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REVIEW

ENTPD1/CD39 is a promising therapeutic target in oncology

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Regulatory T cells (Tregs) are a subpopulation of $CD4^+$ T cells that are essential for maintaining the homeostasis of the immune system, limiting self-reactivity and excessive immune responses against foreign antigens. In cancer, infiltrated Tregs inhibit the effector lymphocytes and create a favorable environment for the growth of the tumor. Although Tregs mediate immunosuppression through multiple, non-redundant, cell-contact dependent and independent mechanisms, a growing body of evidence suggests an important role for the CD39–CD73–adenosine pathway. CD39 ectonucleotidase is the rate-limiting enzyme of a cascade leading to the generation of suppressive adenosine that alters CD4 and CD8 T cell and natural killer cell antitumor activities. Here, we review the recent literature supporting CD39 as a promising therapeutic target in oncology. *In vitro* and *in vivo* experiments involving knockout models and surrogate inhibitors of CD39 provide evidence in support of the anticancer activity of CD39 inhibition and predict a favorable safety profile for CD39 inhibitory compounds. In addition, we report the ongoing development of CD39-blocking monoclonal antibodies as potential anticancer drugs. Indeed, CD39 antagonistic antibodies could represent novel therapeutic tools for selectively inhibiting Treg function without depletion, a major limitation of current Treg-targeting strategies.

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REGULATORY T CELLS AND CANCERS

Regulatory T cells (Tregs) are classified within the CD4 T lymphocyte subset and are characterized by the expression of the forkhead box P3 (FOXP3) transcription factor. They are highly beneficial for immune system homeostasis because they prevent self-responsiveness of T lymphocytes that have escaped 'central tolerance' in the thymus, thereby avoiding potential chronic inflammation and autoimmune manifestations against self constituents. They also have an important homeostatic function, regulating effector lymphocyte expansion during the immune response. However, in certain conditions, they may be detrimental for the host, because they can limit the immune response against some pathogens. In addition, in a cancer setting, Tregs can block spontaneous or vaccine-induced antitumor responses by inhibiting effector T or natural killer (NK) lymphocytes.

It has been widely reported that Tregs are enriched in numerous cancer types, where they often are correlated with poor prognosis.[1–4] The Tregs in the tumor environment may be naturally occurring Tregs (natural Tregs) that originate from the thymus and can proliferate and expand upon activation by the tumor.[5] Tregs may also develop at the periphery from conventional $CD4^+$ T cells through a process termed 'conversion'. Such converted Tregs are called 'induced Tregs'.[6,7] Both recruited and induced Tregs are activated within the tumor or the draining lymph node upon encountering tumor-associated antigens. They then inhibit the immune antitumor response mediated by NK cells or effector T cells, thereby allowing tumor progression (for a review, see Nishikawa and Sakaguchi[8]). Treg infiltration of cancerous lesions and expansion are promoted by different arrays of chemokines, cytokines and growth factors. For instance, CCL22 is released by cancer cells or macrophages and attracts Tregs at the tumor site,[1,9] whereas TGF-β (transforming growth factor beta) promotes Treg-cell proliferation.[10,11]

MECHANISMS OF TREG-MEDIATED SUPPRESSION

Tregs suppress the immune system through multiple mechanisms that are dependent or independent of cell-contact. These mechanisms can be grouped into at least four basic modes of action: suppression mediated by gap junctions that leads to increased cytoplasmic cAMP levels in the target lymphocytes; suppression by cytolysis *via* the granzyme A and B/perforin pathway; suppression through the inhibition of dendritic cell (DC) maturation and/or function; and suppression by the generation of adenosine *via* a pathway that involves the ENTPD1/CD39 ectonucleotidase.[12] Two cytokines, IL-10 and TGF-β, also have an important role in the generation of the induced-Treg populations, Tr1 and Th3, which in turn mediate suppression through IL-10 and TGF-β.[13–15] How many of these mechanisms are required for Treg-mediated suppression *in vivo* remains unclear, but it seems likely that multiple non-redundant mechanisms coexist, with each pathway contributing to the mechanistic whole, while the importance of each pathway may vary and be context specific. Regardless of the mechanism, mouse models have clearly demonstrated that Tregs can shut down the immune system and thus foster tumor progression and that targeting Tregs can alleviate immunosuppression and restore an efficient immune antitumor response. Curiel *et al.*,[1] for example, demonstrated that adoptive transfer of effector T cells into NOD/SCID mice drastically reduced tumor growth, while this beneficial immune response was abrogated by additionally co-transferring Tregs. Therefore, targeting Tregs to restore an efficient spontaneous or

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vaccine-induced antitumor response appears to be a promising therapeutic strategy.

CURRENT STRATEGIES TARGETING TREGS FOR CANCER THERAPY AND THEIR LIMITATIONS

Tregs are defined as $CD4^+$ $CD25^{high+}$ $FOXP3^+$ T cells that express other markers, such as the glucocorticoid-induced TNF (tumor necrosis factor) receptor family-related gene (*GTIR*) and the cytotoxic T-lymphocyte antigen 4 (CTLA-4). Although these markers are widely used to define the Treg subpopulation, none of these markers is specific to Tregs, but rather defines activated T cells (for a review, see Nishikawa and Sakaguchi[8]). Even the FOXP3 transcription factor might not be specific to Tregs in humans.[16] However, depleting strategies have targeted these cell surface molecules in attempt to restore an efficient immune antitumor response. For instance, depletion of the entire $CD4^+$ subpopulation (including Tregs) led to CD8-mediated rejection of fibrosarcoma in mice.[17] Based on these studies, preclinical and clinical studies combining tumor vaccines and Treg-depleting strategies were conducted, with some obvious successes.

