

Hostile, Hypoxia–A2-Adenosinergic Tumor Biology as the Next Barrier to Overcome for Tumor Immunologists

Michail V. Sitkovsky, Stephen Hatfield, Robert Abbott, Bryan Belikoff, Dmitriy Lukashev, and Akio Ohta

Abstract

Hypoxia-driven, A2A adenosine receptor (A2AR)–mediated (hypoxia–A2-adenosinergic), T-cell–autonomous immunosuppression was first recognized as critical and nonredundant in protecting normal tissues from inflammatory damage and autoimmunity. However, this immunosuppressive mechanism can be hijacked by bacteria and tumors to provide misguided protection for pathogens and cancerous tissues. Inhibitors of the hypoxia–A2-adenosinergic pathway represent a conceptually novel type of immunologic adjuvants that could be combined with cancer vaccines, adoptive cell transfer, and/or blockade of negative immunologic regulators to further prolong patient survival and to minimize treatment-related side effects. In support of this approach are preclinical studies and findings that some human cancers are resistant to chemotherapies and immunotherapies due to the tumor-generated extracellular adenosine and A2AR on antitumor T and natural killer (NK) cells. Among the adjuvants are (i) antagonists of A2AR, (ii) extracellular adenosine-degrading drugs, (iii) inhibitors of adenosine generation by CD39/CD73 ectoenzymes, and (iv) inhibitors of hypoxia–HIF-1 α signaling. Combining these adjuvants with CTLA-4 and/or PD-1 blockade is expected to have additive or even synergistic effects of targeting two different antitumor protective mechanisms. It is expected that even after multicombinatorial blockade of negative immunologic regulators, the antitumor T and NK cells would still be vulnerable to inhibition by hypoxia and A2AR. Yet to be tested is the potential capacity of adjuvants to minimize the side effects of CTLA-4 and/or PD-1 blockade by decreasing the dose of blocking antibodies or by eliminating the need for dual blockade. *Cancer Immunol Res*; 2(7); 598–605. ©2014 AACR.

Introduction

Recent advances in cancer vaccines, adoptive cell transfer, and blockade of negative immunologic regulators CTLA-4 and/or PD-1 are reflected in approvals by the FDA and represent a significant hope for many patients (1–7). However, there is still room for improvement in terms of further prolongation of patient survival and lessening of the immune-related adverse side effects (5, 6, 8–10). These goals may be accomplished only after careful and rigorous considerations and testing of other important and yet to be targeted immunosuppressive pathways. These immunosuppressive mechanisms may limit the clinical benefits of current cancer immunotherapies even after the depletion of all known negative immunologic regulators, such as CTLA-4/PD-1 or regulatory T cells (Treg).

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Hypoxia–A2-Adenosinergic Immunosuppression, Transcription, and Redirection of Antipathogen and Antitumor Immune-Cell Effector Functions

The concept of targeting the normally protective but sometimes usurped physiologic mechanisms, such as cellular metabolism and local tissue oxygen tension-dependent A2A and A2B adenosine receptor (A2AR/A2BR)–mediated immunosuppression in inflamed and cancerous tissues, is the basis of the therapeutic strategy and the subject of this Crossroads article (Fig. 1; refs. 11–18). This type of immunosuppression in the tumor microenvironment (TME) seems to be a misguided application of the evolutionarily critical and nonredundant negative feedback immunosuppressive mechanism that is otherwise lifesaving by protecting normal tissues from excessive collateral damage during the antipathogen immune response (13, 14, 18).

The identification of this indispensable immunoregulatory pathway may have provided one of the explanations for the paradoxical coexistence of tumors and antitumor immune cells in the same patient with cancer (19), which could be due to the A2AR-mediated inhibition of tumor-reactive T cells in the TME (12, 15).

