Targeting Cancer-Derived Adenosine: New Therapeutic Approaches

Arabella Young1,2, Deepak Mittal1,2, John Stagg3, and Mark J. Smyth1,2

ABSTRACT
CD73 generation of immunosuppressive adenosine within the hypoxic tumor microenvironment causes dysregulation of immune cell infiltrates, resulting in tumor progression, metastases, and poor disease outcomes. Therapies targeted toward the adenosinergic pathway, such as antibodies targeting CD73 and CD39, have proven efficacy in mouse tumor models; however, humanized versions are only in preliminary development. In contrast, A2a adenosine receptor antagonists have progressed to late-stage clinical trials in Parkinson disease, yet evidence of their role in oncology is limited. This review will compare the merits and challenges of these therapeutic approaches, identifying tumor indications and combinations that may be fruitful as they progress to the clinic.

Significance: High concentrations of immunosuppressive adenosine have been reported in cancers, and adenosine is implicated in the growth of tumors. This brief review delineates the current treatment strategies and tumor subtypes that will benefit from targeting adenosinergic pathways, alone or in combination with contemporary approaches to cancer treatment.

INTRODUCTION
Immunosuppression within the tumor microenvironment causes inability of host immune effectors to eliminate transformed malignant cells. Although immune infiltrate is present in a proportion of most tumor types, variability in immune constituents may dictate disease outcome. The presence of regulatory T cells (Treg), tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC) drives a suppressive environment, which inhibits antitumor immune defenses (1, 2). Furthermore, the ratio between effector T cells and Tregs appears an important predictor of tumor prognosis (3). Generation of anti-inflammatory cytokines and metabolites by the tumor together with these suppressive cell types antagonize tumor immunity by inhibiting the cytotoxic functions of natural killer (NK) and CD8+ T lymphocytes, instead enhancing angiogenesis and promoting tumor cell survival and progression.

Currently, the development of therapeutic modalities to overcome effector immune cell dysfunction seems promising. Targeted therapies and immunotherapies offer advantages over conventional cytotoxic cancer treatments. Targeted therapies limit the availability of critical metabolites necessary for tumor survival, reducing off-target effects and minimizing unrestrained tissue damage. Immunotherapies aim to improve the patient’s own antitumor immune responses by releasing the suppressive restraints on effector immune cell populations. The advantages of such an approach include reduced toxicity and the development of memory responses leading to long-term tumor eradication.

Promising preclinical investigations into the blockade of immune checkpoint receptors, reduction of angiogenesis, and inhibition of immunosuppressive metabolite signaling in the tumor microenvironment have translated to oncology practice. Inhibitory immune checkpoint molecules cytotoxic T-like antigen-4 (CTLA4) and programmed death-1 (PD1), expressed on dysfunctional tumor-infiltrating lymphocytes, have been highlighted as targets to reset the effector T-cell immune response. The monotherapies ipilimumab (anti-CTLA4) and nivolumab (anti-PD1) have shown success in enhancing patient survival in a growing number of cancer types (4, 5). Nevertheless, tumors can deploy multiple mechanisms to allow their survival by avoiding immunosurveillance. Increasingly evident is the need to target multiple immunosuppressive mechanisms to overcome the high level of redundancy that exists in eliciting immunosuppression, if we are to increase patient life expectancy and quality. This need was highlighted by the clinical effects of combining ipilimumab and nivolumab, which showed even greater tumor clearance for patients with nonresectable metastatic melanoma (6).
Targeting the immune system to instigate enhanced effector immune cell function through blockade of inhibitory receptor signaling is not without risk. Immune checkpoint receptors are critical regulators of immune system activity, enabling self-tolerance and preventing the development of autoimmune pathologies. Administration of ipilimumab and nivolumab significantly enhances tumor regression; however, the risk of developing toxicity in the form of immune-related adverse events (irAE) from unrestrained stimulation of the immune response is also increased (6). Selection of compatible therapies that present synergistic antitumor efficacy in combination with minimal toxicity and autoimmune is a current clinical challenge. Identification of alternate immunosuppressive pathways, which may be targeted to enhance clearance of malignancies with limited associated irAEs, will be advantageous.