Targeting CD25/IL-2

The activation of T cells results in upregulation of CD25, the alpha chain of the IL-2 receptor, and IL-2 binding to its receptor triggers T-cell activation and proliferation. Historically, anti-CD25 antibodies (such as daclizumab) were developed to deplete alloreactive effector T lymphocytes and prevent graft rejection during renal transplantation.[18] However, treatments with anti-CD25 antibody were shown to suppress tumor growth in mice, and it was later shown that anti-CD25 antibodies also deplete Tregs.[19,20] Anti-CD25 antibody administration before cancer cell grafting efficiently depleted $CD25^{high+}$-Tregs without interfering with subsequent CD4 T-cell activation, leading to TCD4-mediated rejection of melanocytic tumors in mice.[20] A diphtheria toxin fused to IL-2 (denileukin diftitox, ONTAK), an FDA-approved treatment for cutaneous T-cell lymphoma, has been tested as a way to eliminate Tregs. In clinical trials involving patients challenged with a cancer vaccine, combined vaccination and denileukin diftitox reduced the number of circulating Tregs and increased the frequency of tumor-specific cytotoxic T lymphocytes compared with individuals receiving vaccination alone.[21–23] However, transient depletion of CD4 and CD8 T cells was also observed, and only a few patients with metastatic melanoma experienced tumor regression.[22]

Targeting CTLA-4

Upon engagement with its ligands CD80 and CD86, CTLA-4 expressed by activated effector T cells mediates a negative signal that attenuates effector T-cell activation. CTLA-4 is also expressed by Tregs and may be required for their suppressive function. In preclinical studies, CTLA-4 blockade resulted in the rejection of established tumors and long-term immunity in mice.[24] Concomitant blockade of CTLA-4 from both effector T cells and Tregs seems to be required for an optimal antitumor response.[25] Several clinical trials have been initiated with the anti-CTLA-4 monoclonal antibodies, ipilimumab and tremelimumab, in a variety of tumors, but with a special tropism for advanced, unresectable melanoma (for a review, see Tarhini and Iqbal[26]). Ipilimumab demonstrated clinical responses with frank tumor regression (including complete and partial responses) and improvement of overall survival, which led to FDA approval for unresectable stage III–IV melanomas in March 2011.[27–29] However, both antibodies were associated with severe (grade III–IV) autoimmune manifestations (see below, 'limitations') and the phase III trial with tremelimumab was prematurely stopped by the Data Safety Monitoring Board.[30]

Low-dose cyclophosphamide

Tregs are more sensitive to cyclophosphamide-mediated cell death than conventional T cells, possibly due to reduced intracellular adenosine triphosphate (ATP) levels in Tregs.[31] A single dose of cyclophosphamide depleted $CD4^+$ $CD25^+$ T cells in a rat colon cancer model and delayed tumor growth, whereas its combination with immunotherapy (which had no effect alone) led to regression of established tumors.[32] In a clinical trial, patients with hepatocellular carcinoma treated with low-dose cyclophosphamide had a decreased frequency of Tregs in the peripheral bloodstream and a tumor antigen-specific T-cell response was observed in many patients.[33]

Limitations of current strategies

Although promising responses were observed and some successes were obtained (for example, in advanced melanoma), the preclinical and clinical data highlight the limitations of current Treg-depletion strategies. First, the depletion of both Tregs and effector T cells by agents such as anti-CD25 antibodies or IL-2-toxin potentially masks the tremendous potential of Treg-depleting therapeutic strategies.[22,34,35] It is very likely that the success rate of these therapies would be increased if effector cells were not depleted in the course of the treatment. Furthermore, it has been reported that the elimination of Tregs is followed by peripheral conversion of conventional T cells into Tregs. This phenomenon results in replenishment and expansion of a population of newly converted Tregs with a more diversified TCR repertoire.[34] For these reasons, Treg-inactivating strategies, such as OX40 (CD134) triggering[36] or blockade of the CD39–CD73–adenosine pathway,[37,38] have been proposed as replacements for the elimination of Tregs. Finally, therapies that target membrane inhibitory receptors expressed on both Tregs and effector T cells are associated with severe autoimmune adverse effects, especially for anti-CTLA-4 therapies, in which treatment-related deaths were reported in 3 out of 324 patients treated with tremelimumab as a single agent.[30] Autoimmune manifestations result from the hyperactivation of the immune system, and can be viewed as an expected side effect of these therapies. Rather than depletion, fine and specific modulation of Treg activity, for example, *via* targeting the CD39–CD73–adenosine pathway, may prevent autoimmune side effects and should not be associated with the generation of newly converted Tregs. In support of this hypothesis, although Tregs from CD39 null mice have impaired suppressive activity,[38,39] the mice do not exhibit such autoimmune manifestations.

CD39 AND TREGS: A ROLE IN REGULATING ANTITUMOR IMMUNITY

The CD39–adenosine pathway

A growing body of evidence supports an important role for the Treg-mediated CD39–CD73–adenosine pathway in regulating T cell and NK-cell activity, as well as its importance in regulating antitumor response. The key role of this pathway was uncovered by the seminal work published by two groups in 2007.[37,38] ENTPD1/CD39 (ectonucleoside triphosphate diphosphohydrolase-1) is the main ectonucleotidase expressed by human and murine Tregs. CD39 hydrolyzes extracellular ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP).[38] AMP is then processed into immunosuppressive adenosine by the CD73 ecto-5′-nucleotidase. Adenosine is a critical regulator of both innate and adaptive immune responses. Upon binding to A2A receptors (the main adenosine receptor), adenosine causes the accumulation of intracellular cAMP, thereby preventing TCR-triggered CD25 upregulation and inhibiting effector T-lymphocyte proliferation and the secretion of inflammatory cytokines,[40] as well as cytotoxic activity and cytokine production by activated NK

cells.[41] Therefore, the CD39–CD73–adenosine pathway may be important for the balance between activation and regulation of effector immune responses (Figure 1).