It must be emphasized that hypoxia–A2-adenosinergic signaling is not only an immunosuppressive pathway that inhibits

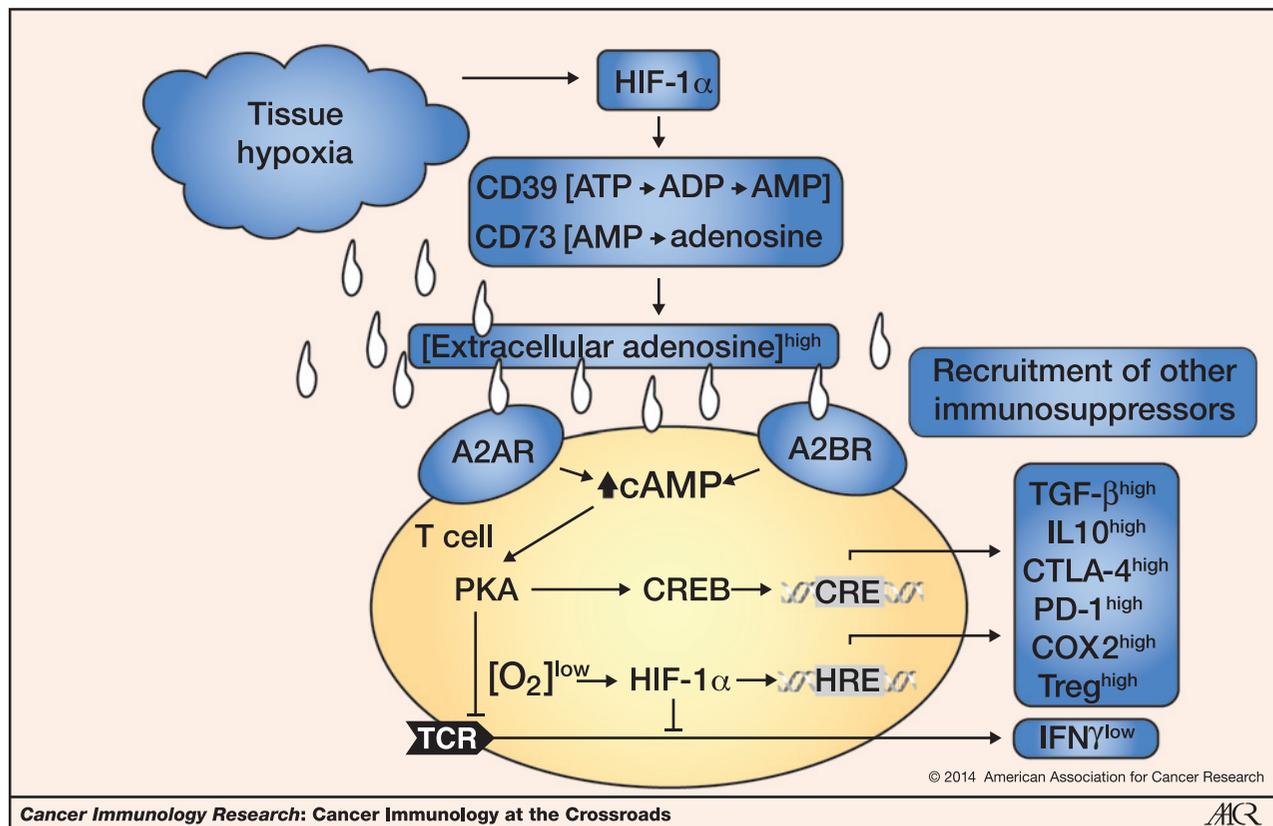


Figure 1. Schematic diagram of tissue hypoxia in inflamed and cancerous tissues. The hypoxia-driven stabilization of HIF-1 α transcription factor leads to CD39/CD73 ectoenzyme–mediated generation of extracellular adenosine, which signals through the G_s-protein–coupled A2A and A2B adenosine receptors (A2AR and A2BR) and triggers the accumulation of intracellular cAMP. The increased expression of CD73 on the surface of Tregs (78) may generate extracellular adenosine that would further enhance their suppressor activities and add to the immunosuppressive effects of tumor-produced adenosine on CD8⁺ T cells. The binding of cAMP to the regulatory subunit of cAMP-dependent PKA results in a cascade of phosphorylation events that inhibits the TCR-triggered signaling pathway and therefore inhibits the proinflammatory effects of T cells. After phosphorylation by PKA, CRE-binding protein CREB participates in the transcription of genes containing the CRE (79). In addition, HIF-1 α participates in the transcription of genes containing HREs.