Recently, the adenosinergic pathway has risen to prominence as a therapeutic target for immune activation. This review details the effects of adenosine-generated immunosuppression in the tumor microenvironment, highlighting the potential use of associated enzymes and receptors as predictive biomarkers for treatment response and progression. Further, we dissect the pros and cons of targeting the generation of adenosine versus adenosine receptors as a therapeutic strategy. Finally, we identify potential synergistic combinations that might reduce tumor growth and metabolic burden.

ADENOSINE-ABUNDANT TUMOR MICROENVIRONMENT

Adenosine is generated in response to proinflammatory stimuli such as cellular stress initiated by hypoxia or ischemia (7). Release of extracellular ATP undergoes conversion to AMP by the enzyme CD39 and subsequent dephosphorylation of AMP to adenosine is catalyzed by CD73 (Fig. 1). Landmark studies by Ohta and colleagues (8) have highlighted the importance of adenosine for tumor escape. Adenosine interacts with four G-protein–coupled receptor subtypes (A1, A2A, A2B, and A3), allowing for diversity and cellular specificity in signaling pathway activity. Each receptor possesses variable affinity to the adenosine ligand indicative of different sensitivities and responsiveness to adenosine levels. In particular, the low-affinity A2B adenosine receptor initiates signal transduction in comparatively limited physiologic conditions. Together, the A1, A3, and A2B adenosine receptors stimulate Gs/Go-α-dependent adenyl cyclase-driven cAMP accumulation, enabling signal transduction to target genes. A2A adenosine receptors are also coupled to G proteins, which can activate JNK and p38 via phospholipase C-dependent or –independent pathways (9). Positioning of adenosine receptors, particularly A1a and A2B, adenosine receptors that promote an anti-inflammatory response, on immune cells reduces tissue injury in conditions of cellular stress, enabling maintenance of tissue homeostasis. The persistence of increased adenosine concentrations beyond the acute-injury phase can, however, become detrimental to tissues by activating pathways that trigger immunosuppression or promote an unremitting wound-healing process. Thus, the accumulation of adenosine in the hypoxic tumor microenvironment mediates immunosuppression, causing dysregulation of effector immune cell subsets, dampening the antitumor immune response. Rapid proliferation of tumor cells in a manner unsupported by surrounding vasculature leads to the development of hypoxic regions within solid tumors (Fig. 1). Although hypoxia increases cell death due to oxygen and nutrient deprivation, it also promotes selection of resistant, aggressive cancer cells able to survive in unfavorable conditions. Hypoxia-resistant malignant cells typically possess stem-like features with greater proliferative and migratory capacity. This correlates with increased risk of metastasis, reduced responsiveness to conventional therapies, and ultimately poor prognosis for patients. Hypoxia-inducible factors (HIF) initiate differential transcriptional regulation in response to hypoxic conditions, influencing cellular metabolism and angiogenesis, in an effort to ease oxygen requirements. Identification of HIF1α-driven CD73 (NT5E) and CD39 (ENTPD) transcriptional activation in epithelia and endothelia provides an important link for hypoxia-mediated adenosine production (Fig. 1; ref. 10). Modification of the HIF1α binding site situated in the CD73 promoter abrogated transcriptional response indicative of a direct regulatory relationship (10). Similarly, hypoxia-controlled HIF1α-induced expression of the A3h adenosine receptor was identified, representing a coordinated response that may contribute to autocrine and paracrine adenosine signaling (Fig. 1; ref. 11). Although modulation of CD73, CD39, and A3h adenosine receptor (ADORA2B) gene expression represents a potential global mechanism of hypoxia response, interaction between these markers and HIF1α within the tumor microenvironment is yet to be confirmed.

Apart from the mobilization of adenosine via concentrative and equilibrative nucleoside transporters, adenosine production within the tumor microenvironment is essentially mediated by CD39 and CD73 ectonucleotidase expression on both non-hematopoietic and hematopoietic cellular subsets. Predominant cell types expressing CD39 and CD73 include endothelial cells and lymphocytes including immunosuppressive Tregs, as well as the tumor itself (Fig. 1). In humans, only a small fraction of Tregs express CD73 in steady-state (12). In response to IL2, however, the majority of human Tregs express both CD39 and CD73 (13). Increased Treg abundance in response to hypoxia via upregulated FOXP3 gene expression represents an additional mechanism of HIF1α regulatory control. Consequently, increased Treg infiltration within the hypoxic tumor microenvironment is likely to enhance adenosine-mediated immunosuppression (14). However, tumors produce comparatively greater adenosine levels than those generated by endogenous host CD73 (15, 16). Release of tumor-derived exosomes expressing catalytically functional CD39 and CD73 represents an additional mechanism of tumor-mediated immunosuppressive adenosine generation (17). This is indicative of the critical importance and reliance on adenosine production to enable immune evasion and enhance tumor survival.

