Published in final edited form as:

Expert Opin Biol Ther. 2011 September ; 11(9): 1203–1214. doi:10.1517/14712598.2011.581225.

Targeting human inducible regulatory T cells (Tr1) in patients with cancer: blocking of adenosine-prostaglandin E₂ cooperation

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Abstract

Introduction—Emerging data suggest that human inducible Treg (Tr1) produce adenosine and prostaglandin E₂ and that these factors cooperate in mediating immune suppression.

Areas covered—Human Tr1 present in human tumors or blood of cancer patients express ectonucleotidases, CD39 and/or CD73, hydrolyze ATP to adenosine and are COX-2⁺. Expression of CD39 and/or CD73 on human tumors favors expansion and suppressor functions of Tr1. Adenosine and PGE₂ signal via A_{2A}R and EP₂R expressed on effector T (Teff) cells, suppressing their anti-tumor functions by a common mechanism involving up-regulation of cytosolic cAMP levels and PKA type I activation. The frequency and activity of circulating CD4⁺CD39⁺ and CD4⁺COX-2⁺ Treg subsets increase in advanced disease and also following oncologic therapies.

Expert Opinion—Pharmacologic blocking of adenosine/PGE₂ collaboration provides a clinically-feasible strategy for disarming of Treg. Used in conjunction with conventional anti-cancer drugs or immune interventions, pharmacologic inhibitors could improve outcome of oncologic therapies.

Keywords

adenosine; cancer; CD39; CD73; PGE₂; Treg

1. Introduction

Human tumors are known to produce a variety of immunosuppressive factors, including adenosine [1, 2], prostaglandin E₂ [3–5], cytokines such as TGF-β [6], tumor-associated gangliosides [7] and others [8, 9]. These factors induce functional abnormalities in those immune cells that are expected to target and eliminate tumor cells [10]. It is important to realize that tumor-induced immune dysfunction is not generic but is restricted to tumor-specific immune responses. While anti-viral or anti-bacterial immune responses are largely unimpaired in most cancer patients, anti-tumor immune responses are selectively compromised. As a result, cancer patients are unable to mount effective anti-tumor immune responses. Tumor-induced dysfunctions are: (a) responsible for abnormalities in a variety of cellular activities such as signal transduction, activation, cytokine production, proliferation, cytotoxicity and cell migration [11]; (b) concomitantly present in a variety of immune cell

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Declaration of Interest

Supported in part by NIH grant PO1 CA109688 to TL Whiteside.

types, including T cells, natural killer (NK) cells, monocytes, dendritic cells (DC) and B cells [11]; (c) variable in the profile of dysfunctions demonstrable in immune cells and in the severity of these dysfunctions [12]; (d) not only local (i.e., involving immune cells in the tumor or tumor-involved lymph nodes) but also systemic [13]. Immune abnormalities in cancer patients are thought to contribute to cancer progression by enabling the tumor to escape from immune control [14].

Human tumors are also infiltrated by immune cells, especially T lymphocytes and myeloid cells, which are recruited to the site by tumor-derived factors and which may be subverted by the tumor to promote its growth [15–17]. Alternatively, infiltrating immune cells may retain a limited degree of anti-tumor effector functions, depending on the tumor aggressiveness in implementing immune suppression [18, 19]. Among mononuclear cells infiltrating solid tumors are CD3⁺ T cells, with various proportions of CD4⁺ and CD8⁺ lymphocytes, which may be CD25⁺ [11]. These activated, IL-2R-expressing T cells accumulate in the tumor milieu, and often contain substantial proportions of CD4⁺CD25^{high} T cells which also express FOXP3, confirming that they are regulatory T cells (Treg). It has been reported that Treg accumulations are common in many, but not all, human solid tumors [20–22]. However, it appears that the greater the frequency of activated CD8⁺ T cells in the tumor microenvironment, the higher is the proportion of Treg [22]. This suggests that the ratio of activated T effector cells (Teff)/Treg could be an important measure of immune activity in the tumor milieu. Also, an increased frequency of Treg at the tumor site or in the peripheral circulation of patients with cancer appears to positively correlate with tumor progression and poor prognosis [21, 23]. On the other hand, in colorectal cancer, Treg infiltrates are associated with a better prognosis [24, 25]. In the gut, where brisk chronic pro-inflammatory responses favor tumor development, the presence of Treg may be beneficial to the host [25]. This type of data suggests that the local tissue environment influences Treg frequency and probably their activity. Most human solid tumors are able to create and maintain the microenvironment favorable for tumor escape from the host immune system by counter-balancing accumulations of activated Teff by an enrichment in Treg. In addition, other unique features of the tumor microenvironment, including the presence of cell necrosis, “aerobic glycolysis,” acidosis and hypoxia [26], favor dysfunction of the Teff, but not Treg recruited to the tumor site. In contrast to Teff, tumor-infiltrating Treg are not debilitated but rather activated, are resistant to apoptosis, mediate high levels of suppressor functions and, in general, appear to thrive [27].