the T-cell receptor (TCR)–triggered production of proinflammatory cytokines, such as IFN γ (Fig. 1). This pathway may also redirect the immune response, as we discussed in detail in a model proposed previously (ref. 16 and Fig. 1). Briefly, the local tissue hypoxia and the A2AR signaling–mediated increase in intracellular cAMP and cAMP-dependent protein kinase inhibit the production of proinflammatory cytokines such as IFN γ in CD8⁺ and CD4⁺ T cells, while promoting transcription of genes that express the cAMP-response elements (CRE) or the hypoxia-response elements (HRE). This, in turn, may lead to the synthesis of immunosuppressive molecules and the development of Tregs (Fig. 1; ref. 16). Thus, the generation of anti-inflammatory mediators or the development or functions of immunosuppressive Tregs could be facilitated by hypoxia–A2-adenosinergic signaling. This may provide an explanation for the “infectious tolerance” of Tregs (16) in hypoxic and extracellular adenosine-rich inflamed tissues and in the TME (16, 20, 21).

The power and versatility of A2-adenosinergic immunosuppression is usurped by *Staphylococcus aureus* and other bacteria to suppress and escape host immune responses. These pathogens have acquired the ability to synthesize

extracellular adenosine, leading to the inhibition of antibacterial effector functions of neutrophils through A2AR signaling (22).

Key Molecules of the Hypoxia–A2-Adenosinergic Signaling Pathway

Descriptions of the upstream and downstream stages of the hypoxia–A2-adenosinergic pathway in Figs. 1 and 2 and in this Crossroads article follow the history of the search for the molecular mechanism of physiologic immunosuppression, which started with the bet on the importance of intracellular cAMP. cAMP is a high-fidelity intracellular immunosuppressor that inhibits virtually all tested TCR-triggered effector functions of T cells through the activation of the cAMP-dependent protein kinase A (PKA), albeit with different efficacy (23–29).

Elevation of intracellular levels of cAMP in T cells, natural killer (NK) cells, or myeloid cells in hypoxic and extracellular adenosine-rich tissues is triggered by the binding of extracellular adenosine to G-protein–coupled, cAMP-elevating A2AR (high-affinity) and A2BR (low-affinity) adenosine receptors (30, 31). Our focus on studying A2AR in T cell–mediated