ADENOSINE-DRIVEN CELLULAR SIGNALING

As demonstrated in Fig. 1, elevated adenosine is instrumental in regulating an abundance of cellular responses in
the tumor. Essentially, adenosine disables cytotoxic effector functions of both NK and CD8+ T cells predominantly via A3A adenosine receptor signaling, enabling tumor immune evasion and escape (7). Furthermore, signal transduction through the A3A adenosine receptor inhibits the Th1 CD4+ T-cell response, limiting the cytokine environment necessary to support these effector cell types. Adenosine polarizes myeloid cells to develop into immunosuppressive phenotypes such as M2 macrophages and tolerogenic dendritic cells (DC) due to A3A and A2A adenosine receptor signaling, respectively (18). In addition, adenosine enhances proliferation of Tregs and granulocytic MDSCs, which further affect T effector cell proliferation and function (14, 19, 20). Release of cytokines and immune modulatory factors, such as VEGF, IL6, IL10, and TGFβ, by these suppressive cell types enhances tumor survival via heightened angiogenesis and inhibited immunosurveillance, and thus the majority of evidence suggests that adenosine is favorable to tumor survival.

Figure 1. Adenosine-mediated effects in the hypoxic tumor microenvironment. Release of extracellular ATP in the hypoxic tumor microenvironment is converted to adenosine by CD39 and CD73 ectonucleotidases. CD39 and CD73 are broadly expressed across a number of cell types. Modulation of their distribution can vary dependent on cellular activation and tissue localization. Adenosine enhances polarization of myeloid and T-cell subsets to proangiogenic and immunosuppressive phenotypes, enhancing tumor growth and survival. High adenosine levels affect effector immune cells, NK cells, and CD8+ T cells, responsible for cytotoxic killing of aberrant malignancies due to inhibited expression of molecules that mediate cell death. DC, dendritic cells; IDO, indoleamine 2,3 dioxygenase; TCR, T-cell receptor.

ADENOSINE MEDIATORS AS PUTATIVE BIOMARKERS

Overexpression of the ectonucleotidase enzyme CD73 has been shown in different types of cancer, and several studies reported an association between high CD73 levels and poor prognosis, increased risk of metastasis (21), and resistance to chemotherapy (22, 23). In breast cancer, hypoxia-independent mechanisms further drive CD73 upregulation. Spychala and colleagues (24) first described that the absence of estrogen receptor (ER) expression in human breast cancer cell lines was inversely associated with high CD73 expression, but the underlying mechanism is still not fully understood. Using methylation-specific PCR and DNA pyrosequencing, CD73 expression in breast cancer cell lines was inversely associated with CD73 promoter CpG methylation (25). Interestingly, CD73 promoter methylation was associated with increased disease-free and overall survival and better outcome after adjuvant chemotherapy. These results suggest that
CD73 promoter methylation could be used as a breast cancer biomarker to predict metastasis and global clinical outcome.

We recently reported a large meta-analysis of CD73 gene expression in breast cancer, gathering more than 6,000 patients, where CD73 gene expression is significantly associated with poor prognosis in patients with triple-negative breast cancer (TNBC, ref. 23). CD73 expression in patients with TNBC was also correlated with an increased resistance to anthracycline chemotherapy due to adenosine-mediated suppression of antitumor immunity. In addition to its immunosuppressive effects, we showed that CD73-derived adenosine promotes tumor cell migration in vitro and metastasis in vivo via the A<sub>2B</sub> adenosine receptor (26). Most interestingly, the A<sub>2B</sub> adenosine receptor has recently been identified as a target of the metastasis-inducing transcription factor FOS-related antigen 1 (FRA1) in TNBC (27). Ntantie and colleagues (28) further unraveled a signaling pathway linking A<sub>2B</sub> adenosine receptor signaling and tumor cell adhesion. Taken together, these studies converge to suggest an important role for the CD73–A<sub>2B</sub> axis in metastatic dissemination, independently of immunosuppression. These findings also highlight the importance of developing these and other biomarkers of adenosine generation and signaling as putative biomarkers of TNBC, before any new therapeutic approaches.

