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Regulatory T cell subsets in human cancer: are they regulating for or against tumor progression?

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Abstract

Regulatory T cells (Treg) play a key role in maintaining the balance of immune responses in human health and in disease. Treg come in many flavors and can utilize a variety of mechanisms to modulate immune responses. In cancer, inducible (i) or adaptive Treg expand, accumulate in tissues and the peripheral blood of patients and represent a functionally-prominent component of CD4⁺ T lymphocytes. Phenotypically and functionally, iTreg are distinct from natural (n) Treg. A subset of iTreg expressing ectonucleotidases, CD39 and CD73, is able to hydrolyze ATP to 5'-AMP and adenosine (ADO) and thus mediate suppression of those immune cells which express ADO receptors. iTreg can also produce prostaglandin E₂ (PGE₂). These iTreg, expanding in response to tumor antigens and cytokines such as TGF- β or IL-10, are presumably responsible for suppression of anti-tumor immune responses and for successful tumor escape. On the other hand, in cancers associated with prominent inflammatory infiltrates, e.g., colorectal carcinoma or certain types of breast cancer, iTreg down-regulate excessive inflammation by producing ADO and/or PGE₂ and protect the host from tissue injury and tumor development. Thus, iTreg utilizing the same adenosine pathway play a key but dual role in cancer, and their plasticity is controlled and driven by the microenvironment. Thus, monitoring for the frequency and functions of iTreg rather than nTreg is important in cancer. In addition, elimination of iTreg by various available strategies prior to immunotherapies may not be beneficial in all cases and needs to be undertaken with caution.

Keywords

Immune regulation; inducible Treg; adenosine; PGE₂; tumor microenvironment; CITIM 2013

Introduction

Regulatory T cells (Treg), which represent about 5% of circulating CD4⁺ T lymphocytes in the human peripheral blood, comprise several functionally distinct cell subsets [1]. Treg are responsible for maintaining immune responses in balance and preventing excessive and dangerous immune reactivity. Several human diseases including cancer, chronic infections and autoimmune syndromes have been associated with Treg imbalance, which seems to contribute to the disease process and to have an impact on patient survival [2–4]. For this reason, disease-associated changes in the Treg frequency and function have been of considerable interest. In healthy individuals, thymus-derived natural (n)Treg are responsible for peripheral tolerance and immune homeostasis. In pathologic situations, nTreg are largely

Conflict of Interest

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replaced by inducible (i)Treg, which develop and function in response to unique microenvironmental stimuli and regulate various types of T helper (Th) responses during inflammation. In cancer, Treg are thought to be involved in tumor escape from the host immune system [3, 5]. Treg accumulate in tumor tissues and the peripheral circulation of cancer patients [5, 6]. However, the role of these cells in tumor progression or regression has not been clear, especially since their frequency in patients' tissues or blood does not always correlate with outcome [7]. For example, in OvCa patients, increased Treg frequency and function are associated with poor prognosis [8], while in patients with colon Ca, increased Treg frequency is predictive of better prognosis and improved overall survival [9]. These discrepant findings suggest that Treg accumulations in pathologic tissues and Treg functions *in situ* might be under the control of a complex regulatory network orchestrated by factors that largely remain unidentified. Currently, there are numerous unanswered questions in respect to Treg, including the fundamental question of whether Treg subsets regulating immune responses in human diseases are the same or different from Treg subsets operating in healthy subjects. Little is known about the origin of human iTreg, and suppressor mechanisms utilized by these cells appear to be numerous and varied. Phenotypic and/or functional differences between human Treg accumulating at inflammatory sites and those present in the circulation remain unclear, and the extent to which the local microenvironment modulates Treg activity is of considerable current interest. Assuming that suppressor activities of iTreg are contextual, the question of which factors or signals in the microenvironment regulate Treg functions becomes the central issue.

How are Treg functions regulated in inflammatory microenvironments?

In inflammatory lesions and in tumors infiltrated by immune cells, Treg usually are a prominent component of the infiltrate [10, 11]. It is not clear, however, whether these cells regulate in favor of the host or in favor of the tumor. It is known that inflammation may lead to tissue injury, and Treg, which down-regulate activities of immune cells and suppress inflammation, may protect the host from injury. In this situation, Treg are "good citizens." However, in tumors, Treg suppress anti-tumor functions of immune cells migrating to the tumor site, thus promoting tumor escape from the host immune system. In this situation, Treg are "bad citizens." From the clinical perspective, this is an important distinction, as the elevated frequency and functions of Treg infiltrating human tumors appear to correlate with poor outcome in many but not all human tumors [8, 12–14]. If Treg promote cancer progression by ablating anti-tumor immunity, they need to be controlled or removed. But if Treg down-regulate pro-inflammatory responses that favor tumor progression, then their therapeutic removal is clearly contraindicated.