Treg are a small subset of CD4⁺ T cells which interact with Teff suppressing their functions [28, 29]. Since their first descriptions in the mid 1990's, several Treg subsets have been recognized which utilize distinct mechanisms of suppression. Thus, Treg are functionally heterogeneous comprising subsets of phenotypically similar cells able to suppress functions of Teff via distinct and often unexpected mechanisms [30, 31]. At least two Treg subsets have been recognized in humans: (a) natural Treg (nTreg), which originate in the thymus, mediate suppression by cell contact-dependent mechanisms involving the Granzyme B/perforin or Fas/FasL pathways and constitute the major regulatory T-cell subset responsible for maintaining peripheral tolerance to self [32, 33]; (b) inducible Treg (iTreg) also referred to as type 1 regulatory T cells (Tr1), which are induced in the periphery following chronic antigenic stimulation in the presence of IL-10 derived from tolerogenic antigen-presenting cells [34]. They differentiate into active suppressor cells which mediate suppression via contact-independent mechanisms largely involving TGF-β1, IL-10 or other soluble immunoinhibitory factors [35, 36]. While the phenotype of human Treg is not yet firmly defined, most nTreg are CD3⁺CD4⁺CD25^{high}FOXP3⁺, while Tr1 cells are CD4⁺CD25^{low}CD132⁺TGF-β⁺IL-10⁺IL-4⁻ [35]. The frequency of Treg is increased in the peripheral circulation of most patients with cancer or chronic viral infections [28, 38–40], and it is decreased in individuals with autoimmune diseases [41]. Today, the role of Treg in

suppression of anti-viral or anti-tumor immunity appears to be firmly established, and they may represent a key participant in mediating tumor escape from the host immune system. At the same time, it should be remembered that Treg-mediated regulation of activated immune cells represents a physiologically normal response designed to maintain a homeostatic balance and prevent undesirable immune activation.

High levels of suppression mediated by Treg found in the tumor microenvironment, e.g., among tumor infiltrating lymphocytes (TIL), relative to suppression mediated by autologous Treg in the peripheral blood [37], imply that the tumor-derived factors might be involved in conditioning of Treg or regulating their recruitment to the tumor site or in their generation and differentiation. In this review, we describe the evidence for intercellular interactions between the tumor and Treg that involve two well known soluble immunosuppressive factors found in the tumor microenvironment, adenosine and prostaglandin E₂ (PGE₂). Human tumors may express and produce both these factors and suppressive activities of both have been well documented in the literature [42, 43]. However, the ability of human Treg to utilize adenosine and PGE₂ for inducing Teff suppression is a new and important finding. In addition, the paucity of adenosine deaminase (ADA) in Treg and the dependence of Teff on ADA expression for protection from adenosine-mediated suppression illustrate the diversity of mechanisms employed by immune cells in regulating resistance/sensitivity to suppression. Insights into molecular mechanisms driving interactions between Treg and the tumor provide us with an opportunity to devise novel strategies for silencing Treg-mediated suppression in cancer through pharmacologic or immunologic means. The same therapeutic strategies could potentially eliminate tumor escape from the host immune system by directly inhibiting adenosine and PGE₂ production by the tumor.