CD39 is expressed by Tregs

A study by Deaglio *et al.*[38] demonstrated that CD39 and CD73 are strongly and constitutively expressed by a subset of murine T cells that also express FOXP3 and CTLA-4, that is, Tregs, as well as by most monocytes. CD39 and CD73 enzymes on $CD4^+$ $CD25^{high+}$ T cells generate adenosine; CD39 is the rate-limiting component of this machinery. Indeed, $CD4^+$ T cells from CD39 knockout (KO) mice have a markedly decreased ability to hydrolyze ATP and ADP into AMP and are unable to generate subsequent adenosine. Furthermore, although other NTPDases exist, CD39 appears to be the main NTPDase, at least in T cells, because $CD4^+/CD25^-$ T cells (that do not express CD39) or Tregs from CD39 KO mice have essentially no ATPase/ADPase enzymatic activity and do not produce adenosine.[38] Our own results in human immune cells support this conclusion, as $CD39^-$ cell-sorted human peripheral blood mononuclear cells have negligible ATPase/ADPase activity (manuscript in preparation). Adenosine generated through this pathway is a potent suppressor of effector T-cell proliferation and function, because T cells upregulate A2A receptor upon activation. It is important to note that Tregs from CD39 null mice are aberrantly constitutively activated, proliferate excessively and have lost their suppressive function.[38] CD39 null Tregs failed to suppress effector CD4 T cells *in vitro* and were unable to prevent skin allograft rejection in immune reconstituted mice *in vivo*, whereas wild-type (WT) Tregs conferred long-term graft survival.

Borsellino *et al.*[37] reached a similar conclusion in both mouse and human Tregs. CD39 is constitutively expressed in virtually all $CD4^+$ $CD25^{high+}$ T cells in mice; it was shown that the FOXP3 transcription factor drives CD39 expression in these cells. In humans, CD39 is also expressed on Tregs. However, although virtually all murine Tregs express CD39, its expression on human Tregs varies among healthy donors, for example, from 2 to 60%.[37] Our own work with peripheral blood mononuclear cell from healthy subjects reached the same conclusion (unpublished data). Importantly, $CD39^+$ Tregs are markedly enriched in disease settings, for example, in HIV[42] or in cancer patients.[43–45] Thus, CD39 is expressed at the cell surface of Tregs and participates in the generation of immunosuppressive adenosine, suggesting that $CD39^+$ Tregs may participate in tumor progression as a key mechanism of tumor escape from immune surveillance.

One intriguing observation is that several immune cell populations express CD39, albeit at different levels. Monocytes, neutrophils and B cells constitutively express CD39.[46–48] Among $CD4^+$ cells, both $FOXP3^+$ T cells (Tregs) and $FOXP3^-$ T cells express CD39. However, only $FOXP3^+$ $CD39^+$ Tregs mediate suppression through the generation of adenosine, whereas $CD4^+$ $FOXP3^-$ $CD39^+$ T cells were shown to exhibit a memory phenotype and did not mediate suppression.[49,50] These observations suggest that $FOXP3^+$ cells may express one or more additional factors that participate in CD39-mediated immunosuppression. One candidate is CD73, which is not expressed on $CD4^+$ $FOXP3^-$ $CD39^+$ memory T cells.[49] It was therefore proposed that the lack of expression of CD73 could impair adenosine generation. However, CD73 is widely expressed on immune and non-immune cells, including some epithelial cells and fibroblasts,[51] and is not the rate-limiting enzyme of the CD39–CD73–adenosine pathway. Therefore, CD73 activity may not explain these observations. Another candidate is IL-10. Indeed, IL-10 is secreted by Tregs and it was observed that Tregs from IL-10 KO mice fail to exert suppressive function, due to impaired adenosine generation.[52] Further work is needed to clarify which factor(s) cooperate with CD39 in the generation of immunosuppressive adenosine.

CD39-mediated immunosuppression via adenosine

The importance of the CD39–CD73–adenosine pathway in regulating effector T-cell or NK-cell antitumor response has long been unclear. In 2007, Ohta *et al.*[53] shed new light on the crucial