Newer data suggest that Treg functions and their polarization from the "good" to "bad" status is environmentally regulated by a finely tuned system of molecular interactions and cellular crosstalk. For example, Vignali and co-workers working in the mouse have identified a molecular pathway that regulates Treg survival and functions in the tumor microenvironment *in vitro* and *in vivo* [15]. Apparently, binding of semaphorin-4a, which is expressed on immune cells, to neuropilin-1 (Nrp-1), its receptor on Treg, potentiates survival and suppressive activity of Treg. Remarkably, the Nrp-1/sema-4a pathway is **absolutely necessary** for protecting Treg and prolonging their survival in the tumor microenvironment but, in other inflammatory environments, e.g., autoimmune infiltrates, this pathway is dispensable. The Nrp-1/sema-4a pathway processes tolerogenic signals delivered to Nrp-1, leading to decreased activation of PI3 kinase, restraining of Akt phosphorylation and the recruitment of PTEN to the immunologic synapse. As immune cells (e.g., plasmacytoid DC) are one of the major sources of semaphorin-4a, activation of this tolerogenic pathway depends on the cellular content of the infiltrate. Solid tumor infiltrates often contain plasmacytoid DC [16], which promote tolerance and Treg differentiation and

suppressor functions, presumably via the Nrp-1/sema-4a pathway. Silencing of this pathway could result in selective elimination of “bad” Treg in tumors thereby benefiting the host, while preventing adverse effects of chronic inflammation or autoimmunity [15].

The environmentally-regulated character of Treg, as illustrated above, suggests that other molecular pathways might also be involved in modulating Treg activity *in situ*. The adenosine pathway has long been viewed as especially active in tumors, because of an excess of ATP produced and its brisk hydrolysis to adenosine in diseased tissues. Unexpectedly, expression of ectonucleotidases, CD39 and CD73, which hydrolyze ATP to 5'-AMP and adenosine, was described in mouse and then in human Treg [17–19]. This discovery firmly placed Treg in the metabolic circuit involving adenosine and operating in the inflammatory milieu.

The adenosine pathway and inducible (i) Treg in cancer

The emerging view of events mediated by Treg accumulating in human tumors suggests that inducible (i)Treg are the major participants in shaping immune responses *in situ*. These regulatory T cells develop and function in response to unique microenvironmental stimuli and represent a “tailor made” system of brakes and balances needed to modulate different types of T helper responses during inflammatory responses. To be able to learn more about the precise mechanisms responsible for the generation, phenotype and functions of human iTreg, we developed an *in vitro* assay system for their expansion [20]. Human CD4+CD25^{neg} T cells and autologous immature dendritic cells (iDC) were co-incubated with irradiated tumor cells and a cytokine mix containing IL-2, IL-10 and IL-15 (20IU/mL of each) for 10 days at 37°C [21]. The cells that outgrew in these cultures gradually acquired phenotypic and functional characteristics consistent with those of iTreg or Tr1 cells as described in the literature [21]. By day 10, most of proliferating T cells were CD3+CD4+CD25+IL-2R β +IL-2R γ +FOXP3+IL-10+TGF- β +IL-4(-), and they strongly suppressed proliferation of autologous responder CD4+ T cells [20]. Using this well-defined model system for the Tr1 generation, we investigated CD39 and CD73 ectonucleotidase expression on Tr1 cells and their potential contribution to adenosine-mediated suppression of T effector (Teff) functions. By flow cytometry and Western blots, most Tr1 cells co-expressed CD39 and CD73 and efficiently hydrolyzed exogenous ATP to adenosine, as shown in ATP consumption assays and by mass spectrometry for adenosine [19]. Upon the addition of ARL67156, a selective CD39 antagonist, or $\alpha\beta$ -methylene ADP, an inhibitor of CD73, Tr1-mediated suppression of proliferation of autologous CFSE-labeled CD4+CD25(-) responder T cells was inhibited, restoring the ability of these cells to proliferate or produce cytokines [19]. These *in vitro* data indicated that human Tr1 co-expressing CD39 and CD73 could produce immunosuppressive adenosine. Tr1 appeared to be able to exert suppressive effects on Teff functions via engaging A_{2A}R, as ZM241865, a selective A_{2A}R antagonist, reversed Tr1-mediated suppression [19]. However, it remained unclear whether *in vivo* generated Tr1, presumed to represent the major subset of Treg in cancer patients, also co-expressed these ectoenzymes.

