties in classifying human Tregs into distinct subsets using metrics generally applied to other immune cells. The currently adapted nomenclature for Tregs, reflects their diversity: natural (n) Tregs are now called thymic-derived (t) Tregs; iTregs are now referred to as peripheral (p) Tregs to reflect their differentiation in the periphery as opposed to the thymus; within pTregs, it is necessary to distinguish *in vivo*-generated Tregs from *in vitro*-induced Tregs [5]. *In vitro*-generated and *in vivo*-detectable Tr1 cells, which produce inhibitory cytokines, for example, IL-10 that kill targets using GrB/perforin and express markers of Tregs, represent yet another subset of iTregs [1]. In this review, the older terminology is being used: it better defines the inducible versus naturally acquired character of the Treg subsets present in cancer. Although other lymphocyte subsets, for example, $CD8^+$ T cells, have been reported to mediate immune suppression [6], this review focuses on $CD3^+CD4^+$ Tregs.

Intuitively, Tregs' diversity can be interpreted as function of their environment or, to be more precise, of Tregs' adaptation to the environmental landscape they occupy. Today, at the time when various microenvironmental signals and factors are recognized as critical in shaping immune responses [7,8], this view is not without merit. But then, it becomes necessary to consider which environmental factors are responsible for Treg recruitment to tissue sites, how these factors regulate Treg activities or survival and which molecular and cellular mechanisms are devoted to regulate Treg accumulations and functions *in situ*. Also, the involvement of nTregs versus adaptive or iTregs in orchestrating immune responses in the presence of cancer requires special attention. Considerable amount of information has accumulated in recent years in support of the microenvironment-driven regulation of Tregs

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in various disease states, including cancer [9,10]. Tregs prevent autoimmunity, limit immune pathology and maintain immune homeostasis. But in cancer, they suppress antitumor immunity, and in chronic viral infections, they suppress antiviral immunity [11,12].

The main objective of this review is to present evidence of the role of contextual Treg regulation and to provide some understanding of molecular pathways operating in the environment of human cancer that the accumulating Tregs tend to utilize. Treg-mediated activity during cancer development and progression is likely to be under the control of tumor-derived factors. Future immune therapies of cancer are increasingly likely to incorporate measures designed to eliminate or partially silence Treg activities. However, because these measures may not always benefit cancer patients, it might be necessary to discriminate between 'bad' and 'good' Tregs. To clarify, 'bad' Tregs mediate suppression of antitumor effector cell (Teff) functions, promote tumor growth and thus need to be muted; 'good' Tregs benefit the host by extinguishing chronic inflammatory responses which lead to cancer development and thus need to be spared. How functions of 'good' and 'bad' Tregs are regulated or how their control might facilitate immunotherapy of cancer remains undetermined.

2. Measurements of human Treg

In contrast to murine Tregs, where CD25 and FOXP3 have served as the consistent and reliable markers for Treg identification, human Tregs are difficult to phenotype. This reflects their greater diversity and the paucity of markers that are uniquely expressed on functionally defined Treg subsets. At the recent international workshop (29 October 2013) organized in Amsterdam as a part of the Wallace Coulter Project on Harmonization of Immunomonitoring Assays, experts in the field gathered to define the most appropriate assays/markers to measure Treg phenotype, frequency and function. Perhaps the most interesting result that has emerged from the canvassing of 22 participants in the workshop was a list of markers used for flow-based phenotyping of Tregs that included 21 distinct markers. Of these, only four (CD3, CD4, CD25 and FOXP3) were used by 95–100% of participants, whereas CD127 was used by 77% and CD45RA was used by 27% only. These six markers were considered to be the 'backbone' markers. All other markers, including CTLA-4, CD39, CCR7, HELIOS or CD69 were considered as 'optional'. In contrast to this large list of phenotypic markers, the defining Treg functions contained just two entries: inhibition of proliferation [13] and inhibition of activation of effector T cells [14,15]. Importantly, Ki-67, the universal proliferation marker, proved to be useful as functional Treg marker: *in vivo*, albeit not *in vitro*, Tregs proliferate vigorously and are Ki-67^{+} [16,17]. In view of the lack of Treg-specific markers and the very broad phenotypic profile of human Tregs, their suppressive activity remains the only reliable means of identification regardless of the phenotypic subtype. To indicate a large number of phenotype markers currently in use for Treg identification, Table 1 is provided. Table 2 lists functional assays available for assessments of suppressor activity of Tregs.

Not only the presence but also the absence of certain markers in Tregs might be informative, as for example, in the case of CD127 [18] or CD26 [19]. As always with phenotypic studies, it is necessary to remember that the marker absence could simply be due to the poor quality

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of antibodies used for detection or to fixation procedures employed prior to staining. Today, however, the commercially available mAbs and standardized fixation procedures for intracytoplasmic marker detection largely have eliminated these concerns. More likely explanation for the presence or absence of a certain marker on Tregs is their clonal diversity, as indicated by early studies with human as well as murine Tregs [20,21]. Further, it is important to remember that permanent versus transient expression of certain markers on Tregs might be informative. For example, FOXP3, a transcription factor considered to be the lineage marker for nTregs [22], has been reported to be also transiently expressed in activated conventional $CD4^+$ T cells or even $CD8^+$ T cells, as previously discussed [2]. This finding has been used to more or less discredit FOXP3 as a marker specific for human Tregs [3]. More recently, special AT-rich sequence-binding protein-1 (SATB-1), a transcription factor with the role in T-cell development and maturation, was identified and shown to be repressed in Tregs [23]. Induction of its expression in Tregs results in a loss of suppressor functions and conversion of Tregs into Teffs [23]. Since FOXP3 regulates repression of the SATB-1 gene [23,24], downregulated SATB-1 expression in $FOXP3^+$ T cells could potentially be used as a negative marker of Tregs. On the other hand, the absence of FOXP3 in a $CD39^+$ subset of peripheral human iTregs, which are unable to mediate suppression of proliferation in activated conventional T-responder cells, might indicate an incomplete or delayed conversion of iTreg precursors into mature fully functional iTregs [25]. Similar situation exists in respect to $CD25^+$ Tregs, where high levels of CD25 expression have been long considered as their relatively stable feature, although activated conventional $CD4^+$ T cells are often equally high CD25 expressors. Further, human-activated iTregs tend to be low in CD25 but high in CD122 (IL-2Rβ) and CD132 (IL-2Rγ) expression, as previously reported [26]. Although neither FOXP3 nor CD25 can be solely relied on as Treg markers, the presence of both characterizes the subset of $CD4^+CD39^+$ iTregs capable of suppressing functions of autologous Teff cells [25]. Today, the CD25 and FOXP3 pair still remains the most frequently used phenotypic signature for human Tregs.