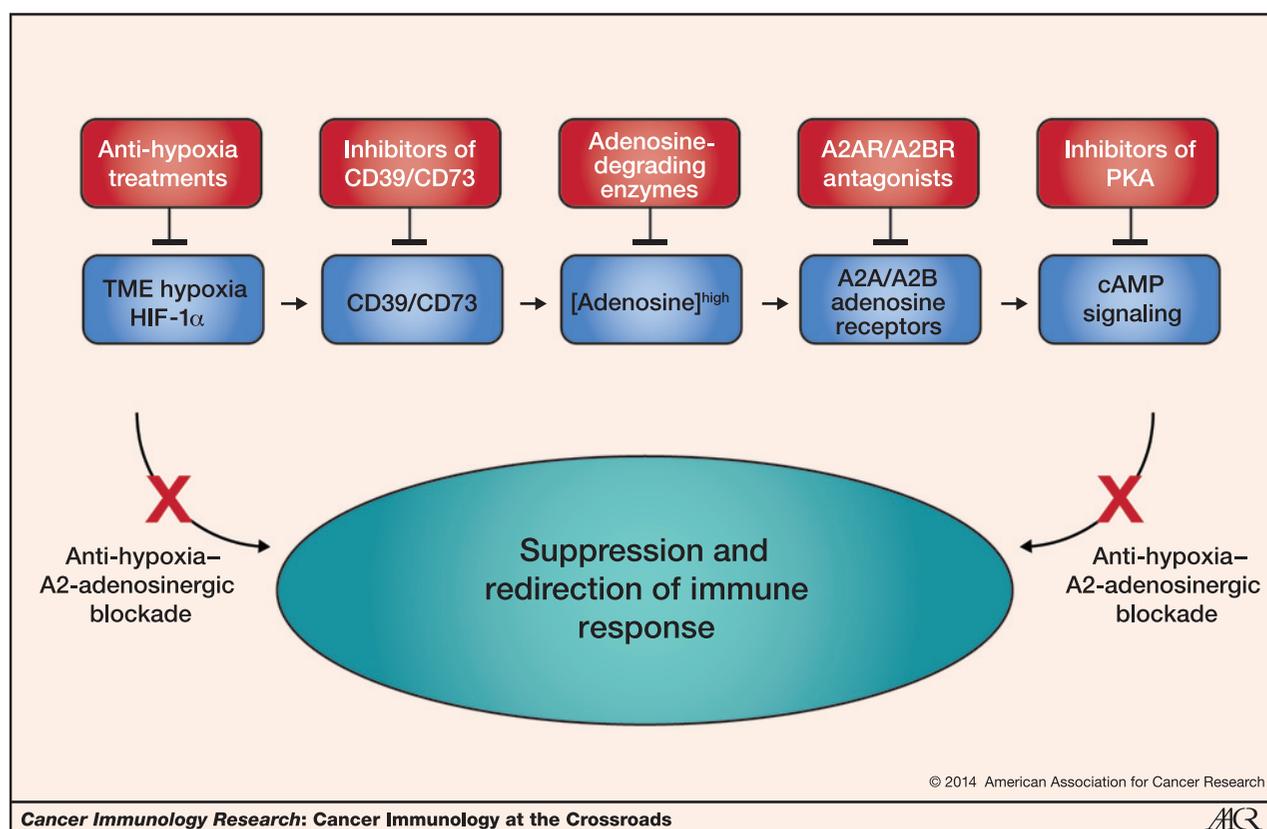


Figure 2. Anti-hypoxia-A2-adenosinergic coadjuvant-mediated inhibition of the hypoxia-A2-adenosinergic pathway. The anti-hypoxia-A2-adenosinergic drugs shown were developed for other therapeutic purposes. Inhibitors of the hypoxia→HIF-1 α early stage, including inhibitors of HIF-1 α , can weaken hypoxia and promote destabilization and degradation of HIF-1 α in antitumor T and NK cells. Inhibitors of CD39 ecto-ATPase/ADPase and CD73 5'-nucleotidase may prevent the accumulation of extracellular adenosine in the TME, thereby decreasing the intensity of immunosuppressive signaling through A2AR or A2BR. Commercially available drugs such as stabilized adenosine deaminase may be tested to degrade extracellular adenosine. Alternatively, enzymes such as adenosine kinase may be tested for the ability to re-phosphorylate adenosine to generate AMP, thus decreasing the levels of extracellular adenosine. Antagonists of A2A/A2B adenosine receptors compete with the tissue-produced adenosine for binding to the adenosine receptor. The antagonist-bound adenosine receptors are not activated and do not increase intracellular cAMP levels. Inhibitors of cAMP-dependent PKA could decrease cAMP signaling, but the associated side effects may outweigh potential benefits.

immunity was based on the original observations that murine T cells preferentially express A2AR (32–34), which is likely to be activated in the lower ranges (~50 nmol/L) of extracellular adenosine in inflamed and cancerous tissues *in vivo*. Human antitumor T cells also express both A2AR and A2BR (Sitkovsky et al.; unpublished data).