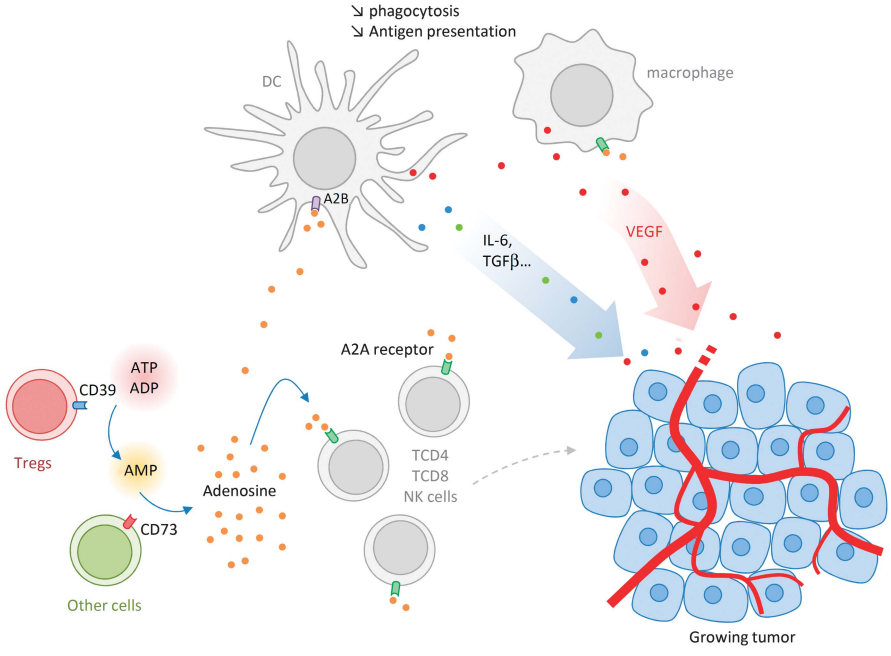


Figure 1. Treg-mediated immunosuppression and tumor growth *via* the CD39–CD73–adenosine pathway. CD39 is expressed at the cell surface of Tregs and converts ATP and ADP into AMP. In turn, AMP is converted by CD73 into adenosine, which is a potent immunosuppressor. Adenosine binds to its receptors (for example, A2A receptor) at the surface of CD4, CD8 T cells and NK cells and inhibits effector cell response, leading to tumor progression. Adenosine also binds to A2A or A2B receptors on macrophages and DCs, inhibits phagocytosis and antigen presentation and increases secretion of pro-tumorigenic factors, such as VEGF, TGFβ and IL-6.

role of A2A receptor stimulation by adenosine in controlling the effector T-cell response. They demonstrated that the genetic deletion of the A2A receptor in the host resulted in T-cell-mediated rejection of established immunogenic melanomas. The use of various antagonists or small interfering RNA targeting the A2A receptor enhanced the immune response of transferred CD8 T cells, resulting in inhibition of primary tumor growth, elimination of metastases and prevention of neoangiogenesis. Recently, the same group continued to investigate the underlying mechanisms, and demonstrated that A2A receptor agonists inhibited the development of cytotoxicity during T-cell activation, in particular by modulating cytokine release by both CD4 and CD8 T cells.[54] Importantly, the adenosine pathway not only impaired the establishment of fully functional effector T cells but also exhibited a long-lasting modulatory activity, because the immunosuppression persisted after A2A receptor stimulation was stopped.

CD39-generated adenosine may also modulate NK-cell lytic activity. In 1990, it was reported that adenosine or several adenosine receptor agonists inhibited lysis mediated by NK cells *in vitro*, whereas further treatment with an adenosine receptor antagonist abrogated these effects.[55] Adenosine-mediated inhibition of NK-cell activity was then additionally confirmed, although there may be some discrepancies regarding the adenosine receptor(s) involved.[56] The involvement of CD39 in this process was elegantly illustrated by Sun *et al.*[39] using CD39 KO models. Alhough WT Tregs suppressed NK-cell function, Tregs from CD39 KO mice were unable to suppress NK-cell lytic activity, both *in vitro* and *in vivo*. Thus, the CD39-mediated generation of adenosine is an important regulator of effector TCD4, TCD8 and NK-cell functions (Figure 1).

Adenosine also affects phagocytosis by the mononuclear phagocyte system. For instance, adenosine binds A2A receptors and inhibits phagocytosis by macrophages,[57] as well as decreasing the secretion of various proinflammatory mediators, such as TNFα (for a review, see Hasko and Pacher[58]). This decrease in proinflammatory cytokines is accompanied by a concomitant increase in secretion of VEGF (vascular endothelial growth factor),[59,60] a process termed the 'angiogenic switch.' As neoangiogenesis is required for tumor development, this 'angiogenic switch' certainly participates in carcinogenesis, and could at least partially explain the anti-angiogenic effect of CD39 inhibition (see below). Adenosine was also reported to affect the differentiation of monocytes into DCs.[61] Upon binding to A2B receptors, adenosine skews DC differentiation towards aberrant differentiated DCs secreting various pro-tumorigenic factors, such as VEGF, IL-6, IL-8 and TGFβ. Importantly, adoptive transfer experiments revealed that these cells strongly supported tumor growth with an increased number of blood vessels.

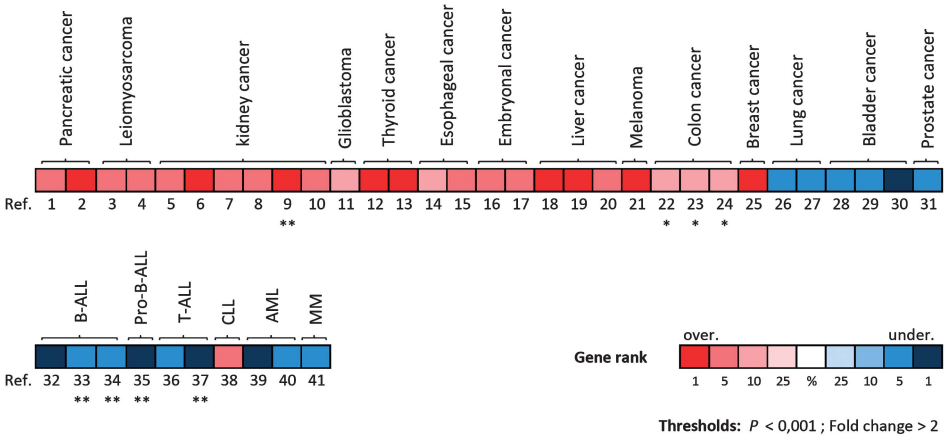
CD39-mediated immunosuppression via degradation of ATP