Considerable efforts have been expanded to search for a marker specific for human Tregs. At present, only HELIOS merits attention as a potentially specific marker for human $FOXP3^+$ T cells [27]. Recent studies suggest that HELIOS defines Treg subsets with distinct phenotypic and functional characteristics [28,29]. However, there is considerable controversy as to HELIOS expression on nTregs and iTregs [27], and additional studies are needed to verify its involvement in the Treg development and functions. inducible T-Cell co-stimulator (ICOS), a costimulatory molecule, is found to be overexpressed on the surface of strongly suppressive IL-10-producing Tregs in melanoma-infiltrating T cells [30]. Another potentially significant Treg marker is CD134 [31,32] which decorates activated Tregs, that is, Tregs capable of mediating suppressor functions. Neuropilin (NRP1) was identified as nTreg marker in the mouse, but in humans, this marker appears to be expressed on Treg populations present in lymph nodes and on plasmacytoid dendritic cells (pDC) [33,34]. Tregs, especially activated iTregs, also express several chemokine receptors (notably CCR4, CCR5, CCR6, CCR7 and CCR10), which mediate trafficking of Tregs to tissue sites [35]. However, the same chemokine receptors are present on other lymphocytes capable of migration to tissue sites, and their expression on Tregs is a measure of function but not a measure of identity. Other surface markers linked to suppressive functions of

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human Tregs are discussed below in the context of molecular pathways they mediate. Today, the search for the marker specific for human Tregs continues with an objective of defining a functionally relevant surface marker that is not only specific but also useful for isolation of these cells.

In the absence of a well-defined and stable marker for all human Tregs, it might be useful to consider a combination of markers that could perhaps help define Tregs preferentially recruited to specific compartments such as the tumor site, lymph nodes or peripheral blood. Alternatively, certain marker combinations could discriminate activated Tregs from their precursors or serve to distinguish iTregs from nTregs [2]. It has been known for some time, for example, that Tregs differ from Teffs by the utilization of the phosphoinositide 3-kinase (PI3K) pathway in preference of the mammalian target of rapamycin (mTOR) pathway [36] and that certain other markers, such as CD39 and CD73, are expressed on the Treg subsets which mediate suppression via adenosine production [37]. Expression of markers such as latency-associated peptide (LAP) and/or glycoprotein A repetitions predominant (GARP) on Tregs suggests the involvement of the TGF-β pathway in Treg-mediated suppression [38–41]. Similarly, the presence of FasL on the surface of activated Tregs [42] or intracellular expression of granzyme B/perforin granules in Tregs [43] has been associated with suppressive functions of these cells. These functional markers, although not specific for Tregs, when combined with the constellation of phenotypic Treg markers, such as CD25 and FOXP3, for example, allow for measurements of human Tregs in assays that do not require isolation of Tregs in numbers required for the performance of conventional carboxyfluorescein succinimidyl ester (CFSE)-based suppressor assays (Table 2). When confronted with a task of correlating the Treg phenotype with their function in selected tissue locations, it is best to resort to flow cytometry-based assessments. These assays provide a means for practical and feasible quantification of human Treg-mediating suppression *in situ* based on expression levels of functional markers such as a cytokine, an enzyme or a metabolite (Table 2).

3. Regulation of human Tregs in the tumor microenvironment

The possibility of defining the profile of phenotypic markers that may be directly linked to Treg-mediated suppression or their survival and proliferation in a given microenvironment is worth of attention. The question of how Tregs are regulated in the tumor microenvironment (TME) remains unanswered. Given the diversity of mechanisms Tregs employ to mediate immune suppression [44], it is not unreasonable to concentrate on studies of those subsets of Tregs that utilize distinct molecular pathways for implementing immune suppression in a local microenvironment. Several of such pathways have been identified, and the role of Tregs in mediating environmentally driven suppression of immune responses via these pathways is illustrated in Figure 1. None of these pathways are unique to the TME; however, in the setting of cancer and the presence of tumor-derived signals, they can be and are subverted to promote tumor progression and to suppress antitumor immune responses.

3.1 IL-2/IL-2R pathway

The cytokine IL-2 is important for the activation of several types of immune cells [45]. Among T cells, Tregs express high levels of IL-2R (CD25) and are dependent on IL-2 for

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their development, growth and suppressor functions [45–47]. In the TME, levels of IL-2 available to immune cells may vary depending on the tumor type or stage [48]. At low concentrations, IL-2, which is considered a stimulatory cytokine, promotes the development, function and homeostasis of Tregs [49]. In many but not all cancers, the frequency of IL-2R^{high} Tregs with strong suppressive activity is increased in the peripheral blood and at tumor sites [26,50,51]. Human tumors differ enormously in the numbers and activation stage of Tregs present in inflammatory infiltrates, and it can be surmised that Treg numbers as well as Treg suppressor functions might reflect the local availability of IL-2. The IL-2 balance is a sum of the cytokine production and utilization, which at inflammatory sites depends on the intensity and nature of the immune infiltrate. As immune cell migration to tumors and activity of tumor-infiltrating lymphocytes are regulated by tumor-derived factors, it follows that the availability of IL-2 in the environment is dependent on the same factors. In the tumor, IL-2 levels might be just sufficient to support CD25^{high} Tregs, which accumulate and consume the available IL-2, depriving infiltrating Teffs of this essential cytokine. Indeed, it has been suggested that IL-2 consumption by Treg is one of the mechanisms responsible for suppression of Teff antitumor activity [52]. More recent studies provide evidence that low-dose IL-2 used as therapy can suppress immune reactivity by increasing the number of Tregs [53,54]. Thus, IL-2 now emerges as a therapeutically useful tolerogenic agent in, for example, graft-versus-host disease or post-transplant rejection episodes [52,54].