**TARGETED THERAPEUTICS AGAINST ADENOSINE GENERATION**

Because adenosine is a metabolite, genetics can only be used to study the adenosinergic pathway by targeting the genes encoding for CD39, CD73 (that generate adenosine), or the adenosine receptors (that consume adenosine). Initial investigations determined that CD39- or CD73-deficiency improved antitumor immunity and survival by significantly reducing tumor growth and metastasis (29–32). Development of bone marrow chimeras confirmed that critical CD39 and CD73 expression on both nonhematopoietic endothelial and hematopoietic Treg cellular lineages mediated tumor growth (30, 32, 33). However, formation of experimental pulmonary metastases in CD73-deficient mice appeared dependent on nonhematopoietic CD73 expression (30). Nonhematopoietic endothelial expression of CD73 regulates angiogenesis, a necessary requirement for the promotion of metastasis (29, 34, 35). Reduction of immunosuppressive adenosine within the tumor microenvironment enhances effector functions of immune cell infiltrate in a tumor-dependent manner. Expansion of tumor antigen–specific CD8<sup>+</sup> T cells infiltrating the tumor and circulating within the periphery was evident in CD73-deficient mice (33). In contrast, deletion of CD39 reduced Treg-mediated immunosuppression, enabling increased NK cell infiltration and cytotoxic activity in the hepatic tumor microenvironment (32).

 Mimicking these enhanced antitumor responses through pharmacologic targeting of CD73 and CD39 identified similar reductions in cancer progression. Small-molecule inhibitors APCP and POM-1, which directly abrogate the catalytic functions of CD73 and CD39, respectively, inhibited tumor growth and metastasis (30, 32, 33). Efficacy was negated when treating their respective gene-targeted mouse strains, indicative of inhibitor specificity (32, 33). Intratumoral injection of CD39 inhibitors also reestablished the prommunogenic activity of anthracyclines in mice (36). Blockade of CD39 enzymatic activity may lead to an increase in extracellular ATP levels, enabling coactivation of inflammasomes, promoting tumor immunity. Antibody-directed immunotherapies targeting CD73 demonstrated similar antitumor properties (26). Therapeutic efficacy required the presence of a competent immune system, indicating that antibody blockade of immunosuppressive adenosine generation enhanced immunosurveillance. Cocultured immune cell subsets, NK, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells, with ovarian carcinoma cell lines expressing CD39 and CD73, displayed enhanced proliferative and lytic capacity in vitro following the addition of anti-CD39 or anti-CD73 (15). These effects were directly attributed to reduced adenosine production within the culture system, enabling improved immune responsiveness.

In addition to mediating the rate-limiting step for adenosine production, CD73 also promotes cellular adhesion, particularly lymphocyte interactions with the endothelium, and angiogenesis (34, 35, 37). Furthermore, CD73 enhances the migratory capacity of malignant cells, resulting in increased risk of metastatic progression (26, 38, 39). Recently, anti-human CD73 monoclonal antibody (mAb) therapy was shown to reduce spontaneous metastasis of human breast cancer xenografts, independent of primary tumor growth (39). Lack of a functional immune system within these models implies that metastasis was attenuated by an alternative mechanism to the improved antitumor immunity previously determined. An epitope-specific function was identified, in which recognition of CD73 on cancer cell lines promoted clustering and internalization of CD73, preventing CD73-mediated extravasation and colonization of potential metastatic clones (39). Determining which patients will benefit from anti-CD73 treatment or contraindicated circumstances will be necessary as this therapy moves forward to clinical utility. In the setting of ER<sup>+</sup> breast cancer, the use of anti-CD73 may facilitate the accumulation of extracellular AMP, directly activating the A<sub>1</sub> adenosine receptor, potentially enhancing ER signaling and breast cancer growth (40, 41).

Although CD73-deficient mice present no overt phenotypic abnormalities, global CD39 deficiency is associated with various physiologic disturbances, including glucose intolerance, insulin resistance, and increased levels of circulating fatty acids (42). Importantly, CD39-deficient mice exhibit significantly increased bleeding times (>20 minutes compared with 1 minute) resulting from P2Y1 receptor desensitization, and a vascular prethrombotic state in basal conditions (43). Perhaps most worrisome is the propensity for CD39-deficient mice to spontaneously develop hepatocellular carcinoma, with a 70% incidence at 18 to 24 months of age (44). This seems to result from enhanced mTOR signaling in hepatocytes as a consequence of high extracellular ATP levels and P2 receptor activation. RAS and PI3K may also be involved, because both are regulated by P2 receptors. These studies emphasize the need to further investigate the role of CD39 in cancer.