2. Adenosine in the tumor microenvironment

Adenosine is an anti-inflammatory product of enzymatic hydrolysis of extracellular ATP by CD39 (ecto-ATPase) and CD73 (ecto-5'-AMP nucleotidase). The source of adenosine in the tumor microenvironment can be tumor cells, infiltrating immune cells and epithelial cells. The tumor microenvironment is characterized by tissue disruption and numerous dying tumor cells resulting in ATP release, high levels of ectonucleotidase activity and the accumulation of extracellular adenosine (e.g., 10–20 fold increase in tumor vs. normal tissues) [2]. Local hypoxia greatly favors ectonucleotidase activity and adenosine metabolism [26]. Adenosine has well-documented immunosuppressive activity, which contributes to the inhibition of local and systemic anti-tumor immune responses [1, 2]. Overexpression of CD39 and, especially CD73, in human tumor cells, including melanoma, breast carcinoma and HNSCC has been reported [40, 44, 45], suggesting that tumors are a major source of endogenous adenosine. In this respect, we have shown that a HNSCC cell line, PCI-13, which expressed CD73 but little CD39 on the cell surface efficiently converted exogenous AMP to adenosine, while ANT-1, another HNSCC cell line, expressed no CD39 or CD73 and produced no adenosine (Figure 1). In the presence of $\alpha\beta$ -methylene ADP, a specific inhibitor of CD73 activity, adenosine production was significantly inhibited in PCI-13 cells. These data illustrate the variable capabilities of HNSCC to produce adenosine from exogenous ATP or ADP, depending on the expression of exonucleotidases in the tumor. It is reasonable to suggest that because of high levels of extracellular ATP generated in the tumor microenvironment, ectonucleotidase activity might be extremely important for removing its excess and protecting the tumor from ATP-associated toxicity [46]. At the same time, tumor growth is promoted by adenosine-mediated inhibition of anti-tumor T-cell immunity [47]. In support of this view, it has been shown that ectonucleotidase expression in tumor cells correlated with tumor progression, invasiveness, neoangiogenesis and metastasis formation [48, 49].

More recently, it has become clear that Treg also express CD39 and CD73 proteins which are enzymatically active [50]. Initially described in murine Treg [51, 52], ectonucleotidases were found by us and others to be present on human Treg. We have demonstrated that Treg sorted from human PBMC hydrolyze exogenous ATP and generate adenosine [50, 53]. In human lymphocytes, expression of CD39 and CD73 is not limited to Treg as CD8⁺ T cells, and B cells are positive as well. Up to 5% of CD4⁺ T cells express CD39 and of these, 50% or more co-express CD73 as determined by flow cytometry (Figure 2A). In humans, CD39 is strongly expressed on Treg and can serve as a surface marker for the isolation of CD4⁺CD39⁺ human Treg [54, 55]. Our recent data show that Treg isolated from the peripheral blood based on CD39 expression contain a subset of CD4⁺CD39⁺CD25⁺FOXP3⁺ cells which mediate suppression and a subset of CD4⁺CD39⁺CD25^{neg} FOXP3⁻ cells which do not [54]. This finding suggests plasticity of Treg that may be shaped by events in their milieu [56].

While we and others consistently show expression of CD39 and CD73 on human Treg in the peripheral circulation of patients with cancer, the presence of these ectonucleotidases on Treg accumulating in tumor tissues has not been documented. Therefore, we searched for CD39 and CD73 expression in HNSCC tissue sections stained for these markers. As shown in Figure 2B, T cells co-expressing CD39 or CD73 and other phenotypic markers usually associated with Treg were detected *in situ* [50], confirming that ectonucleotidases are present on Treg in human tumors and, if enzymatically active, could be responsible for adenosine production. While we have documented the presence of enzymatically-active ectonucleotidases in Treg obtained from human peripheral blood [50, 53], the level of enzymatic activity mediated by CD39 and CD73 expressed on Treg present in tumors awaits further studies with tumor-infiltrating lymphocytes in suppression assays.

Adenosine binds to A₁, A_{2A}, A_{2B} and A₃ receptors (R) expressed on the surface of various cell types, including lymphocytes and dendritic cells (DC). In immune cells, suppressive effects of adenosine are largely mediated through A_{2A}R and A_{2B}R signaling [57] with a concomitant activation of adenylyl cyclase and up-regulation of cAMP resulting in a functional paralysis of responding Teff cells (Figure 3). Thus, Treg-generated adenosine binds to A_{2A}R or A_{2B}R liberally expressed on CD4⁺ Teff, which results in the inhibition of T-cell proliferation and cytokine production. An elevation of cAMP levels that follows A_{2A}R triggering in these cells leads to activation of protein kinases which mediate protein phosphorylation. Studies have shown that immunosuppressive effects of adenosine binding to its receptor on T cells appear to be largely mediated by protein kinase A type I (PKA I) [53, 58].