Interestingly, iTregs that accumulate in cancer may not be uniform with respect to IL-2R expression. We have shown earlier that Tr1 cells as well as iTregs in cancer patients peripheral circulation express IL-2Rβ (CD122) and IL-2Rγ (CD132) rather than CD25. This suggest that Treg subpopulations with distinct requirements for IL-2 are present [26]. Subsets of $CD4^{+}CD25^{hi}CD45RA^{+}$ and $CD4^{+}CD25^{hi}CD45RA^{neg}$ Tregs are known to differ in their stability, survival and ability to produce IL-10 [55]. Further, the ability to mediate suppression was recently shown by us to be restricted to the subset of human pTreg coexpressing CD25 and FOXP3 in our hands [25]. Also, CD25 blockade with anti-CD25 mAbs (daclizumab), one of the strategies adopted in therapy of patients with cancer in order to restore Teff functions, was reported to result in a loss of suppressor function and FOXP3 expression in a CD45RA^{neg} Treg subset, which is prominent in cancer patients but not in CD45RA^{+} Treg subset [56]. This study points to the differential IL-2R expression on human Tregs in the microenvironment of metastatic breast cancer. Further, daclizumab did not induce global depletion of Tregs but rather selectively reprogrammed a subset of $FOXP3^{+}CD25^{high}CD45RA^{neg}$ Tregs, so that they downregulated FOXP3, could no longer mediate suppression and now secreted IFN-γ [56]. This reprogramming was likely due to IL-2 deprivation in the presence of daclizumab, suggesting that the IL-2/IL-2R pathway plays a key role in maintaining Treg functionality and survival. Thus, changes in the TME, including those induced by immunotherapies such as vaccines, that impact on the integrity or efficiency of this pathway are likely to regulate Treg-mediated suppression.

3.2 TGF-β pathway

Human Tregs upregulate expressions of LAP and GARP on *in vitro* activation [25]. These surface markers are detectable on freshly isolated human $CD4^{+}CD25^{high}$ Tregs and have

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been used in discriminating Treg from Teff [40,57]. More recently, these markers have served as surrogates for Treg suppressor activity [41,57]. LAP is a pro-peptide which noncovalently associates with the amino-terminal domain of TGF-β and prevents mature TGF-β from binding to its receptor by forming a latent LAP–TGF-β complex [58]. Only when TGF-β is released from LAP, can it bind to its receptor and mediate biological activity. GARP is a cell-membrane anchor for LAP [39]. Although LAP and GARP are not selective markers for Treg, as their expression has been observed in other lymphocyte subsets [59], their upregulation on the surface of Tregs present in tumor inflammatory infiltrates and in the circulation of cancer patients serves as a surrogate marker for the TGF-β pathway activation in iTregs.

TGF-β is an anti-inflammatory cytokine-regulating activities of many different cell types, including immune cells [60], and it plays a key role in immune homeostasis [61]. In mice, ablation of T-cell signaling via the TGF-β receptor II leads to spontaneous activation of $CD4^+$ T cells and the development of wasting multiorgan inflammatory disease [62]. TGF-β inhibits proliferation and effector functions of T cells [61,63]. In human peripheral blood mononuclear cell, TGF-β induces expression of FOXP3 and promotes differentiation of T conventional to regulatory cells [25]. TGF-β also converts conventional $CD4^+CD25^{neg}$ T cells to 'induced' $FOXP3^+$ Tregs that are capable of mediating suppression *in vivo* [64]. Further, as indicated above, Tregs express surface-bound TGF-β and secrete it, so that TGF-β signaling is one of the mechanisms through which Treg mediate suppression in mice and humans [65,66]. Human tumors are avid producers of TGF-β [67]. In the hypoxic TME, which is enriched in inducible nitric oxide synthase, arginase and indoleamine 2,3 dioxygenase, TGF-β interaction with these catabolic enzymes significantly contributes to the inhibition of Teff functions [64]. Further, hypoxic conditions promote expansion of Tregs and upregulate their TGF-β secretion and immunoinhibitory activity [68]. In addition, recent data suggest that Treg-generated TGF-β is instrumental in inducing CD73 expression on immune cells thus enhancing adenosine production [69]. Differentiation of iTreg, upregulation of Treg functions and concomitant inhibition of Teff activity by TGF-β is an excellent example of how regulatory microenvironments are created in cancer and how they modulate Treg functions.

3.3 Adenosine/prostaglandin E2 pathway

Adenosine is a well-known mediator of a wide variety of physiological effects in the body. It mediates diverse regulatory activities in the endocrine, neurological, vascular, renal, pulmonary, immunological systems and in diseases such as cancer, infections and autoimmunity disorders [70]. Exogenous adenosine is a product of ATP hydrolysis by two ectoenzymes acting in sequence: CD39, an ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1) which hydrolyzes ATP to ADP and AMP and CD73, an ecto-5′-nucleotidase, which catalyzes AMP conversion to adenosine. This nucleoside, signaling via its four surface G-protein-coupled receptors, A_1, A_{2A}, A_{2B} and A_3, which are widely distributed throughout tissues, mediates regulatory effects via upregulation or downregulation of intracellular levels of 3′5′-cAMP. In the immune system, adenosine inhibits functions of immune cells and is considered to be an anti-inflammatory factor [71,72]. In cancer, however, in addition to promoting migration of immune cells to the

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tumor and inhibiting antitumor functions of accumulating Teffs, this pathway promotes differentiation, expansion and suppressor activities of Tregs and myeloid-derived suppressor cells (MDSCs), as recently reviewed [71,72]. The adenosine pathway in the TME ceases to be a protective pathway guarding against tissue damage by activated immune cells and becomes a tool for suppressing antitumor immune functions and, through its effects on the vasculature, for promoting metastasis [73]. Importantly, these pro-tumor activities of Tregs occur in cooperation with the prostaglandin E2 (PGE_2) pathway [74,75]. Most tumors are COX-2^+ and produce PGE_2, which signals via four prostaglandin E receptors expressed on immune as well as various tissue cells [76]. PGE_2, like adenosine, acts via adenylate cyclase (AC), upregulates 3′5′-cAMP levels in lymphoid cells and mediates suppression [77]. The two pathways converge at the AC level and together deliver powerful immunoinhibitory signals to responder cells.