In summary, preclinical studies targeting CD39 or CD73 identified the therapeutic potential and validity of these approaches as strategies for improving antitumor immune response (Table 1). Both targets display enhanced expression in a broad spectrum of cancer types, as well as on
### Table 1. Advantages and disadvantages for targeting adenosine generation (CD39 and CD73) and adenosine signaling (A₁, A₂A, A₂B, and A₃ adenosine receptors) pathways

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<tr>
<th>Therapeutic target—localization</th>
<th>Potential advantages</th>
<th>Potential disadvantages</th>
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<td><strong>CD39</strong>&lt;br&gt;• B cells, NK cells, activated T cells, particularly Tregs, endothelial cells, solid tumors, and certain leukemias.</td>
<td>• Rate-limiting step converting ATP to AMP&lt;br&gt;• Increased expression in some cancer types&lt;br&gt;• Antibody therapies and small-molecule inhibitors [polyoxometalate-1 (POM-1)] improve antitumor immunity and in some cases display anticancer potential in murine models.</td>
<td>• Lack humanized antibodies&lt;br&gt;• POM-1 displays proven selectivity for CD39, difficult to determine potential off-target effects in humans.</td>
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<td><strong>CD73</strong>&lt;br&gt;• Endothelium and epithelium, stromal cells, Tregs, MDSC, B cells, CD8⁺ T cells, tumor correlating with increased metastasis.</td>
<td>• Multifunctionality of CD73&lt;br&gt;• Generation of adenosine from AMP&lt;br&gt;• Immunosuppressive environment&lt;br&gt;• Angiogenesis&lt;br&gt;• Involved in lymphocyte adhesion&lt;br&gt;• Migration of immune and tumor cells&lt;br&gt;• Inhibition promotes antitumor immunity.&lt;br&gt;• Direct relationship with several types of cancers and proven anticancer properties.&lt;br&gt;• Use as a predictive biomarker to dictate therapeutic efficacy of adenosine-mediated therapies.</td>
<td>• Current lack of humanized antibodies.&lt;br&gt;• Development of antibodies that target multiple functions of CD73 will be necessary for maximal efficacy.&lt;br&gt;• Highly abundant CD73 expression may lead to alternative deleterious effects not identified in murine models.</td>
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<td><strong>A₁</strong>&lt;br&gt;• Broad distribution, particularly abundant in nerves, heart, and kidneys.</td>
<td>• A₁ adenosine receptor antagonists and agonists have undergone clinical testing, i.e., Rolofylline, Tonapofylline, and GW493838.&lt;br&gt;• A₁ adenosine receptor shown to regulate ER expression in certain breast cancer subtypes.</td>
<td>• Disappointing clinical outcomes and discontinuation of testing in the treatment of nerve injury and prevention of heart and renal failure.</td>
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<td><strong>A₂A</strong>&lt;br&gt;• Broad distribution, particularly immune cells NK, CD4⁺, and CD8⁺ T cells, macrophages as well as endothelium.</td>
<td>• High-affinity adenosine receptor expressed on immune cells.&lt;br&gt;• Antagonism promotes antitumor immunity, enhancing cytotoxic functions of CD8⁺ T cells and NK cells.&lt;br&gt;• A₂A adenosine receptor antagonists and display excellent safety profiles in clinical testing for neurodegenerative diseases.</td>
<td>• Small-molecular inhibitors show appropriate tissue penetration and bioavailability but short half-life ranging in hours.&lt;br&gt;• Therapeutic efficacy specific to highly hypoxic adenosine-mediated tumors.</td>
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<td><strong>A₂B</strong>&lt;br&gt;• Broad distribution, particularly endothelium, MDSCs, DCs also apparent in some cancer types.</td>
<td>• HIF1α-driven A₂B adenosine receptor expression in response to the hypoxic tumor microenvironment.&lt;br&gt;• Antagonists display anticancer, antiangiogenesis, and enhanced immune efficacy.</td>
<td>• Low-affinity adenosine receptor, may be necessary to block others, particularly A₂A adenosine receptor, in concert for efficacy.</td>
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<tr>
<td><strong>A₃</strong>&lt;br&gt;• Broad distribution, particularly the nervous and cardiovascular system as well as immune cell subsets, highly expressed in liver and colon cancers.</td>
<td>• CF101 A₃ adenosine receptor agonist in clinical trial for the treatment of autoimmune disorders such as psoriasis and rheumatoid arthritis.&lt;br&gt;• CF102 A₃ adenosine receptor agonist in clinical trial for treatment of liver cancer.&lt;br&gt;• Good safety profile and increased survival in initial clinical testing.</td>
<td>• Development of therapeutic resistance by directly targeting cancer cells.&lt;br&gt;• Off-target effects have not been established, potential agonists of immune cells can lead to activation-induced cell death or autoimmunity.</td>
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immunosuppressive targets within the tumor microenvironment. However, given that these molecules collectively regulate inflammation in cancer and other chronic diseases, the safety of these therapeutic modalities in patients must be met with caution. Nonetheless, there is currently a lack of humanized anti-human CD39 and CD73 antibodies available for immediate progression toward clinical translation. Because of the multifunctionality of CD73 (promoting tumor-associated angiogenesis, and cellular adhesion and migration), CD73 appears to be a most promising target. It is our contention that therapies able to target both its catalytic and noncatalytic roles would be desirable and are likely to display the greatest efficacy in the treatment of cancer.