In a series of *ex vivo* studies with lymphocytes obtained from the peripheral circulation of normal donors and patients with cancer, we showed that CD39 is expressed on the surface of nearly all Treg identified by flow cytometry as CD4+CD25^{high}FOXP3+ T cells [22]. However, CD73 is expressed on only a small subset (~1%) of these cells [23]. Furthermore, CD4+ CD39+ T cells are functionally heterogeneous and may be broadly subdivided into “activated” (CD25+FOXP3+) cells which express CD45RO (i.e., have a memory phenotype) and “resting” CD45RA+ cells which do not mediate suppression [1, 23–25]. Different subsets of iTreg have been recently identified that appear to be phenotypically and functionally distinct from each other. In addition to CD4+CD39+ Treg involved in the

adenosine pathway, there are IL-35 producing iTreg (iTreg35), which do not express FOXP3 and are independent of IL-10 or TGF- β [26], and the iTreg subsets that express select chemokine receptors and mediate suppression of only those Teff lineages that utilize the corresponding chemokines [1]. The absence of CD73 on most circulating CD39+ Treg, in contrast to its presence on many *in vitro* generated Tr1 [27], is of concern, because CD73 is the end-step in adenosine production. Its absence can be, in part, explained by the propensity of CD73 to aggregate on the cell surface, forming “caps,” which are rapidly stripped off the cells. As numerous CD73+ granules are present in the cytoplasm of circulating T and B lymphocytes (demonstrable by confocal microscopy in our preliminary experiments), this enzyme may be readily albeit transiently expressed on iTreg surface when needed. In tumor tissues, for example, we have shown that CD4+ T cells infiltrating tumors (HNSCC) co-expressed CD39 and CD73, and that at least some infiltrating CD4+CD25+ Treg were CD39+CD73+ *in situ* [28]. There also exists a possibility that CD4+CD39+ iTreg producing 5'-AMP could signal via A₁R and directly modulate activities of Teff, because 5'-AMP has been shown to be an A₁R agonist independent of ectonucleosidases and capable of binding to A₁R with an affinity equal to or better than that of adenosine [29]. Yet another mechanism for ensuring the availability of CD73 to iTreg can be proposed by recalling that a CD4+CD73+CD39(-) subset of T cell as well as CD19+ B cells, nearly all of which are CD39+CD73+ [22], and CD39+CD73+ exosomes isolated from the plasma of cancer patients are all good adenosine producers in the presence of exogenous ATP (P. Schuler and T.L. Whiteside, unpublished data). As T cells, B cells and exosomes are ubiquitous components in the blood, body fluids and tissues, we suggest that they could expeditiously deliver membrane-tethered CD73 to enable CD39+ Treg to produce adenosine.

Tumor microenvironment and human iTreg

Tumor-derived soluble factors, such as VEGF, SDF-1, IL-10 and TGF- β , have been acknowledged to be responsible for expansion of iTreg in tumor-bearing hosts [30, 31]. Recently, the number and variety of these factors have been increased to include tumor-derived exosomes which carry death receptor ligands contributing to apoptosis of activated CD8+Teff [32] as well as a number of other cytokines, chemokines and enzymes able to directly induce expansion of Treg [33, 34]. In addition, these factors induce accumulation of immature DC which, in turn, promote the expansion of Treg, thereby contributing to inhibition of anti-tumor immune responses [35]. An enzyme, indoleamine 2, 3-dioxygenase (IDO), produced by DC is one of the most potent inducers of Treg differentiation in the tumor milieu [36]. The IDO activity results in the tryptophan depletion, leading to activation of the GCN2 kinase and to Treg expansion [37]. The ligation of CTLA-4, which is highly expressed on Treg, also leads to enhanced IDO production and favors Treg expansion [36]. In addition, the transcription factor, STAT3, as well as the immunosuppressive cytokine, TGF- β , are abundant in the tumor microenvironment and can also contribute to maintaining elevated IDO expression in DC or tumor cells.