The adenosine pathway operating in cancer plays an important role in Treg-mediated suppression [78]. Expression of CD39 and CD73 on Tregs was first reported by Borsellino *et al.* [79] and Deaglio *et al.* [80] in 2007. Since then, human Tregs were shown to be capable of hydrolyzing ATP to 5′-AMP and adenosine and mediating suppression of Teff functions via the $A_{2A}R$ engagement [37,81]. In contrast to nTregs, which express CD39 on the cell surface and mainly produce 5′-AMP, *in vitro*-generated human Tregs (Tr1) and iTregs upregulate surface expression of CD73, co-express CD39 and CD73 and produce copious levels of adenosine [38]. These iTregs also strongly suppress Teff functions in standard *in vitro* CFSE-based proliferation inhibition assays [82]. In contrast to $CD4^+$Teffs, human Tregs do not express CD26 which is linked to adenosine deaminase at the cell surface and thus are unable to convert adenosine to inosine [19,37]. Increased pericellular levels of adenosine in Tregs might facilitate autocrine signaling, potentially augmenting their suppressor activity. As $A_{2A}R$ is highly expressed on Tregs, adenosine generated by Tregs may signal via $A_{2A}R$ and promote Treg functions [83]. In CD73 knockout (KO) or A_{2A}RKO mice, this autocrine loop does not operate, and Tregs are unable to protect the animals from kidney ischemia-reperfusion injury [83]. Also, a selective $A_{2A}R$ agonist or adenosine was shown to upregulate programmed cell death (PD-1) expression in Tregs, suggesting autocrine activation of this suppressive pathway [83]. These data indicate that the adenosine pathway may be important not only for Treg proliferation but also for regulation of their suppressor functions. Together with tumor-derived PGE_2, adenosine regulates Treg signaling in the TME.

3.4 Neuropilin–semaphorin pathway

A recent report introduced the NRP1/semaphorin-4a (sema-4a) pathway as an example of an environmentally driven molecular program that regulates Tregs *in situ* [84]. NRP1 is a receptor present on murine (and perhaps also human) Tregs accumulating in inflammatory sites; its ligand, sema-4a, is expressed on a variety of immune cells. The receptor–ligand interaction in the inflammatory milieu potentiates Treg functions and promotes Treg survival [34]. Using elegant *in vitro* and *in vivo* tumor models in which NRP1 or sema-4a signaling was silenced, Vignali's group demonstrated that the NRP1/sema-4a pathway was absolutely necessary for Treg protection and survival in the tumor, although in other inflammatory environments, this pathway was dispensable [84]. The pathway is orchestrated

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by the environment in which Tregs reside and by activation signals that are processed via the PI3K pathway. Vignali's group showed that NRP1–sema-4a interaction was responsible for recruitment of the phosphatase and tensin homolog to the immunologic synapse in Tregs. This resulted in blocking of Akt phosphorylation, restraining Akt-mTOR signaling, facilitating nuclear localization of Foxo transcription factors and thereby potentiating Treg suppression [84]. In this scenario, sema-4a ligation to NRP1 expressed on Tregs was responsible for potentiating Treg suppression. Interestingly, sema-4a is expressed not only on lymphoid cells but also on cells in the nervous system, intestinal mucosa or the eye, where Treg tolerogenic activity is desirable. In mouse tumors, pDC are especially rich in the sema-4a ligand expression, suggesting that Treg–DC interactions may be implicated in mediating antitumor activities of Tregs. In aggregate, these data convincingly indicate that the TME is very special in that it promotes immune tolerance by driving and promoting the NRP1/sema-4a pathway, favoring suppressive activity and survival of Tregs without disturbing Treg ability to migrate to other tissue sites. In effect, only Tregs in the tumor milieu acquire the license to suppress anti-tumor immunity without perturbing those Tregs that are needed for keeping autoimmunity in check. Aside from emphasizing the 'division of labor' among Tregs, this study suggests that the NRP1/sema-4a pathway represents a potential therapeutic target as discussed below. As exciting and far-reaching as these data are, additional studies are clearly in order to investigate whether the NRP1/sema-4a pathway, described for mouse tumors, is utilized in human tumors and whether it operates in only some or all human tumors. Also, interactions of this molecular pathway with other pathways regulating functions of Tregs in the tumor need to be studied. Nevertheless, potential immunological consequences of targeting the NRP1/sema-4a pathway are promising for immunotherapy of cancer and represent an exciting new venue for explorations of Tregs present in the TME.

4. Cooperation between regulatory pathways in the tumor microenvironment

Assuming that Tregs in the TME can utilize the above-described molecular pathways for inducing immune suppression, the question arises as to which of the pathways predominates in modulating Treg functions and why. It is also conceivable that different human tumors instruct Tregs to preferentially utilize one pathway. If so, this would be important for selection of future therapeutic approaches to silence Tregs in cancer. Concerning the pathways listed above, it appears that a considerable molecular crosstalk exists between them. The adenosine/PGE_2 signal convergence at the level of AC activity illustrates the receptor-AC-cAMP-mediated control of Treg–Teff interactions [77,82]. Concentrations of IL-2 in the microenvironment are strictly related to the numbers and activation of lymphocytes accumulating *in situ* and are likely to be influenced by suppression exerted via Tregs using any one of the described pathways [85]. Recent data indicate that CD73 expression at the mRNA and surface protein levels are significantly upregulated by TGF-β1 [61], potentially leading to amplification of the adenosine pathway in environments enriched in this cytokine. Finally, preliminary evidence suggests that NRP1 expression on the Treg surface, a requirement for its interaction with the sema-4a ligand, is dependent on the activation state of Tregs and the ligand expression on immune cells infiltrating the TME

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[84]. In aggregate, the view that emerges suggests that not one but all the suppressive pathways may be used in tandem to regulate Tregs at the tumor site and that crosstalk or cooperation between these pathways determines the degree of prevailing immune suppression. It is not known whether some human tumors preferentially depend on any one pathway for regulating immune suppression. However, given the evidence that TGF-β1, COX-2 and ectonucleotidases expression and their functions differ from tumor to tumor [86,87], it seems reasonable to conclude that, not surprisingly, the ultimate control is exerted by the tumor.