3. Adenosine deaminase (ADA) and its importance for Treg suppressor function

Although ectonucleotidases, CD39 and CD73, are present in most human Treg, and CD39 has been considered as a potential Treg marker useful for Treg isolation [53, 54], CD26 is absent/low on the surface of these cells (Figure 4). CD26 is a 110 kD glycoprotein with intrinsic dipeptidyl peptidase IV activity whose extracellular domain is associated with ADA [59]. CD26 is highly expressed on the surface of all conventional CD4⁺ T cell subsets (Tconv), where it serves as an anchor for ADA and, therefore, localizes ADA to the cell surface [60, 61]. In contrast to Treg, Tconv are CD4⁺ non-activated CD25^{neg} T cells. Signaling via CD26 on Tconv cells involves CD45 molecules and links CD26 and ADA to T-cell differentiation into Teff which mediate helper functions [62]. ADA hydrolyzes adenosine to inosine, decreasing its pericellular concentration in CD4⁺CD25^{neg} Tconv. On the other hand, Treg, which do not express ADA, can accumulate adenosine in the pericellular space and use it to inhibit functions of other T cells. It is likely that the ability of

Teff to deaminate adenosine is a protective mechanism, allowing these cells to in part escape from adenosine-mediated suppression. Because Treg have fewer A_{2A}R relative to Teff, they might be less sensitive to inhibitory activity of adenosine. In contrast, activated Teff which upregulate A_{2A}R are very sensitive to adenosine-mediated inhibition and require ADA for protection from Treg. Due to the increased expression of ectonucleotidases, the absence of the CD26/ADA complex and low ADA activity, Treg are equipped with a complete set of tools to not only generate adenosine but to maintain high levels of extracellular adenosine in their microenvironment.

Thus, we have shown that human CD4⁺CD25^{high}FOXP3⁺ Treg, which are positive for CD39 and CD73 but negative for CD26 are able to sequentially hydrolyze exogenous ATP to adenosine, accumulate it pericellularly and utilize it to induce suppression of Teff cell proliferation [50].

4. Human inducible Treg (Tr1) produce adenosine and use it for mediating suppression

To address the possibility that activated inducible Treg can also produce adenosine, we utilized an *in vitro* assay system [63]. Co-cultures of naïve, CD4⁺CD25^{neg} T cells obtained from the peripheral blood of normal donors were co-incubated for 10 d with autologous immature dendritic cells (iDC) and irradiated tumor cells in the medium containing low doses of cytokines (IL-2, IL-10 and IL-15) and cross-linked anti-CD3 antibody. The T cells outgrowing in these co-cultures gradually acquired the Tr1 phenotype and suppressor activity. By day 10, most of the proliferating T cells were CD3⁺CD4⁺CD25^{low}IL-2Rβ⁺IL2Rγ⁺FOXP3⁺IL-10⁺TGF-β⁺IL4⁻, and they effectively mediated suppression of autologous responder cell proliferation [63]. These cells also hydrolyze exogenous ATP to adenosine and are themselves adenosine producers [53]. Thus, our data indicated that not only nTreg but also Tr1 cells could generate and release adenosine. Using CFSE-based suppression assays, in which naïve autologous CD4⁺CD25^{neg} T cells were responding to TcR-mediated signals, we evaluated the involvement of adenosine in Treg-mediated suppression of proliferation [53]. We observed that in the presence of ARL67156, a selective CD39 antagonist or αβmethylene ADP, an inhibitor of CD73, as well as an agonist of A_{2A}R, ZM241865, Tr1-mediated suppression of proliferation was significantly inhibited [53]. Furthermore, not only proliferation but also cytokine secretion by T cells responding to TcR-mediated signals was inhibited by Treg-derived adenosine and was relieved in the presence of the above mentioned antagonists. These data suggest that Treg producing adenosine can suppress not only the expansion of responder cells but also their effector functions.