**BLOCKADE OF ADENOSINE SIGNALING ENHANCES ANTITUMOR RESPONSE**

Expression of the A2A adenosine receptor on immune cell subsets has led to this receptor being primarily investigated as a target to disrupt adenosine-mediated immunosuppression in the tumor microenvironment (Table 1). Immunogenic CD8+ T-cell–regulated tumors were shown to display reduced growth in A2A receptor–deficient mice (8). In addition, T-cell–specific knockdown of the A2A and A2B adenosine receptors in combination reduced metastasis and enhanced survival (8). Similarly, antagonism of both A2A and A2B adenosine receptors with small-molecule inhibitors suppressed metastatic nodule formation (38) against CD73-expressing tumors, indicative of adenosine receptor reliance on adenosine generation within the tumor microenvironment. A3 adenosine receptor antagonism mediated its response via a lymphocyte-dependent perforin-mediated mechanism, whereas metastatic progression was inhibited via the A3 adenosine receptor independent of NK and CD8+ T-cell functions (38). In contrast, another study showed that orthotopic murine breast cancer growth and subsequent development of spontaneous lung metastasis was reduced by intratumoral A3b adenosine receptor antagonism in an IFNγ–activated T cell–dependent manner (45). Recently, A2A adenosine receptor expression was localized in the cell membrane of malignant lung adenocarcinoma tissue (46). Blockade of the A2A adenosine receptor was shown to reduce tumor growth by enhancing apoptotic cell death in vivo in an immunocompromised human xenograft model (46), indicative of possible alternate functions of A2A adenosine receptor antagonism. Similarly, A2B adenosine receptor inhibitors are able to directly influence tumor cell growth and migration (30). Prostate cancer cell lines, in which A3b was the dominant adenosine receptor, demonstrated reduced proliferative capacity following blockade of A2b adenosine receptor or endogenous reduction via siRNA knockdown indicative of direct anticancer effects (47). Alternatively, promotion of endothelium-derived angiogenesis by the A3b adenosine receptor in response to hypoxia is an alternative mechanism for impeding tumor development (11). Gene-targeted A2b adenosine receptor mice display impacted VEGF secretion in the tumor microenvironment, resulting in reduced neovascularization and repressed tumor growth (48).

In contrast to the A2A and A2B adenosine receptors, which activate signal transduction cascades via cAMP, the A1 and A3 adenosine receptors downregulate cAMP, enhancing a proinflammatory response. Consequently, targeting these receptors via a different approach is necessary, with agonists perhaps more suitable for abrogating tumor cell survival. Currently, the A3 adenosine receptor agonist CF102 is undergoing clinical testing for safety and efficacy in treatment of hepatocellular carcinoma, in which A3 adenosine receptor is highly expressed (Table 1; ref. 49). Current indications suggest this agonist is well tolerated by patients, inducing modest survival benefit through initiation of cancer cell apoptosis (49, 50). However, targeting malignant cells directly induces selection of A2A adenosine receptor–null clones, which potentially may escape therapeutic intervention. Although A2A adenosine receptors are present on immune cell subsets, the effect of this therapy on the immune system has not been distinguished. As adenosine receptors show overlapping cellular specificity and redundancy in pathway activity, it may be necessary to target multiple receptor subtypes concomitantly to potentiate an effective treatment response.