In addition to its role in generating immunosuppressive adenosine, CD39 may also impact the immune response and tumor growth by decreasing levels of extracellular ATP in the tumor microenvironment. Phagocytosis of apoptotic bodies by monocytes, macrophages and DCs is important for the clearance of dying cancer cells and (cross) presentation of tumor antigens by antigen-presenting cells to initiate or sustain the antitumor immune response. Chemotherapy efficiency also relies on its ability to induce immunogenic cancer cell death, and ATP was discovered to be a major 'find-me' signal released by apoptotic cells responsible for the recruitment of phagocytes, and required for cancer cell death to be immunogenic. Dying cancer cells release ATP that binds to the $P2Y_2$ receptor on monocytes and macrophages and promotes their recruitment to the tumor site.[62] CD39-mediated degradation of ATP abrogated the ability of apoptotic cells to recruit monocytes both *in vitro* and *in vivo*. ATP released by apoptotic cancer cells also binds to $P2RX_7$ purinergic receptors on DCs, thereby inducing the IL-1β release required for the priming of INFγ producing CD8 T cells.[63] Efficient ATP release appears to be critical for the immunogenic response after chemotherapy. In cancer cells with an impaired release of ATP, chemotherapeutic agents fail to elicit an immunogenic response unless CD39 inhibitors were used.[64] Beyond its central role in initiating an immune response, extracellular ATP is also known for inhibiting tumor cell (but not normal cell) proliferation[65,66] and for promoting cancer cell death.[67,68] ATP induces changes in membrane permeability, resulting in Na^+ and Ca^{2+} influx into the cell, which may participate in growth inhibition. Therefore, CD39-mediated decreases in extracellular ATP may render the tumor microenvironment permissive to cancer cell growth and may impede the immune response induced following chemotherapy. Altogether, it seems that CD39 may have a double immunosuppressant activity, that is, by generating adenosine, which is a major inhibitor of effector T- and NK-cell antitumor activities, and by removing ATP, an inhibitor of tumor cell proliferation and an essential sensor molecule that attracts antigen-presenting cells to the tumor site (Figure 1 and for an excellent review on adenosine, ATP and cancer, refer to Stagg and Smyth[69]). However, increasing ATP levels following CD39 blockade may also exhibit some toxicity towards peripheral lymphocytes *via* P2 × 7 receptor-mediated cell death,[70] which could possibly limit the immune response, a possibility that would require further investigation.

CD39 IS A POTENTIAL THERAPEUTIC TARGET FOR CANCER IMMUNOTHERAPY

Increased CD39 expression and activity in human cancers

CD39-expressing Tregs mediate potent immunosuppression, and CD39 may thus be upregulated in human malignancies. In an attempt to evaluate whether CD39 is involved in human cancers, we analyzed *ENTPD1* expression in public microarray data using the Oncomine software. As illustrated in Figure 2, CD39 is strikingly upregulated in a large number of solid tumors (with the exception of lung, bladder and prostate cancers). CD39 transcripts are also upregulated in chronic lymphocytic leukemia. Surprisingly, however, CD39 is downregulated in the other hematological tumors included in the analysis. Along similar lines, patients with head and neck cancer have higher frequencies of $CD39^+$ Tregs (with increased expression of CD39 at their cell surface) compared with normal subjects.[43] This is associated with increased enzymatic activity, enhanced production of adenosine and the strongest immunosuppressive activity.[43,44] In chronic lymphocytic leukemia, patients have a higher percentage of $CD39^+$ CD4 and CD8 T cells,[45] which is in accordance with Oncomine data. Among chronic lymphocytic leukemia patients, high levels of CD39 correlated with disease stage and severity. Interestingly, in contrast to CD39, CD73 was expressed but not increased in cancer patients. Similarly, in follicular lymphoma, CD39-expressing T cells were increased in follicular lymphoma nodes, and they mediated anergy of tumor-derived T cells through the generation of adenosine.[71] Interestingly, $CD39^+$ cells were Tregs as well as other T cells. In pancreatic cancer, a significantly elevated expression of CD39 in tumor stroma has been reported, whereas its expression was restricted to endothelial cells in normal tissues.[72] Yet, high expression of CD39 was, surprisingly, associated with better outcome. More recently, examination of ovarian cancer specimens revealed expression of CD39 in the tumor stroma and in tumor cells.[73] Using cancer cell lines, the authors demonstrated unambiguously that ovarian cancer cells express CD39 and generate adenosine that suppresses T- and NK-cell antitumor response, similar to Tregs. Further work is needed to know whether this result applies



* 1 reporter (A_23_P24260) out of 6 showed contradictory results and was therefore removed for analysis
** 1 reporter (243111_at) out of 5 systematically showed contradictory results across datasets and was therefore removed for analysis