5. PGE₂ in the tumor microenvironment

PGE₂ is a major product of cyclooxygenase 2 (COX-2) activity [64]. COX-2 is overexpressed by many human tumors, and its expression has been linked to tumor progression and poor patient survival [65]. PGE₂ mediates its immunoinhibitory activity via four different G protein-coupled receptors (EP₁–EP₄) expressed on various cells. PGE₂ signaling leads to an intracellular increase and activation of cAMP, with a concomitant decrease in cell proliferation and suppression of other functions in immune cells [66, 67]. PGE₂ also induces Tr1 cells and modulates their activity thus contributing to creating a tolerogenic milieu [20]. In a series of *in vitro* experiments in which tumor cells were engineered to express COX-2 or were either COX-2^{neg} or had COX-2 expression inhibited (the COX-2 gene knock out with siRNA; Diclofenac, a generic COX inhibitor), we demonstrated that the outgrowth of Tr1 cells and their suppressive activity mediated via released IL-10 and TGF-β were dependent on COX-2 expression in the tumor cells [20].

Using the same type of *in vitro* assays with COX-2⁺ and COX-2^{neg} tumor cells, we showed that the former induced a significantly greater number of Tr1 cells than the latter. Also, Tr1 generated in co-cultures with COX-2⁺ tumor cells were significantly more suppressive, hydrolyzed more exogenous ATP, and produced higher levels of adenosine and PGE₂ ($p < 0.05$ for all) than Tr1 induced by COX-2^{neg} tumors. Tr1 cells induced in the presence of COX-2⁺ tumors expressed COX-2 themselves and were able to produce PGE₂. These COX-2⁺ Tr1 co-expressed CD39 and CD73 and in addition to PGE₂, they also produced adenosine [53]. Their suppressor function was inhibited in the presence of ectonucleotidase antagonists and also in the presence of indomethacin, confirming that both adenosine and PGE₂ contributed to Tr1-mediated suppression.

6. Effects of Treg-derived adenosine and PGE₂ on Teff functions

Since tumor cells as well as Treg produce adenosine and PGE₂, it has been suggested that these two suppressive factors cooperate in mediating immune suppression in the tumor microenvironment [67]. Both factors activate respective G protein-coupled receptors which mediate intracellular signaling via cAMP. PGE₂ mediates its biologic effects through EP₁, EP₂, EP₃ and EP₄ receptors [68], while adenosine binds to A_{2A}R and A_{2B}R expressed on immune cells. By adding AH6809, an EP₂R antagonist, to co-cultures of Tr1 and Teff, we determined that the inhibitory signal mediated by PGE₂ is largely delivered via the EP₂R. The addition of AH23848, an EP₄R antagonist, or all other EPR antagonists had no effect on Teff proliferation in these co-cultures [53]. Similar studies were performed with an A_{2A}R and A_{2B}R antagonist, ZM241385, to illustrate that Tr1-derived adenosine mediates suppression of Teff proliferation by binding to its receptors on Teff cells. In aggregate, these studies do not explain the differential contribution of each factor to Tr1-mediated suppression. However, we have determined that the total level of PGE₂ produced in co-cultures of Tr1 with Teff cells is approximately 100-fold greater than the level of adenosine. Yet, the use of receptor antagonists in the proliferation inhibition assays showed an almost equal reversal of suppression for both PGE₂ and adenosine. These preliminary studies with receptor antagonists need to be extended to consider factors such as the density of A_{2A}R and EP₂R on responding Teff cells as well as the respective receptor affinities to be able to adequately evaluate cooperation between Tr1-derived adenosine and PGE₂ in mediating suppression of Teff cell proliferation. We have determined that PKA type I in Teff cells is involved in inhibitory activities of adenosine and PGE₂ [50]. Using Rp-8-Br-cAMPS, an agent which prevents binding of cAMP to the regulatory subunit of PKA type I, we showed that Tr1-mediated suppression of Teff proliferation was significantly inhibited, suggesting that blocking of PKA type I activity in Teff cells protects them from suppression by PGE₂- and adenosine-producing Tr1 cells [53].