5. Therapeutic strategies for Treg regulation

Immunotherapy of cancer aims at: i) recovery of effective anti-tumor immune responses generally in the setting of minimal residual disease; and ii) elimination or reduction of mechanisms promoting tumor escape. The latter strategy targets suppressor cells such as tumor-associated macrophages, pDC, MDSC and, of course, Tregs.

5.1 Treg depletion

Various approaches have been devised to target Tregs, Treg depletion being one of them, especially prior to administration of cancer vaccines or adoptive cell transfers. To date, low-dose cyclophosphamide, daclizumab (anti-CD25 Ab), denileukin diftitox (ONTAK) or tyrosine kinase inhibitors such as sunitinib have all been utilized in the clinic to deplete Tregs [88–91]. These depletion strategies and their shortcomings have been extensively reviewed [92]. Current data suggest that attempts at Treg elimination may have to be more finely tuned to target not all but rather specific subsets of Tregs, taking advantage of their functional diversity.

The notion that not all Tregs are the same and that Treg subsets responsible for tumor escape are distinct from those mediating tolerance to self-antigens is supported by experimental evidence. For example, Vignali's group [84] suggest that blocking of the NRP1/sema-4a pathway with Abs or soluble antagonists allows for depletion of Tregs that suppress antitumor immunity without disturbing Treg subsets that regulate autoimmunity. They propose that targeting the NRP1/sema-4a pathway in Tregs could provide a novel therapeutic strategy aimed at a selective removal or silencing of 'bad' Tregs but not 'good' Tregs necessary for control of auto-immune diseases. Similarly, a recent human clinical trial in which a single dose of daclizumab was given prior to a tumor vaccine in patients with breast cancer provides support for differential drug effects on Treg subsets [56]. Daclizumab-mediated CD25 blockade resulted in acute and prolonged depletion of circulating Tregs. However, it was not a global but selective depletion of $CD4^+FOXP3^+$ $CD45RA^{neg}$ Tregs, which were not depleted but reprogrammed to Teffs producing IFN-γ, lost FOXP3 expression and could no longer mediate suppression. Significant recovery of Teff functions and peptide-specific cytotoxic T lymphocyte activities accompanied this reprogramming induced by IL-2 deprivation by daclizumab. Importantly, the $CD45RA^+$ Treg subset was not affected by the CD25 blockade. The authors hypothesize that the daclizumab-resistant subset of $CD45RA^+$ Tregs may be poised to guard against systemic autoimmunity [56].

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This ‘division of labor’ among Tregs is further illustrated by our studies describing the presence in the peripheral circulation of two subsets of $CD4^+CD39^+$ Tregs [25]. One subset is $CD25^+FOXP3^+$ and suppresses proliferation of Teffs, whereas the other is $CD25^{neg}FOXP3^{neg}$ and not effective in inhibiting Teff proliferation, although it is $CD39^+$ and potentially is capable of producing adenosine [38]. These two subsets are always detectable and distinguishable in the blood of normal donors and patients with cancer and also at tumor sites and are always present at the 1:1 ratio [25]. We speculate that the $CD4^+CD39^+CD25^+FOXP3^+$ Treg subset is ready to suppress, whereas the other ‘resting’ subset serves as a guardian always prepared to step in and upregulate CD25 and FOXP3 and activate the suppressive machinery. Because this Treg subset expresses CD39, we suspect that the adenosine pathway as well as IL-2 levels may control the conversion of resting Tregs to actively tolerogenic cells when they are needed. Further, we suspect that these Treg subsets may be differentially sensitive to depletion whether by mAbs or drugs, and that the plasticity of Tregs allows for their reprogramming depending on the microenvironmental landscape.

5.2 Checkpoint blockade

Another strategy currently widely used for reducing tumor-induced immune suppression is the immune checkpoint blockade [93,94]. Molecules targeted by the checkpoint blockade are negative inhibitors of immune responses and, more specifically, of T-cell activation. A number of such regulatory molecules have been identified, including CTLA-4, PD-1, LAG-3 and TIM-3 [95–98]. These molecules control the magnitude of immune responses and are instrumental in downregulation of excessive or tissue-damaging immune responses. They are also expressed by Tregs and are implicated in Treg functions [97,98]. The mAbs to these molecules, especially anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) have been tested in Phase I –III clinical trials and have shown efficacy against solid and hematological malignancies [99–102]. Although blocking of negative signaling in Teffs and restoration of their activities is considered to be the mechanism responsible for efficacy, these mAbs may also recognize and bind to Tregs, thereby reducing their numbers and suppressor functions [103].

5.3 Blocking of inhibitory pathways

Neutralizing Abs and pharmacological inhibitors have been available for many components of the molecular pathways discussed above. Interactions between iTregs and Teffs could be addressed and corrected through antagonizing one or more than one of these pathways using specific Abs or pharmacological interventions or both, thus preventing cancer-associated immune suppression. This therapeutic strategy has been previously referred to as ‘blocking the inhibitors’ [104]. For example, inhibitors of the PGE_2 pathway (celecoxib, indomethacin, diclofenac, ibuprofen) have been clinically used in patients with cancer, with an intent to block suppressive PGE_2 production by $COX\text{-}2^+$ tumors [105]. Our *in vitro* studies with human Tr1cells showed that expression by tumor cells of COX-2 had pronounced effects on the Tr1 generation as well as suppressive functions of these cells [106]. Thus, Tr1 cells generated in co-cultures with $COX\text{-}2^+$ tumor cells were more suppressive, hydrolyzed more exogenous ATP, expressed high levels of CD39 as well as CD73 and produced more adenosine and PGE_2 than Tr1 cells induced by $COX\text{-}2^{neg}$ tumors

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[106]. Suppressive functions of Tr1 cells were blocked by pharmacological antagonists of ectonucleotidases and also in the presence of the above-mentioned inhibitors of the PGE_2 pathway. These studies suggest that a combined inhibition of the adenosine and PGE_2 pathways was highly effective in eliminating immune suppression mediated by Tr1 cells [107]. Also, we reported that iTregs accumulating in human cancers, overexpress CD39, upregulate CD73 and produce elevated levels of adenosine [108]. Blocking of adenosine synthesis via CD73/CD39 inhibition has been evaluated in preclinical murine models of cancer [109]. These studies showed that silencing of CD73, a terminal enzyme in the ATP hydrolysis pathway, with anti-CD73 mAbs resulted in a delay of tumor growth and reduced metastasis [109,110], Various pharmacological inhibitors of CD73 enzymatic activity as well as siRNA have been used in mice and have been shown to be effective in inhibiting tumor growth [71,111]. CD39 also appears to be a promising therapeutic target in oncology [87]. Experiments with the CD39 KO cells or mouse models and with CD39 antagonistic Abs or pharmacological inhibitors of CD39 activity provide convincing evidence in support of anticancer effects of CD39 inhibition [87]. It is important to point out that these therapies target not only Tregs but also tumor cells which often express CD39 and/or CD73 [87,110,112]. Importantly, these therapies aim at the selective inhibition of adenosine-mediated suppressive functions without depleting all Tregs and disturbing immune homeostasis [87].