Ref.		
1	Pancreatic Ductal Adenocarcinoma vs. Normal	Badea Pancreas, Hepatogastroenterology, 2008
2	Pancreatic Adenocarcinoma vs. Normal	Iacobuzio-Donahue Pancreas 2, Am J Pathol, 2003
3	Leiomyosarcoma vs. Normal	Barretina Sarcoma, Nat Genet, 2010
4	Leiomyosarcoma vs. Normal	Detwiller Sarcoma, Cancer Res, 2005
5	Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	Beroukhim Renal, Cancer Res, 2009
6	Non-Hereditary Clear Cell Renal Cell Carcinoma vs. Normal	Beroukhim Renal, Cancer Res, 2009
7	Clear Cell Renal Cell Carcinoma vs. Normal	Gumz Renal, Clin Cancer Res, 2007
8	Clear Cell Renal Cell Carcinoma vs. Normal	Jones Renal, Clin Cancer Res, 2005
9	Clear Cell Renal Cell Carcinoma vs. Normal	Lenburg Renal, BMC Cancer, 2003
10	Clear Cell Renal Cell Carcinoma vs. Normal	Yusenko Renal, BMC Cancer, 2009
11	Glioblastoma vs. Normal	Bredel Brain 2, Cancer Res, 2005
12	Follicular Variant Thyroid Gland Papillary Carcinoma vs. Normal	Giordano Thyroid, Clin Cancer Res, 2006
13	Thyroid Gland Papillary Carcinoma vs. Normal	Giordano Thyroid, Clin Cancer Res, 2006
14	Barrett's Esophagus vs. Normal	Hao Esophagus, Gastroenterology, 2006
15	Esophageal Adenocarcinoma vs. Normal	Hao Esophagus, Gastroenterology, 2006
16	Embryonal Carcinoma, NOS vs. Normal	Korkola Seminoma, Cancer Res, 2006
17	Teratoma, NOS vs. Normal	Korkola Seminoma, Cancer Res, 2006
18	Cirrhosis vs. Normal	Mas Liver, Mol Med, 2008
19	Cirrhosis vs. Normal	Wurmbach Liver, Hepatology, 2007
20	Hepatocellular Carcinoma vs. Normal	Wurmbach Liver, Hepatology, 2007
21	Cutaneous Melanoma vs. Normal	Riker Melanoma, BMC Med Genomics, 2008
22	Cecum Adenocarcinoma vs. Normal	TCGA Colorectal, No Associated Paper, 2011
23	Colon Adenocarcinoma vs. Normal	TCGA Colorectal, No Associated Paper, 2011
24	Rectal Adenocarcinoma vs. Normal	TCGA Colorectal, No Associated Paper, 2011
25	Invasive Ductal Breast Carcinoma vs. Normal	Turashvili Breast, BMC Cancer, 2007
26	Squamous Cell Lung Carcinoma vs. Normal	Garber Lung, Proc Natl Acad Sci U S A, 2001
27	Large Cell Lung Carcinoma vs. Normal	Hou Lung, PLoS One, 2010
28	Superficial Bladder Cancer vs. Normal	Blaveri Bladder 2, Clin Cancer Res, 2005
29	Infiltrating Bladder Urothelial Carcinoma vs. Normal	Sanchez-Carbayo Bladder 2, J Clin Oncol, 2006
30	Superficial Bladder Cancer vs. Normal	Sanchez-Carbayo Bladder 2, J Clin Oncol, 2006
31	Prostate Carcinoma vs. Normal	Welsh Prostate, Cancer Res, 2001
32	B-Cell Acute Lymphoblastic Leukemia vs. Normal	Andersson Leukemia, Leukemia, 2007
33	B-Cell Acute Lymphoblastic Leukemia vs. Normal	Haferlach Leukemia, J Clin Oncol, 2010
34	B-Cell Childhood Acute Lymphoblastic Leukemia vs. Normal	Haferlach Leukemia, J Clin Oncol, 2010
35	Pro-B Acute Lymphoblastic Leukemia vs. Normal	Haferlach Leukemia, J Clin Oncol, 2010
36	T-Cell Acute Lymphoblastic Leukemia vs. Normal	Andersson Leukemia, Leukemia, 2007
37	T-Cell Acute Lymphoblastic Leukemia vs. Normal	Haferlach Leukemia, J Clin Oncol, 2010
38	Chronic Lymphocytic Leukemia vs. Normal	Rosenwald Lymphoma, N Engl J Med, 2002
39	Acute Myeloid Leukemia vs. Normal	Haferlach Leukemia, J Clin Oncol, 2010
40	Acute Myeloid Leukemia vs. Normal	Stegmaier Leukemia, Nat Genet, 2004
41	Multiple Myeloma vs. Normal	Zhan Myeloma, Blood, 2002

Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization.

Figure 2. CD39 is overexpressed in human cancers. The hit-map displays CD39 expression in various human tumors from public microarrays. Oncomine (Compendia Bioscience, Ann Arbor, MI, USA) was used for analysis and visualization. In this unbiased approach, all data sets in which CD39 expression is significantly differentially expressed between normal and cancer tissues were included in the analysis (thresholds: fold change >2, $P<0.001$).

to other cancers. Similarly, cancer exosomes (small vesicles 'secreted' by cancer cells) released by various cancer cell lines were shown to hydrolyze ATP and generate immunosuppressive adenosine in a CD39–CD73 dependent manner.[74] Taken together, these results illustrate the role of $CD39^+$ Tregs that infiltrate tumors, and potentially cancer cells themselves, in promoting immunosuppression through decreasing extracellular ATP and generating adenosine. Therefore, it is anticipated that blockade of the CD39 enzyme would have a beneficial impact by restoring the immune antitumor response.