7. Functionally distinct subsets of human Treg

While the above described *in vitro* studies indicated that adenosine and PGE₂ play a major role in suppression mediated by Tr1 cells, the *in vivo* involvement of these factors in Treg activities in cancer-related immune suppression has not been documented. To this end, we studied PBMC of HNSCC patients for the presence of Tr1 subsets by quantitative flow cytometry, including PGE₂⁺, CD39⁺/CD73⁺, IL-10⁺ and TGF-β1⁺ subpopulations. In the circulation of patients with HNSCC, the frequency of CD39⁺ and COX-2⁺ Treg was significantly increased relative to that in normal controls (NC). We have also shown that the percentage of CD39⁺ Treg in the patients' blood was significantly greater in patients with later than early stage disease, suggesting that the increased frequency of CD39⁺ Treg in the peripheral circulation of HNSCC patients is related to disease progression [23]. Further, we compared the frequency of CD39⁺ Tr1 cells in the peripheral blood of HNSCC patients prior to and after oncologic treatments and consistently observed a significant increase after

oncologic therapy (Figure 5A) [53]. The proportions of IL-10⁺ and TGF- β 1⁺ CD4⁺ T cells were also elevated in the patients' blood relative to those in NC, but their frequency was significantly reduced after therapy (Figure 5B). Also, we found that while CD39, CD73 and COX-2 were co-expressed in CD4⁺ T cells, IL-10 and TGF- β 1 were expressed by non-overlapping, distinct CD4⁺ T cell subset (Figure 5B). If these preliminary observations are confirmed, they point to the existence of distinct Treg subsets in the peripheral circulation of patients with cancer: those that produce adenosine/PGE₂ and those that suppress by secreting IL-10 or TGF- β 1. Only the former Treg subset expands following radiochemotherapy. Further, we observed co-expression of CD39 and COX-2 in Tr1 cells within the TIL at the tumor site [55]. At the very least, these data support the conclusion that Tr1 cells present in the blood and tumor tissues in cancer patients co-express CD39 and COX-2 and, therefore, can produce both immunosuppressive adenosine and PGE₂.

8. Clinical significance of the adenosine/PGE₂ cooperation

Adenosine and PGE₂ are well known immunoinhibitory factors present in the tumor microenvironment [4, 26, 47, 49]. Their production by many human tumors has been documented [26, 43]. However, the contribution of Treg, a small subset of CD4⁺ T cells, to adenosine and PGE₂ production is a novel and potentially clinically important finding. Our preliminary data suggest that adenosine and PGE₂ produced by human Treg might synergize in mediating suppression of Teff cell functions. This cooperation produces a more potent immunosuppressive effect. *In vitro*, tumor cells expressing COX-2 drive the development of Tr1 cells that are producers of both adenosine and PGE₂. In patients with cancer, Tr1 cells able to produce both factors are increased in frequency and appear to be resistant to conventional oncologic therapies. While these results are preliminary, they suggest that the tumor/tissue microenvironment determines the frequency, quality and mechanisms of suppression inducible Treg employ. As human tumors are often COX-2⁺ and rich in extracellular ATP because of cell death, opportunities exist for ATP-mediated up-regulation of ectonucleotidase activity and COX-2 expression in Tr1 cells generated in or attracted to the tumor microenvironment. In fact, up-regulation of these molecules is known to occur during inflammation that often is a component of the tumor development [67, 69]. Cooperation between adenosine and PGE₂ creates a powerful and effective milieu for suppression of anti-tumor immune responses and thus for tumor progression. Therefore, the hypothesis we favor is that the most successful human tumors take advantage of cooperative interactions between various suppressive molecules produced in their milieu to create a strongly immunosuppressive network of molecules engaging them in support of tumor growth and metastasis. The existence of such cooperative networks is likely to be responsible for tumor escape and its resistance to available therapies.

9. Pharmacologic interventions to restore immune cell functions

If the cooperation between the immunosuppressive molecules, adenosine and PGE₂, favors tumor progression, then an obvious solution is to prevent or reduce it by utilizing inhibitors or antagonists available in many formats. This may be accomplished by directly interfering with production of either factor or with their binding to respective receptors on immune cells. A number of pharmacologic strategies exist today to accomplish this [70], and many of these strategies have already been utilized in diseases other than cancer [71]. Traditionally, Treg depletion has been used to improve endogenous anti-tumor immunity and the efficacy of immunotherapies using a variety of different strategies, including the administration of low-dose cyclophosphamide, daclizumab (anti-CD25 Ab), denileukin diftitox (Ontak) or tyrosine kinase inhibitors (TKIs) such as Sunitinib [72–74]. These anti-Treg regimens transiently reduce Treg numbers and function in the blood of some patients with cancer [74]. Invariably, however, Treg come back, and blocking of their activity