**COMPARING SMALL-MOLECULE INHIBITORS WITH ANTIBODY-TARGETED THERAPIES**

One of the driving forces behind the development of mAbs in oncology is their historically higher approval rates compared with small molecules. Part of the success of mAbs comes from the fact that they generally have higher specificity and longer half-life, and can exert both direct on-target and indirect immune-dependent antitumor effects, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP).

On the other hand, small molecules are generally associated with a broader biodistribution. Small molecules can easily diffuse across physiologic barriers such as the blood–brain barrier and plasma membranes. In contrast, mAbs largely rely on tumor endothelium permeability to penetrate solid tumors, and endocytosis consumption affects mAbs biodistribution. Although this has not been reported in mice receiving anti-mouse CD73, antiendothelial cell antibodies can induce systemic sclerosis and other vasculitis diseases. In humans, antiendothelial cell antibodies upregulate adhesion molecules, increase leukocyte adhesion, and promote ADCC (51). Through ADCC, anti-CD73 may also deplete lymphocyte subsets. Accordingly, CD73 is expressed on 70% of circulating B cells, 10% of CD4+ T cells, 80% of naïve CD8+ T cells, and 30% of memory CD8+ T cells. Nevertheless, higher CD73 levels in the tumor microenvironment compared with normal tissue might offer a therapeutic window for ADCC-proficient anti-CD73.

Compared with a small-molecule inhibitor, an anti-CD73 mAb offers the possibility to target both adenosine-dependent and adenosine-independent CD73 functions (Fig. 2). Indeed, accumulating evidence suggests that CD73 is more
than just an adenosine-producing enzyme. Early studies have shown that anti-CD73 mAbs (e.g., clone 1E9), in combination with suboptimal mitogenic signals, can stimulate human T-cell activation. Interestingly, immortalized human T cells (Jurkat) expressing enzymatically inactive CD73 can still be coactivated with anti-CD73. These findings raise the possibility that a physiologic ligand for CD73 may be involved during T-cell activation, although this is yet to be demonstrated. Interaction with SRC protein kinases has been suggested as a mechanism of signal transduction by CD73. One study has shown that anti-CD73 mAbs can induce intracellular kinase signaling in T cells, but they are unable to deliver a tyrosine phosphorylation signal in endothelial cells (52). This supports the view that CD73 molecules on different cells can have different functions. Anti-CD73 targeting distinct epitopes can also induce different effects. Although anti-human CD73 mAb 1E9 promotes T-cell signaling and inhibits CD73 enzymatic activity, mAb 4G4 induces CD73 shedding from the T-cell surface and fails to promote mitogenic activity. 4G4 nevertheless enhances human lymphocyte adhesion to endothelial cells (53). The anti-human AD2 mAb, on the other hand, induces clustering and internalization of CD73, but has minimal effect on enzymatic activity (39).

**COMBINATORIAL POTENTIAL OF ADENOSINE METABOLISM INHIBITION**

Combining therapies with distinct abilities to heighten immune effector functions or limit angiogenic potential is likely to enhance treatment efficacy. In this section, we will consider combinations where preclinical data support their rapid translation to the clinic.