Evidence of favorable safety profile from KO models

As a potential therapeutic target, it is important to mention that CD39 null mice are healthy, with a minor phenotypic modification.[75] Crossing heterozygote mice yielded WT, heterozygous and homozygous offspring at Mendelian ratios. Neither heterozygous nor homozygous animals showed signs of developmental issues, and the phenotypes of adult CD39 null mice were normal. The most pronounced phenotype was a prolonged bleeding time with minimally perturbed coagulation parameters, which should be taken in consideration when developing anti-CD39 therapies. In sharp contrast to CD25 (IL-2Rα) and CTLA-4 KO mice, CD39 KO mice do not spontaneously develop overt autoimmune manifestations. IL-2Rα null mice progressively develop severe autoimmune disorders, anemia and inflammatory bowel disease, with a 25% mortality between 8 and 20 weeks of age.[76] Mice lacking CTLA-4 die before 3 weeks of age from massive lymphoproliferative disease and autoimmune tissue destruction.[77] However, a small percentage of CD39 null adult mice develop autoimmune alopecia.[78] CD39 deficiency also exacerbated dextran sodium sulfate-induced colitis, an experimental form of inflammatory bowel disease,[79] consistent with the reported role of purinergic signaling in intestinal inflammation.[80] In conclusion, despite hemostatic disorders, the KO models predict that CD39 inhibition has a favorable safety profile, making CD39 a pharmaceutical target.

Target proof-of-concept from transgenic and KO mouse models

The first proof-of-concept of targeting CD39 in cancer came from experiments using CD39 KO animals. Melanoma growth and metastasis to the lungs were markedly decreased in CD39 null mice compared with WT littermates.[81] The authors reported that loss of CD39 led to severe defects in angiogenesis, which may be another interesting anticancer property of CD39 antagonists. *In vitro* experiments suggested that the defects in angiogenesis might result from the impaired migration of endothelial and inflammatory cells into the tumor site. However, we cannot rule out the possibility that the reduction of tumor growth and metastasis in CD39 null animals resulted from an enhanced immune response, because the contribution of the immune system was not addressed. Recent work reported that experimental liver metastases of B16F10 melanomas or MCA38 colon tumors were abrogated in CD39 KO mice.[39] Combining adoptive transfer experiments and *in vitro* data, it was demonstrated that CD39 expression on Tregs has a major role in controlling melanoma tumor rejection mediated by NK cells. *In vitro* Tregs from CD39 KO mice had no inhibitory activity on NK cells, whereas NK lytic activity was strongly impaired in the presence of WT Tregs. Accordingly, Tregs from CD39 KO mice were unable to block NK cell antitumor activity *in vivo,* leading to NK-mediated tumor rejection, whereas co-transfer of NK cells with WT Tregs led to extensive tumor growth similar to that observed when no NK cells were transferred. Another elegant study has compared the effects of more subtle changes in CD39 expression by challenging WT, heterozygous (that is, decreased expression of CD39) and transgenic (that is, increased expression of CD39) mice with colon cancer cells.[82] Although the volumes of primary tumors were not significantly affected, purinergic signaling modulated the ability of the colon cancer cells to metastasize and colonize the liver. CD39 transgenic mice developed significantly larger metastatic masses compared with heterozygous animals. Taken together, these mouse models clearly show that CD39 disruption has powerful antitumor and anti-metastatic effects, at least in part through alleviating Treg-mediated suppression of effector immune cells.

Therapeutic approaches targeting the CD39-CD73 pathway

The pharmacological inhibitor POM-1 gave outstanding results in a preclinical trial in melanoma-bearing mice.[39] POM-1 is a chemical inhibitor of NTPDase[83] that has been widely used as a CD39 inhibitor *in vitro*. However, POM-1 is not fully specific for CD39/NTPDase 1 because it also inhibits NTPDase 2 and 3[83] and has off-target effects.[84] However, it can be used for a short period *in vivo* to test the effect of pharmacological inhibition of CD39 in immunocompetent animals. Daily i.p. injections of POM-1 at 5 or 10 mg/kg for 10 days prevented B16 melanoma and MCA38 colonic tumor growth.[39] The antitumor effect of POM-1 was similar to that observed in CD39 null mice, and treatment of CD39 null mice with POM-1 did not further reduce tumor growth, suggesting that the effects were a result of POM-1-mediated inhibition of CD39. During this study, no signs of liver or renal toxicity were reported in POM-1-treated animals, providing additional evidence of a favorable safety profile for CD39 antagonists.

The promising value of targeting the CD39–CD73–adenosine pathway as an anticancer therapy was further shown by Stagg and Smyth,[69] who demonstrated that a murine-CD73-blocking monoclonal antibody significantly decreased tumor growth of metastatic 4T1 and E0771 breast cancer cell lines *in vivo*. As no effect of anti-CD73 monoclonal antibody therapy was observed in SCID mice, they concluded that this was largely mediated *via* the modulation of the adaptive immune response. Furthermore, the growth of CD73 knocked-down 4T1 cells (using short hairpin RNA) was similar to that of mice receiving the parental cell line and CD73-blocking therapy. This suggests that, in this immunocompetent mouse model, the main source of CD73 was the tumor cells, which expressed CD73 at high levels. It remains unclear to what extent the 'host' CD73 has a role in mediating immunosuppression, because CD73 KO mice also have decreased primary tumor growth and metastasis.[85] More surprisingly, in this study, the authors reported that CD73 itself promoted cancer cell migration in transwell assays *in vitro* and *in vivo*, thereby promoting metastasis: an unexpected observation that requires further investigation. However, this result clearly validates monoclonal antibody blocking of the adenosine pathway as an attractive strategy for anticancer therapy. Although CD73 is certainly an interesting target as discussed above, there is a strong rationale for targeting CD39 rather than CD73. Indeed, CD39 was shown to be the rate-limiting enzyme of the process.[38] Furthermore, CD39 (but not CD73) further participates in immunosuppression by decreasing levels of ATP released by apoptotic cancer cells. The released ATP serves as a signal for recruitment and function of antigen-presenting cells [62–64] and promotes tumor neoangiogenesis.[81]