Importantly, T cells do not have "spare" A2AR (i.e., there is no receptor reserve; ref. 35), suggesting that the number of A2AR molecules per T cell is the limiting factor in determining the maximal cAMP response of T lymphocytes to adenosine and the extent of the inhibition of T cells by adenosine-A2AR-cAMP signaling. This important property provides yet another mechanism to fine-tune the intensity of immunosuppressive effects of extracellular adenosine. It was also shown that T cells have a "memory" of signaling through A2AR (36). Therefore, T cells experience the A2AR-triggered suppression long after exposure to the short-lived *in vivo* extracellular adenosine has ceased.

Extracellular adenosine is generated by at least two known mechanisms, from intracellular ATP or from extracellular ATP

due to activities of extracellular adenosine-generating tandem ectoenzymes CD39 (ecto-ATPase/ADPase) and CD73 (ecto-5'-nucleotidase), which was recently reviewed in ref. (17). The CD39 and CD73 ectoenzymes were shown to be important in limiting the inflammatory damage (20, 37–40) by generating extracellular adenosine and thereby enabling the downstream adenosine → A2AR-mediated signaling (11).

Hypoxia → hypoxia-inducible factor-1 α (HIF-1 α)-mediated events have been implicated in upstream events that trigger anti-inflammatory CD39/CD73 → [adenosine]^{high} → A2AR/A2BR signaling. The first evidence of the inhibitory role of hypoxia-HIF-1 α in T cells was obtained in HIF-1 α ^{-/-}Rag2^{-/-} chimeric mice, which are characterized by HIF-1 α deficiency only in cells of the adaptive immune system (41). Results from these studies revealed that HIF-1 α not only regulates lymphocyte development and functions, but it also protects against autoimmunity and inflammatory tissue damage. The immunosuppressive role of HIF-1 α in T cells was confirmed using mice with targeted deletion of the *HIF-1 α* gene in T cells. The genetic "knockdown" of HIF-1 α in T cells prevented the

inhibition of T cells in hypoxic inflamed tissues, increased antibacterial response, and improved survival in mice (42). These observations also established that HIF-1 α effects are T-cell autonomous.

The immunosuppressive role of HIF-1 α in T cells was also indirectly supported by studies of the effects of pharmacologic weakening of tissue hypoxia and of hypoxia \rightarrow HIF-1 α signaling by systemic oxygenation (43) that weakens hypoxia and destabilizes HIF-1 α . The observations of increased immune response and exacerbation of collateral inflammatory damage in these experiments extended the earlier genetic evidence. HIF-1 α was shown to induce the expression of CD73, a membrane-bound glycoprotein that generates the immunosuppressive adenosine following binding of HIF-1 α to HRE sites in the *CD73* gene promoter. Thus, the inhibition of *HIF-1 α* expression by antisense oligonucleotides led to the inhibition of hypoxia-inducible CD73 expression (44). Another immunosuppressive molecule, A2BR, was also shown to be regulated transcriptionally by HIF-1 α (45).

Hypoxia–A2-Adenosinergic Protection of Tumors from Antitumor Immune Cells

Previous demonstrations supported the view that inflammatory damage-associated interruption in local blood supply and the ensuing tissue hypoxia may lead to the accumulation of extracellular adenosine and recruitment of anti-inflammatory A2AR on T cells in the adjacent normal tissues (11). This led these authors to a straightforward assumption that hypoxic and extracellular adenosine-rich cancerous tissues may have hijacked this A2AR-based mechanism to inhibit the incoming antitumor T cells by elevating the levels of their immunosuppressive intracellular cAMP (12, 15).

Indeed, many solid TMEs are hypoxic (46), and tissue hypoxia is conducive to the generation of extracellular adenosine (47). Tumors were shown to contain extracellular adenosine (48), although the intracellular cAMP-elevating A2AR or A2BR were explicitly excluded as possible candidates to mediate the immunosuppression by extracellular adenosine in tumors (48–50).