5.4 Treg resistance to therapies

Considerable interest has been focused on Treg sensitivity/resistance to cancer therapies. Reports that immune therapies leading to upregulation of antitumor immune responses expand rather contract Treg populations [113,114] have called attention to the possibility that cancer therapies might influence the frequency and functions of Tregs in patients. It has been known that chemoradiotherapy (CRT) selectively eliminates subsets of immune cells and that $CD4^+$ T cells are especially sensitive to CRT [115]. More recent studies found that the Treg frequency tends to increase after oncologic therapies [116]. *In vivo* studies in mice indicated that radiation and chemoradiation exert strong effects on the host immune system, including Tregs [117]. We have recently evaluated the effects of adjuvant CRT on Treg numbers and functions in patients with head and neck squamous cell carcinoma (HNSCC) [57]. CRT decreased the frequency of circulating $CD4^+$ T cells ($p < 0.002$) but increased that of $CD4^+CD39^+$ Tregs (< 0.001), compared to untreated or surgery-only patients. Treg frequency remained elevated for > 3 years in a subset of patients. CRT increased surface expression of LAP, GARP and CD39 on Tregs. In parallel *in vitro* studies, Tregs were resistant to activation-induced cell death or cisplatin, whereas conventional $CD4^+$ T cells were not. CRT-induced Tregs obtained from patients or normal donors upregulated pro-survival Bcl-2/Bcl-xL, whereas $CD4^+$ T conventional upregulated proapoptotic Bax [57]. This study showed that highly suppressive, cisplatin-resistant Tregs increased in numbers and persisted after CRT. Further, this CRT-resistant Treg subset could be responsible for suppression of antitumor immunity and, ultimately, for tumor recurrence in HNSCC patients who were initially responsive to CRT. Resistance of Tregs to modulation by immunomodulatory drugs has been also observed and is discussed in [104]. These results emphasize that monitoring of Treg frequency and functions prior to and after oncological therapies is a critical part of therapeutic clinical protocols, aiming at the restoration of

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antitumor immune responses. More recent examples of immune cancer therapies that appear to have profound effects on Treg frequency and functions include anti-CTLA-4 or anti-PD-1/anti-PDL-1 therapies [103]. The role of Tregs that either accumulate or decrease after oncological therapies in the promotion or inhibition of tumor progression remains unclear and prospective serial monitoring will be necessary to elucidate the biological and clinical consequences of Treg resistance to cancer therapies.

6. Conclusion

Tregs have been considered to be significant contributors to tumor-associated immune suppression. For this reason, they have been a target for a variety of therapeutic strategies in recent years. Initial attempts at their removal in order to improve antitumor immune responses have not been successful, as only transient Treg depletion occurred, which, in some cases, provoked autoimmune symptoms or induced acute T-cell lymphopenia [118,119]. Recent therapeutic strategies tend to be more discriminating largely because of the perception that Tregs are heterogeneous, comprising a variety of subsets, some of which may be engaged in immune suppression that is not only beneficial to cancer patients but also necessary for maintaining tolerance to self-antigens [120]. The realization that Tregs actively participate in and regulate various inhibitory pathways operating in the TME in part explains the Treg diversity in cancer [121,122]. It appears that the microenvironment dominated by the developing tumor dictates the rules for engagement of molecular pathways which promote the generation and expansion of iTreg subsets with the ability to adapt their suppressive program to these pathways. Should this be the case, further understanding of the molecular pathways operating in the tumor becomes a prerequisite for the selection of therapeutic strategies, which might simultaneously target the tumor as well as Tregs. The adenosine axis is a good example for this option [71,78]. If tumor cells and iTregs overexpress CD39/CD73, then antagonistic agents or Abs will likely target both. Two important caveats should be considered, however. One is selective elimination of Tregs suppressing antitumor immune responses and not all immune responses. It is still unclear that such antigen-specific Tregs can be effectively targeted in human cancer [123]. The second is above-mentioned resistance of Tregs to therapies, including chemotherapy, radiation and potentially immunotherapy [53]. A greater understanding of why some subsets of Tregs are more resistant than others and how such resistance translates into cancer progression or recurrence is needed.

7. Expert opinion

Considerable progress has been made in the studies of human Tregs and their role in disease, including cancer. Despite this, it remains unclear how various metabolic or molecular processes in tissue regulate Treg accumulations and functions. The challenge in this field is to be able to monitor Tregs and their functions in TMEs and thus more accurately ascertain the impact that Tregs exert on prognosis. In view of the emerging phenotypic and functional heterogeneity of Treg subsets, the numbers of infiltrating Tregs or the Treg:CD8^{+} T cell ratios broadly in use today may not be sufficient. Given the diversity of Treg subsets and the potential engagement of multiple molecular pathways by activated Tregs, ascertaining their contribution to cancer progression or regression is likely to be challenging. The lack of

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definitive phenotypic markers that would specifically identify activated Tregs performing a designated function is a problem begging for solution. Activation markers, enzymes, signaling pathways or cytokines expressed by Tregs might offer potentially valuable insights into the functional diversity of Tregs *in situ*. At the time when immune therapies, including various checkpoint inhibitors, are rapidly gaining ground in the clinic, their effects on Treg silencing, elimination or activation are of great interest. Therapy-induced changes in the Treg frequency and expression levels of inhibitory or stimulatory molecules could serve as metrics for their *in situ* functional integrity and, when correlated to clinical end points, as biomarkers of response or relapse. Similarly, systemic alterations in Treg phenotype and functions, if reliably detected in serially obtained specimens, might be informative. For this reason, the development of reliable, readily applicable monitoring tools for human Treg subsets in tissue and in the peripheral circulation of cancer patients is a priority.