Development of anti-CD39 monoclonal antibodies

In an attempt to target CD39 to restore the immune antitumor response, we have developed CD39-blocking monoclonal antibodies. Two promising candidates, BY40 and BA54G, specifically bind human (but not murine) CD39[86,87] and efficiently block CD39 enzyme activity. A first proof-of-concept study using BY40 monoclonal antibody to alleviate Treg-mediated immunosuppression and to restore CD4 and CD8 T-cell response was recently published.[42] $CD39^+$ Tregs are significantly increased in HIV patients compared with healthy donors, and CD39 expression

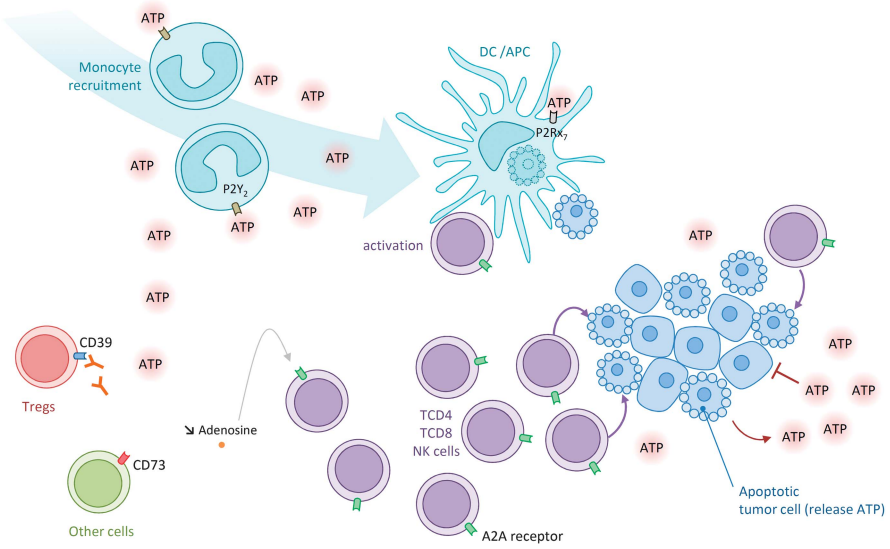


Figure 3. CD39-blocking antibody restores T- or NK-cell antitumor response. The anti-CD39 monoclonal antibody blocks CD39 enzyme activity, leading to an accumulation of ATP without the generation of adenosine. In the absence of immunosuppressive adenosine, effector T cells and NK cells can exert their antitumor response. Furthermore, accumulation of ATP in the tumor milieu is toxic for tumor cells and serves as a 'find-me' signal for the recruitment of DCs, monocytes and macrophages, which present tumor antigens and thereby further participate in the immune response against the cancerous lesion.

correlated with viral load and low CD4 T-cell number. Experiments demonstrated that anti-CD39 antibody BY40 could abrogate CD8 T-cell suppression mediated by Tregs from HIV patients and effectively restored CD8 T-cell proliferation and the secretion of IL-2/IFN-γ/TNF-α. In agreement with those results, we demonstrated that BY40 monoclonal antibody restored CD4 and CD8 T-cell proliferation in a melanoma tumor model *in vitro* (manuscript in preparation). Thus, CD39-blocking antibodies are useful in inhibiting Treg-mediated immunosuppression and restoring an immune response in CD39-associated diseases such as cancer (Figure 3).

CONCLUDING REMARKS

Tregs contribute to tumor progression by suppressing the immune system response to malignancies. Therapies that non-specifically targeted Tregs showed significant results in some cancer patients, including complete responses in advanced stage disease. However, such therapies have major limitations, including serious adverse effects such as autoimmune manifestations and Treg replenishment by the conversion of peripheral T cells due to cell depletion. Furthermore, the overall efficacy of these therapies is modest, because they affect both regulatory and effector T cells, which compromises their net effect. Therefore, there is a need for non-Treg-depleting strategies that do not impair effector T-cell function. Drugs or antibodies that could block the CD39–CD73–adenosine pathway would fulfill such criteria. Indeed, CD39$^+$ Tregs are enriched in cancer and HIV patients and there is now evidence that the adenosine generated through this pathway is an important immunosuppressor. Combined *in vitro* and *in vivo* experiments using KO mice or chemical inhibitors demonstrated that blocking CD39: (1) efficiently restores T- and NK-cell antitumor activity, thereby decreasing both primary tumor growth and metastasis, (2) is safe, although potential adverse effects should be carefully watched for, such as changes in coagulation parameters and (3) is not associated with severe autoimmunity (CD39 KO mice did not evidence overt autoimmune manifestations compared with CTLA-4 or CD25 KO mice). The first proof-of-concept of the use of CD39-blocking monoclonal antibody to alleviate Treg-mediated suppression of CD8 T cells has recently been obtained *ex vivo* in HIV patients. Proof-of-concept in cancer settings is in progress and supports the ongoing development of CD39-blocking antibodies.

CONFLICT OF INTEREST

Drs Bonnefoy, Bensussan, Alberici and Eliaou are cofounders and shareholders of OREGA Biotech. Dr Bastid is an employee of OREGA Biotech. Dr Cottalorda-Regairaz declares no conflict of interest.

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