The key test to confirm or disprove the potential roles of extracellular adenosine and A2AR as mediators of immunosuppression in tumors was the comparison of antitumor immune response in A2AR gene-deficient mice with that of their wild-type (WT) littermates. On the basis of insights about the role of adenosine \rightarrow A2AR in immunosuppression in inflamed tissues, it was expected that A2AR gene-deficient mice would have much stronger and longer-lasting antitumor immunity. This hypothesis was validated by findings that the genetic deletion of A2AR resulted in much stronger antitumor immunity and rejection of established tumors and prolonged survival of A2AR-deficient mice compared with those of control tumor-bearing A2AR-expressing mice (12, 15).

The ability to recapitulate the antitumor effects of genetic deficiency in A2AR by pharmacologic maneuvers pointed to the feasibility and promise of the novel therapeutic approach of using small molecules to unleash the antitumor T cells from hypoxia–A2-adenosinergic inhibition (15).

Anti-hypoxia–A2-Adenosinergic Coadjuvants Enable the Antitumor Capacity of Current Cancer Immunotherapies

The pharmacologic inhibition of A2AR-mediated immunosuppressive signaling in T cells with synthetic and natural A2AR antagonists, or the pretreatment of tumor-reactive T cells with A2AR siRNA before adoptive transfer, led to much stronger antitumor effects of the transferred T cells or of the elicited endogenous antitumor immunity (12). This included reduced neovascularization of tumors, stronger rejection of lung metastases, and stronger inhibition of tumor growth. Similar increases in antitumor effects of transferred T cells are expected with the negatively selected A2AR^{low} antitumor T cells that are more resistant to inhibition by adenosine (51).

The demonstrated ability of A2AR antagonists to interrupt the hypoxia \rightarrow CD39/CD73 \rightarrow [adenosine]^{High} \rightarrow A2A-adenosinergic signaling at the last stage and to unleash the antitumor effects (Fig. 2) provided additional pharmacologic confirmation *in vivo* of the role of A2AR in antitumor immunity. These data also provided a proof-of-principle (12) for the therapeutic use of novel immunologic coadjuvants to block physiologic negative regulators of antitumor immunity (Fig. 2; refs. 11, 12). Figure 2 shows different types of therapeutically feasible treatments that target the individual upstream and downstream stages of the hypoxia \rightarrow CD39/CD73 \rightarrow [adenosine]^{High} \rightarrow A2AR/A2BR \rightarrow cAMP signaling to mitigate immunosuppression in the TME.

Inhibitors of cAMP-dependent PKA

PKA inhibitors were considered first as a potential approach to prevent the inhibition of antitumor T cells (23–29). However, these efforts were abandoned because such inhibitors likely have unacceptable side effects due to the crucial roles of the cAMP binding site and PKA in many fundamental biologic processes.

A2A adenosine receptor antagonists

In contrast with targeting PKA, a much more fruitful approach has been to block the intracellular cAMP-elevating high-affinity A2AR (30, 31) using synthetic or natural antagonists of these receptors. The biologic effects of antagonists of A2AR or A2BR are due to their competition with the tissue-generated, endogenous extracellular adenosine for binding to the same site on A2AR or A2BR. However, the receptor-bound antagonists do not trigger the accumulation of intracellular cAMP.

Thus, the binding of the antagonists to these adenosine receptors prevents the inhibition of T cells by adenosine. In addition, by blocking A2AR, it is expected that A2AR antagonists may also shorten the "memory" of exposure of T cells to immunosuppressive signaling through A2AR (36). Effects of A2AR antagonists are facilitated by the lack of spare A2AR (i.e., no "receptor reserve" in T cells; ref. 35), thereby allowing antagonists to further minimize the immunosuppressive effects of extracellular adenosine. The focus on high-affinity A2AR was because of its pattern of expression

(30–34) and because A2AR will likely be activated even at relatively modest increases in the levels of extracellular tissue adenosine.