becomes again necessary [75]. An alternative strategy of controlling excessive Treg function in cancer is to use pharmacologic inhibitors which specifically target the extracellular 3',5'-cAMP pathway and/or the PGE₂ pathway [64, 76]. As shown here, adenosine binding to A_{2A}R and PGE₂ binding to EP₂R expressed on Teff cells suppressed their anti-tumor functions by a common mechanism involving up-regulation of cytosolic cAMP levels and PKA type I activation. There are numerous clinically available pharmacologic agents that can effectively block these pathways. For example, pharmacologic inhibitors of ectonucleotidase activity, A_{2A}R and EP₂R antagonists or inhibitors of PKA-1 type I decreased Tr1-mediated suppression of Teff proliferation in our experiments [53]. Also, Rolipram, a phosphodiesterase 4 (PDE₄) inhibitor, increased cAMP levels in Teff and consequently increased their susceptibility to Tr1-mediated suppression [53]. Similarly, many drugs blocking COX-2 activity are in clinical use, including indomethacin, diclofenac, ibuprofen, Celecoxib and others [64, 77]. Of particular importance, however, is the fact that adenosine- and PGE₂-mediated signals received by their respective surface receptors on Teff converge at the level of adenylyl cyclase (AC), the key enzyme responsible for cAMP synthesis [78]. The intracellular cAMP concentration is regulated by enzymatic activity of AC, which catalyzes the formation of cAMP and its degradation by PDEs [79]. The AC-7 isoform, which is expressed only in immune cells [80] and is responsible for integrating these signals emerges as a potentially very attractive therapeutic target. The simultaneous blockade of adenosine and PGE₂ signaling at the point of their convergence in Teff might restore anti-tumor immune response in patients with cancer. Currently, the development of AC-7 isoform-selective inhibitors, which would block adenosine-PGE₂ cooperation in Teff and thus restore their anti-tumor competence, is a high priority.

10. Conclusions

In this review we summarize our recent insights into mechanisms utilized by human Treg to induce immune suppression in patients with cancer. It emerges that one of the most prominent mechanisms utilized by activated inducible Treg (Tr1) in the microenvironment of solid tumors such as, e.g., HNSCC, is the production by Tr1 of adenosine as well as PGE₂. The frequency of the CD39⁺ Treg cells in the periphery was related to cancer progression, an indication that their suppressor function may have clinical consequences. The Tr1 subsets present in human tumors or PBMC of cancer patients are COX2⁺/CD39⁺ or IL-10⁺/TGF-β⁺, suggesting that functionally diverse Tr1 subsets may emerge during tumor progression or as a result of therapeutic interventions, both of which might alter the tumor microenvironment. One strategy for overcoming tumor-induced immune suppression and for eliminating tumor escape, involves a pharmacologically-mediated blockade of cooperative interactions between inhibitory factors present in the tumor milieu that suppress immune responses. A potentially promising therapeutic target that recently has emerged is AC-7, an enzyme regulating cAMP synthesis in immune cells. By selectively blocking activity of this enzyme, it may be possible to inhibit adenosine/PGE₂ collaboration, disarm Treg and restore anti-tumor immune functions in Teff in cancer patients.

11. Expert Opinion

It has been recognized that Treg exercise a negative influence on anti-tumor immune responses in patients with cancer. Increased levels of immune activation in response to malignancy as well as tissue damage elicited by the growing tumor and exacerbated by oncologic therapies culminate in the state of chronic inflammation that is characterized by accumulations of activated lymphocytes, including Teff and Treg. Tumor-specific immune responses are thus counterbalanced by activities of suppressor cells, which are empowered by the tumor to inhibit and keep in check anti-tumor immunity. In this situation, where the tumor actively recruits, expands and promotes functions of Treg, it may not be sufficient to

deplete or eliminate Treg expecting to restore anti-tumor immunity. As is well established, cyclophosphamide or antibodies targeting Treg-associated molecules, e.g., anti-CD25 or anti-TGF- β 1 Abs, have been reported to effectively albeit transiently reduce Treg numbers *in vivo* or block their functions without substantial benefit in the disease outcome. The existence of close and intimate cooperation between the tumor and Treg calls for a more complex combinational therapeutic strategy that might be necessary to restore anti-tumor immunity. It might not be enough to deplete Treg prior to delivery of anti-tumor vaccines, for example. The tumor is likely to recruit more Treg and inhibit newly-generated anti-tumor immunity, shifting the balance in its own favor. Chemotherapy or chemoradiotherapy administered concomitantly with anti-tumor vaccines may not improve the results, because they seem to exacerbate rather than relieve chronic inflammation and Treg-tumor cooperation. Further, evidence is emerging that some chemotherapies might promote Treg activity and survival. The future anti-cancer strategies are likely to make use of carefully selected pharmacologic inhibitors, many of which are already in the clinic for treatment of conditions other than cancer. By targeting molecular pathways responsible for immune suppression, this “blocking the inhibitor” strategy in combination with conventional drugs could considerably improve outcome of anti-cancer therapy [70].