In the tumor microenvironment, accumulating CD4+CD39+ iTreg expand upon induction by TA, DC products and selected cytokines and up-regulate CD73, acquiring the capability to utilize adenosine for mediating suppression of other immune cells. In addition to the adenosine pathway, another suppressive pathway is known to operate in the microenvironment of many human solid tumors, which commonly overexpress cyclooxygenase-2 (COX-2). PGE₂ is a major product of COX-2 activity, and it too is a powerful immunosuppressive factor often implicated in human tumor progression and poor outcome [38]. We reported that *in vitro* generated Tr1 were effective producers of PGE₂ [39].

PGE₂ and adenosine crosstalk in the tumor microenvironment

Similar to adenosine, which mediates suppression by engaging one or more of its four receptors (A₁, A_{2A}, A_{2B}, A₃), PGE₂ suppresses immune responses via the four G protein-coupled receptors (EP1-EP4) expressed on the surface of immune cells. Both adenosine and PGE₂ signaling via their respective receptors leads to up-regulation of 3'5'-cAMP levels in target cells and to down-regulation of their functions [40, 41]. Production of PGE₂ in human tumors is regulated by COX-2, which is often overexpressed in tumor cells and contributes to creating an immune-inhibitory milieu dominated by IL-10 and TGF- β , cytokines known to promote Treg differentiation and proliferation [42]. In a series of *in vitro* experiments, we showed that Tr1 cells generated in the milieu of COX-2+ tumor cells expressed PGE₂, were strongly immunosuppressive, hydrolyzed more exogenous ATP and produced higher levels of adenosine and PGE₂ than Tr1 induced by COX-2(-) tumors [42]. Immune suppression mediated by these Tr1 was blocked in the presence of ectonucleotidase antagonists and also in the presence of indomethacin, confirming that adenosine and PGE₂ contributed to Tr1-mediated immunosuppression [39]. Since most human epithelial tumors produce adenosine and PGE₂, cooperation between these two factors appears to be an important mechanism of tumor-induced immune suppression. Both factors down-modulate Teff functions by controlling 3'5'-cAMP levels in responder cells, presumably by engaging adenylate cyclase-7 (Ac-7), an Ac isoform present in lymphoid cells [43, 44]. As a point of convergence for signals processed by EP2 and A_{2A} receptors, this enzyme contributes to the regulation of 3'5'-cAMP levels in responder cells. It thus represents a potential target for blocking inhibitory signals delivered by adenosine- or PGE₂-producing tumor cells or Treg and for preserving functions of Teff.

The adenosine-PGE₂ axis appears to be one of the most prominent immune-suppressive and also tumor-promoting mechanisms operating at tumor sites, and the fact that iTreg are active participants in this pathway firmly establishes their role in the regulation of immune responses in tumor-bearing individuals.

iTreg in cancer patients treated with chemoradiotherapy (CRT)

When studying the frequency and functions of Treg in the circulation of patients with HNSCC we observed elevated percentages and suppressor activity of Treg in a cohort of patients with no evident disease (NED) after CRT [6]. Untreated HNSCC patients with active disease (AD) have a significantly higher frequency of circulating Treg than NC, and serial studies show that treatments with chemotherapy or radiation lead to substantial increase in both the frequency and functions of these cells [45]. The increase is due in part to shrinking of the CD4+ T cell compartment, as a result of CRT (Th cells are more sensitive than CD8+ T cells to chemo) but also to selective inherent resistance of iTreg to drugs such as cisplatin. iTreg resistance to activation-induced cell death (AICD) favors their survival [45]. Preliminary studies indicated that iTreg can up-regulate expression of serpins and of survival molecules, Bcl-2 and Bcl-xL, and down-regulate pro-apoptotic Bax following exposure to cisplatin. Not only prolonged survival but also expression of CD39 and CD73 ectonucleotidases, adenosine and PGE₂ production as well as the GrB/perforin content characterize iTreg after cisplatin therapy. Thus, iTreg in the circulation of patients treated with CRT appear to be substantially different from nTreg present in the peripheral blood of NC. The question of the role of these chemo-resistant iTreg in regulating post-therapy anti-tumor responses remains unclear. Are they contributing to suppression of anti-tumor immune responses thus contributing to cancer recurrence or are they suppressing post-therapy inflammatory responses thus preventing excessive tissue damage? Further studies will be necessary to answer these questions and to determine whether post-therapy iTreg are good or bad for hosts undergoing potentially curative cancer therapies.

Acknowledgments

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