The paucity of information about factors and conditions that govern the conversion of nTregs or $CD4^+$Tconv to Tregs requires attention as well. The tremendous plasticity of Tregs responding to environmental stimuli implies that their differentiation, functional maturation and turnover are rapid and efficient. Tregs must be in the right place at the right time to assume immune control. However, in the case of chronic cancer-associated inflammation, it is probably necessary to think of ongoing, relentless process of Treg mobilization and conversion mediated on the one hand by proinflammatory protective factors and on the other by tumor-driven anti-inflammatory suppressive mediators. Which of the two cascades dominates and which subsets of Treg emerge depends very much on the TME and is related to the tumor aggressiveness and stage. In human cancers, by the time of biopsy or surgery, Tregs are already subverted to play the role assigned by the tumor. Are all or only some Treg subsets subverted and, more importantly, can immune therapy relieve the subversion and reprogram Tregs, so they now acquire the effector phenotype? These are provoking and unanswered questions that are likely to occupy attention of the scientific and clinical communities in the near future.

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Article highlights

- New terminology proposed for regulatory T cells (Tregs):

  (n) natural Treg = (t) thymus-derived Treg

  (i) inducible Treg = (p) peripheral Treg

  In vitro-induced Treg

  In vivo-induced Treg

  Tr1 cells
- The underlying rationale for this terminology is based on Treg diversity evident in their phenotype and functions which depend on the local environment. In tumor-bearing hosts, this environment is created by and dominated by the tumor.
- Treg accumulating and operating in cancer patients are regulated themselves by tumor-derived factors and are a part of inhibitory molecular pathways activated in the presence of cancer.
- The IL-2/IL-2R, TGF-β, adenosine/prostaglandin E2 or neuropilin–semaphorin pathways are examples of normal physiological pathways subverted by the tumor to mediate suppression of antitumor immune responses.
- Tregs actively participate in and regulate various inhibitory pathways operating in the tumor microenvironment which in part explains the Treg diversity in cancer. Tregs can utilize one or more of these pathways to mediate suppression.
- Tregs are induced and expanded *in situ* by signals driving the molecular pathway(s) operating in the tumor microenvironment. Tregs modulate their suppressive activity in the context of inflammatory infiltrates accumulating in the microenvironment they occupy.
- A better understanding of molecular pathways operating in the tumor is necessary for the development of immunotherapies which simultaneously could target the tumor and Tregs.
- Future immunotherapies will aim at selective silencing of Treg subsets which inhibit antitumor responses and sparing Tregs necessary for the maintenance of normal T-cell homeostasis.

This box summarizes key points contained in the article.

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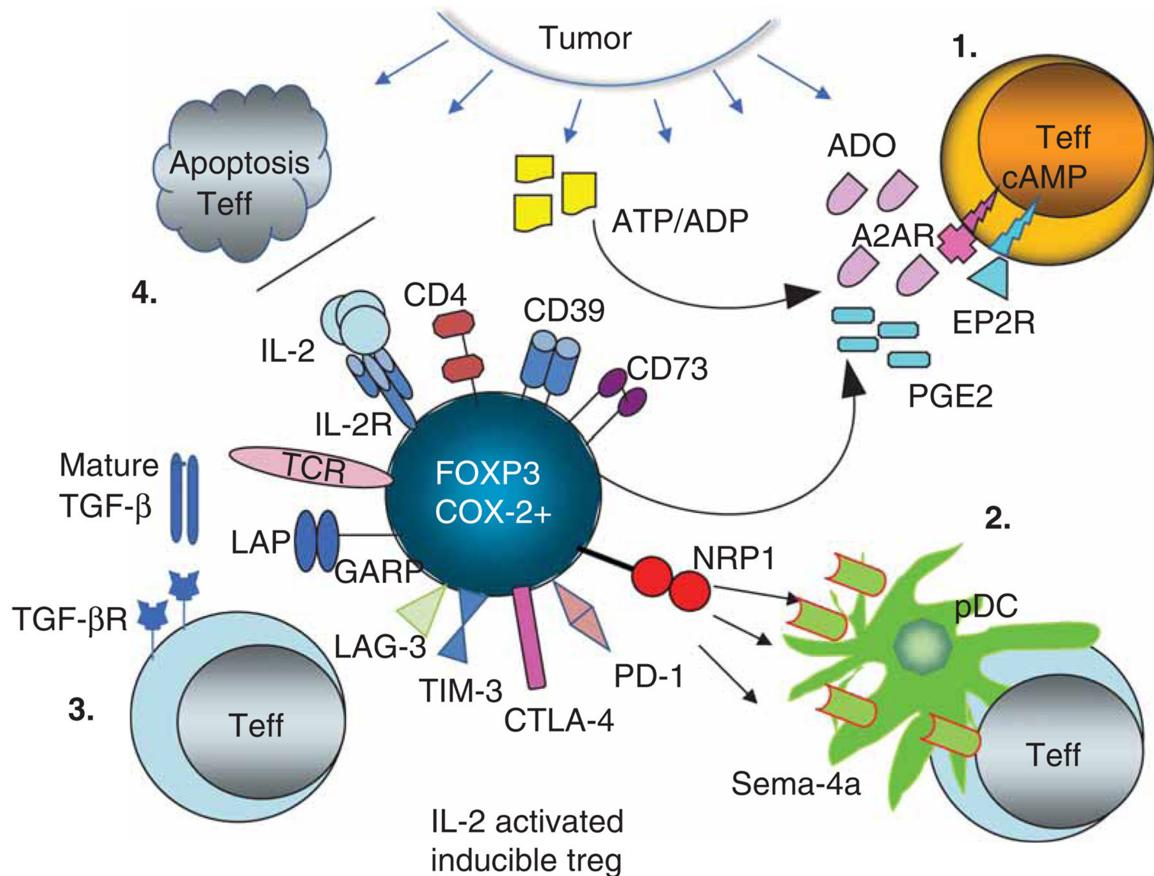

Figure 1. In the TME, activated iTregs operate by engaging several suppressive pathways which downregulate functions or induce apoptosis of immune cells

Shown are: (1) ADO-PGE$_2$ pathway; (2) the neuropilin1-semaphorin4a pathway; (3) the TGF-β pathway; and (4) the IL-2/IL-2R pathway. Cooperation between these pathways might lead to upregulation of immune-suppressive molecules (e.g., TIM3, PD-1, CTLA-4, LAG-3, CD39, CD73, LAP/GARP) on iTregs. Consumption of IL-2 by iTregs deprives tumor effector cell of growth factors inducing apoptosis. A ligand for NRP1, sema-4a, is expressed on lymphocytes and p-DC as well as tissue cells.