List of Abbreviations

A_{2A}R	adenosine 2 _A receptor
ADA	adenosine deaminase
COX-2	cyclooxygenase 2
DC	dendritic cell
EP2R	prostaglandin E ₂ receptor 2
HNSCC	head and neck squamous cell carcinoma
IL-10	interleukin 10
IVA	<i>in vitro</i> assay
Teff	effector T cell
Tconv	non-differentiated conventional CD4 ⁺ T cell
TGF-β	transforming growth factor-beta
TIL	tumor-infiltrating lymphocytes
Treg	regulatory T cell
Tr1	inducible regulatory T cell
PBMC	peripheral blood mononuclear cell
PDE₄	phosphodiesterase 4
PGE₂	prostaglandin E ₂
PKA type I	protein kinase A type 1

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Figure 1.

(A) Ectonucleotidase expression in PCI-13 and ANT-1 tumor cell lines. Tumor cells were surface stained for CD39 and CD73 and analyzed by flow cytometry. One representative experiment of 3 performed is shown. (B) Adenosine production by PCI-13 and ANT-1. 1×10^6 PCI-13 and ANT-1 tumor cells were incubated with either 100 μ M ATP in the presence or absence of ARL67156 (250 μ M), an ecto-ATPase inhibitor, or 100 μ g AMP in the presence or absence of α,β -methylene ADP (100 μ M), a specific CD73 inhibitor. One representative experiment of 3 performed is shown.

Figure 2.

(A) Flow cytometry of human CD4⁺ T cells showing surface expression of CD39 and CD73. (B) Confocal images of a human HNSCC sectioned and stained for CD4⁺CD25⁺CD39⁺ or CD4⁺CD25⁺CD73⁺ T cells. In panel B, CD4⁺ cells are green, CD25⁺ cells are red; in panel C, CD39⁺ cells are blue, CD4⁺CD25⁺CD39⁺ cells are violet and Tconv are red; in panel D, CD73⁺ cells are blue, CD4⁺ cells are green, CD25⁺ cells are red and CD4⁺CD25⁺CD73⁺ cells are violet. Mag × 200; inserts × 400. Reproduced with permission from the AACR Publication Department [23].

Figure 3.

Tr1-derived adenosine and PGE₂ bind to A₂AR and EP2R expressed on T effector cells and suppress functions of T effector cells.

Figure 4.

(A) Western blots showing expression of ectonucleotidases, CD39 or CD73, and of CD26 and ADA in human isolated CD4⁺CD25^{high} Treg and CD4⁺CD25^{neg} conventional T cells (Tconv). (B) Confocal images of Treg and Tconv showing co-expression of 26 and ADA in Tconv. Reproduced from an article originally published in *J. Biological Chemistry* 285:7176, 2010. ©The American Society for Biochemistry and Molecular Biology.

Figure 5.

(A) The frequency of CD4⁺CD39⁺ T cells in the peripheral blood of HNSCC patients is increased compared to that in normal controls (NC). The percentages of CD39⁺ Treg were tested in HNSCC patients prior to or after oncologic therapy. (B) Tr1 cell subsets in the peripheral circulation of patients with HNSCC or normal donors (ND). The frequency of CD4⁺IL-10⁺, CD4⁺TGF-β1⁺, CD4⁺COX-2⁺ and CD4⁺CD39⁺ Treg subsets was evaluated by flow cytometry in patients with active disease prior to therapy (AD), no evident disease (NED) after chemoradiotherapy and in normal controls (NC). Figure 5A is reproduced with permission from the AACR Publication Department [23]. Figure 5B is reproduced from an article originally published in *Journal of Biological Chemistry* 285: 27571–80, 2010. ©The American Society for Biochemistry and Molecular Biology.