Even the short-lived "first-generation" synthetic A2AR antagonists have demonstrated an increase in antitumor immunity *in vivo*, suggesting their use as adjuvants in cancer immunotherapies (12). Subsequent extensive and well-controlled studies revealed the potent antitumor effects of longer-lived A2AR antagonists (52, 53) and provided a strong rationale for clinical trials of existing cancer immunotherapies in combination with the currently available synthetic A2AR antagonists (53).

The use of synthetic A2AR antagonists in combination with cancer immunotherapies was the much desirable outcome of fundamental studies of antipathogen immune responses and autoimmunity (11–18). That alone would be sufficient to justify the large-scale research and development of this class of synthetic drugs. Fortunately, these drugs have been developed by neurobiologists because of the role of A2AR in the central nervous system and their original promise in slowing the progression of Parkinson disease.

Several synthetic A2AR antagonists have been shown to be safe in phase II and III clinical trials of Parkinson disease (30, 54, 55). One such A2AR antagonist, KW6002 (istradefyline), is approved for the treatment of patients with Parkinson disease in Japan. These drugs can be easily re-purposed and tested in combination with existing cancer immunotherapies. In contrast with the use of A2AR antagonists, considerations of the clinical utility of antagonists of A2BR are premature due to insufficient preclinical data, low affinity to adenosine, and potential cardiovascular side effects.

Extracellular adenosine-generating or -degrading enzymes

Approaches to target CD39 and CD73 ectoenzymes, which function in tandem to generate extracellular adenosine, have been developed in innovative research by Robson's research group in studies of CD39 (20, 56, 57), by Smyth and Stagg's research group (58–61), and by Zhang's research group (52, 62) in studies of CD73. Drugs such as adenosine deaminase (ADAGEN; Enzon) that degrade the accumulated extracellular adenosine in the TME (63), and drugs that inhibit the CD39/CD73 ectoenzyme-generated accumulation of extracellular adenosine in the TME, may provide yet another tool to inhibit the CD39/CD73 → A2A/A2B axis. Future studies may reveal relative advantages and disadvantages of using anti-CD39- or anti-CD73-blocking monoclonal antibody compared with small-molecule inhibitors to decrease the intratumoral levels of extracellular adenosine.

Inhibitors of TME hypoxia–HIF-1 α

Drugs that inhibit hypoxia–HIF-1 α signaling are in high demand due to the well-established understanding of the protumor effects of hypoxia (46) and HIF-1 α (64). Promising inhibitors of HIF-1 α , including digoxin and acriflavine (65, 66), were shown to decrease lung metastasis in an orthotopic breast cancer model. Other HIF-1 α inhibitors such as sir-tuin-7 and ganetespib, a new therapeutic candidate targeting

triple-negative breast cancer cells, were also found to have antitumor activities (67, 68), and are candidates for testing as anti-hypoxia–A2-adenosinergic immunologic adjuvants.

Hypoxia–A2-Adenosinergic Immunosuppression in Human Cancers

The original observations of the critical role of hypoxia–A2-adenosinergic immunosuppression in tumor protection (12, 15) have been confirmed in extensive and well-controlled studies by several groups in different models of tumor rejection. These studies have looked at the effects of A2AR genetic deletion in mice, A2AR antagonists and/or genetic deletion or pharmacologic inhibition of upstream stages of adenosine generation by the CD39/CD73 ectoenzymes (52, 53, 60, 62, 69–71).

The most significant are clinical implications of the recent extensive analysis of gene-expression data from more than 6,000 samples of triple-negative breast cancers. These studies provided evidence for the correlation between (i) high levels of expression of extracellular adenosine-generating ectoenzyme CD73 on human triple-negative and chemotherapy-resistant breast cancers, (ii) the inhibition of antitumor T cells and NK cells by A2AR and A2BR, and (iii) the poor prognosis of patients with such tumors (61).