ADO: Adenosine; GARP: Glycoprotein A repetitions predominant; LAP: Latency-associated peptide; NRP1: Neuropilin 1; p-DC: plasmacytoid dendritic cells; PD-1: Programmed cell death; PGE2: Prostaglandin E2; sema-4a: Semaphorin-4a; Teff: Tumor effector cell; TME: Tumor microenvironment.

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Table 1

Phenotypic characteristics of human nTregs, iTregs and $CD4^+$Tconv*.

Markers	nTregs	iTregs	$CD4^+$Tconv	Comment	Ref.
CD3	+	+	+		
CD4	+	+	+		
CD25	High	±	Int/low	Activation marker	[20,26]
CD122/CD132	±	++	±		[26]
FOXP3‡‡	+	±	±	Transient expression in activated T cells	[2,22]
SATB1‡‡	−	±	±	Transcription factor regulated by FOXP3-negative Treg marker	[23,24]
CD45RA	+	−	+ or −	iTregs are $CD45RO^+$	[25,55]
CTLA-4	+	+	+	Upregulated on Tregs	[25]
CD127	−	−	+	Used for negative selection of Tregs	[18]
HELIOS‡‡	+	−‡	±	Transient expression in activated T cells	[27–29]
ICOS**		+	+	iTregs in melanoma TIL upregulate expression	[30]
NRP1**	±	+	±	Restricted expression	[33,34]
CD39**	+	+	−	Ectonucleotidase; hydrolyzes eATP to 5′-AMP; upregulated on Tregs in TIL	[37,28]
CD73‡‡**	+	±§	±¶	Ectonucleotidase; when coexpressed with CD39 on cell surface in activated Tregs, it hydrolyzes 5′-AMP to adenosine	[37,38]
CD26	−	±	+	Peptidase linked to ADA1; used as negative Treg marker	[19]
GITR**	+	+	+		
CD134	±	++	±	Upregulated on Treg	[31,32]
CD137**	+	++	±	Upregulated on iTregs in TIL	[124]
PD-1**	±	++	±	As above	[25]
PD-L1**	±	++	±	As above	[25,99]
TIM-3**	+	++	?	As above	[99]
LAG-3**	+	++	+	As above	[99]
CCR4**	+	++	±	As above	[35]
CCR6**	+	++	±	As above	[35]
CCR7**	+	++	±	As above	[35]

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Markers	nTregs	iTregs	$CD4^+$Tconv	Comment	Ref.
GARP/LAP[#,**]	+	++	−	Upregulated on the surface of circulating Tregs in cancer	[38,41,58]
TGF-β[‡‡,**]	+	++	−		[82]
IL-10[‡‡,**]	+	+	−		[30,42,50]
GrB/perforin[**]	±	++	−		[42]
COX-2[**]	−	+	−		[82]
Ki-67[‡‡,**]	±	+	±	Tregs proliferate *in vivo*	[16,17]

[*] The list is not comprehensive and includes most commonly studied Treg markers. The iTreg column includes data for *ex vivo*-evaluated cells and/or for *in vitro*-generated cells. For a more in-depth commentary on expression of the listed markers on nTregs and iTregs, see Ref. [2].

[‡] Expression of HELIOS in nTregs versus iTregs has been debated and its usefulness for discriminating between nTregs and iTregs is not entirely clear [27].

[§] CD73 is abundantly present in the cytoplasm of Tregs but appears to be readily downregulated (probably enzymatically cleaved) from their cell surface [36] and is seen on the cell surface in highly variable proportions of iTregs and only < 1% of nTregs.

[¶] CD73 is expressed on the cell surface of a small subset (8 – 18%) of $CD4^+CD73^+$ T cells in the peripheral blood of normal donors [12].

[#] GARP or garpin and LAP are TGF-β-associated membrane-bound molecules.

[**] Expression levels variable and dependent on the state of Treg activation.

[‡‡] Intracellular expression.

ADA: Adenosine deaminase; GARP: Glycoprotein A repetitions predominant; ICOS: Inducible T-Cell co-stimulator; GITR: Glucocorticoid-induced TNFR family related; LAP: Latency-associated peptide; NRP1: Neuropilin; TIL: Tumor-infiltrating lymphocytes.

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Table 2

In vitro suppression assays for human Tregs*.

Assay type	Suppressor cell (Ts)	Responder cell (TR)	Measurement	Ref.
Multiparameter flow cytometry	*ex vivo* activated T cells (6 – 12h)	-	Intracellular expression of 1 – 5 cytokines	[25]
-“-	nTregs or iTregs	-	GARP/LAP expression	[25,57,40,41]
-“-	nTregs or iTregs	$CD4^{+}$T conv *ex vivo* activated 7 – 20 h	Downregulation of CD69 or CD154 expression on responder cells	[13,14]
Supernatants	nTregs or iTregs, ± activation	-	ADO, PGE_2, TGF-β, IL-10 production	[38]
Co-culture*	nTregs or iTregs	CFSE-labeled $CD4^{+}$T conv	Proliferation inhibition	[13,50]
FOXP3 demethylation (MS-QPCR)	nTregs	-	Detected TSDR	[125]

*Co-cultures of suppressor with responder cells require assays set up at different Ts/TR ratios.

ADO: Adenosine; CFSE: Carboxyfluorescein succinimidyl ester; LAP: Latency-associated peptide; MS-QPCR: Mass spectrometry; PGE_2: Prostaglandin E2; TSDR: Treg-specific demethylation region.