Anti-hypoxia–A2-Adenosinergic Coadjuvants May Also Block Other Immunosuppressive Pathways

The hypoxia–A2-adenosinergic pathway not only may be the oldest in evolution but also the most influential in recruiting other immunosuppressive pathways. Indeed, it is challenging to come up with older biochemical entities/parameters than the lack of oxygen (anoxia, hypoxia) or adenosine, as discussed in an earlier review (16). It was proposed and confirmed that A2AR and A2BR, CRE and HRE-mediated transcription, and HIF-1 α have key roles in governing the functions of Tregs and effector cells (Fig. 1; ref. 16).

Thus, blocking hypoxia–A2-adenosinergic signaling should block at least partially many other immunosuppressive mechanisms. Indeed, it is already established that the hypoxia–A2-adenosinergic pathway also recruits other immunosuppressive molecules such as cyclooxygenase-2 and eicosanoid mediators (72–74). This pathway is also implicated in the development and functions of Tregs (16, 20, 75). Interestingly, both CD73-mediated generation of extracellular adenosine and A2AR were required for the suppressive effects of Tregs through a PD-1-dependent mechanism in the kidney ischemia–reperfusion injury model (76). It was also shown that the activation of A2AR recruited the negative immunologic regulators PD-1 and CTLA-4 on T cells (77).

Conclusions and Expectations

Hypoxia–A2-adenosinergic immunosuppression negates the antitumor effects of tumor-reactive T cells and NK cells. Published data and insights from yet to be published studies have identified this pathway as an important remaining barrier to more effective tumor rejection. Immunosuppressive

adenosine → A2AR/A2BR-mediated signaling can be weakened by anti-hypoxia–A2-adenosinergic coadjuvants, thereby further enhancing the antitumor potential of current cancer immunotherapies.

As depicted in Figs. 1 and 2 and reviewed here, the inhibition of hypoxia–A2-adenosinergic immunosuppression should improve the antitumor immunity of tumor-reactive T cells that have been induced by other immunotherapeutic protocols, including the mono- or dual immunotherapies with CTLA-4 or PD-1 blockade. This hypothesis was supported by recent observations of stronger antitumor effects of CTLA-4 or PD-1 blockade when combined with reducing levels of extracellular adenosine and inhibiting the adenosine → A2AR and A2BR signaling (53, 60). It was shown that inhibition of the accumulation of extracellular adenosine by anti-CD73 monoclonal antibody did indeed enhance the antitumor activity of dual CTLA-4 and PD-1 blockade in models of transplanted and chemically induced mouse tumors (60). It would be interesting to test whether the efficacy of A2AR antagonists could be further increased by lowering the concentration of extracellular adenosine in the TME by drugs that either inhibit the accumulation or induce the degradation of extracellular adenosine.

It must be emphasized that treatments with cancer vaccine-induced tumor-reactive T cells, adoptively transferred tumor-reactive T cells, or inhibitors of CTLA-4/PD-1, are highly complementary with anti-hypoxia–A2-adenosinergic coadjuvant treatments. Indeed, inhibition of all known

immunologic negative regulators along with the depletion of Tregs will still leave T cells vulnerable to multifaceted and powerful immunosuppression by tumor hypoxia and A2AR that can be weakened by anti-hypoxia–A2-adenosinergic coadjuvants.

Finally, inhibitors of the hypoxia–A2-adenosinergic pathway may have additional favorable anti-immunosuppressive effects by decreasing the intensity of many other immunosuppressive mechanisms such as Tregs, CTLA-4, TGF- β , cyclooxygenase-2, and eicosanoid-mediator immunosuppression. This, in turn, may allow for treatment with lower therapeutic levels of the checkpoint inhibitors of CTLA-4 or PD-1, thereby decreasing the treatment and immune-related side effects. Taken together, the available data strongly justify targeting the hypoxia → adenosine → A2AR/A2BR pathway to prevent the inhibition of antitumor T cells and NK cells in the TME.

Disclosure of Potential Conflicts of Interest

M. Sitkovsky is the founder and president of Redoxtherapies, Inc., has ownership interest (including patents) in a U.S. patent, and is a consultant/advisory board member for NewVac. A. Ohta has ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

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