The mechanism of action for current conventional cancer therapeutics, chemotherapy and radiotherapy, is considered largely reliant on direct cytotoxicity of tumor cells via genome instability and inability to repair vast amounts of DNA damage. In addition, some of these therapies enhance immunogenicity of tumor cells through increased MHC-I
expression and initiate immunogenic cell death, stimulating an immune response, enabling patients to actively clear residual malignant cells as well as distant metastases (54). Cellular stress initiated by these therapeutic modalities increases extracellular ATP release, leading to adenosine production impeding the immune response (55). Induction of CD73 and to a lesser extent CD39 by the anthraclycine doxorubicin was identified in human breast cancer cell lines, predominantly those lacking the ER (23). Patients with TNBC expressing high levels of CD73 display increased risk of relapse and poor prognosis following anthracycline treatment. Similarly, transplantable TNBC murine models with transduced CD73 confer increased anthracycline resistance and reduced survival (23). This was abrogated following combination treatment by doxorubicin and anti-CD73 or A$_{2A}$ adenosine receptor inhibitor. Blockade of CD73 in combination with doxorubicin robustly increases the frequency of tumor-specific CD8$^+$ T cells, correlating with the increased IFN$\gamma$ response essential for chemotherapeutic efficacy (23, 54). Furthermore, chronic lymphocytic leukemia (CLL) expressing CD73 was shown to have greater proliferative capacity, particularly in localized perivascular regions (22). Generation of adenosine by these cells attenuated both spontaneous and drug-induced apoptosis; however, inhibition of A$_{2A}$ adenosine receptor signaling rectified CLL chemosensitivitiy (22). Although antibodies and small-molecule inhibitors against CD73 have yet to be tested in humans, clinically tested therapies against downstream adenosine receptors are available. Clinical investigations targeting A$_{2A}$ adenosine receptors have predominantly related to neurologic and autoimmune disorders (56). Because of the excellent safety profile of these adenosine receptor antagonists, it is feasible to consider a rapid translation toward clinical testing in patients with cancer.

Manipulation of effector T-cell activity through blockade of immune checkpoint receptors provides durable antitumor responses in a proportion of patients (4, 5). Although both ipilimumab and nivolumab extend patient survival, combinatorial treatments targeting multiple immunosuppressive pathways appear necessary for optimal curative benefits. The development of toxicity in the form of irAEs due to systemic stimulation of the immune system is of current concern for clinicians. Because of excessive adenosine production in the tumor microenvironment, it is expected that adenosine-targeted therapies will specifically enhance antitumor immunity with limited autoimmune pathologies. Nevertheless, due to the central role of adenosine in dampening inflammation, great caution must be taken in early-phase trials while targeting this pathway. Immune checkpoint blockade by nivolumab and ipilimumab and inhibition of the adenosinergic system display increased antitumor efficacy via similar, nonredundant enhancement of CD8$^+$ T-cell responses (8, 23, 57). Concurrent treatment schedules may synergistically enhance antitumor effector function allowing for reduction in maximal dosage, potentially limiting development of irAEs. Recently, anti-CD73 therapy in combination with both anti-PD1 and anti-CTLA4 therapy was shown to synergistically reduce syngeneic tumor burden and extend survival via IFN$\gamma$-dependent, Th1 CD4$^+$-driven expansion of tumor-specific CD8$^+$ T cells (57). The adenosine analog NECA appeared to heighten expression of PD1, but not CTLA4, on tumor-specific T cells. However, this was attenuated by antagonism of the A$_{2A}$ adenosine receptor, potentially increasing anti-PD1 potency (57).

Blockade of the A$_{2A}$ adenosine receptor significantly reduces metastatic burden and improves survival in experimental and spontaneous metastases models (38). Use of ipilimumab and nivolumab in advanced cancer settings, such as the treatment of nonresectable metastatic melanoma, parallels these preclinical findings. It is therefore conceivable that immune checkpoint inhibitors and A$_{2A}$ adenosine receptors with corresponding, nonredundant functions may be targeted to provide synergistic antitumoral activity within the clinic. However, screening for CD73 status of disease must be considered to gain maximal efficacy from adenosine-targeted treatments (38). In particular, certain cancer types, such as TNBC, in which CD73 expression correlates with poor prognosis, should be targeted to examine potential efficacy of therapeutic combinations involving the adenosinergic pathway.

CONCLUSION

Generation of extracellular adenosine is greatly dependent on the ecto-enzyme CD73, which seems to be highly abundant in the hypoxic tumor microenvironment.

CD73 acts as a prognostic biomarker for decreased survival, increased metastatic activity, and multidrug chemoresistance in various types of cancer. Protumorigenic effects of CD73 are at least partly attributed to immunosuppression via A$_{2A}$ adenosine signaling in antitumor immune cells. The recent clinical studies of A$_{2A}$ adenosine receptor antagonists in Parkinson disease are indicative of their potential safety for rapid translation. In particular, preclinical evidence suggests that A$_{2A}$ antagonists may potentiate current chemotheraphy and immunotherapeutic strategies. The development of therapies that modulate adenosine receptor signaling in the cancer setting must be taken forward cautiously, but are likely to improve treatment efficacy.

Disclosure of Potential Conflicts of Interest

J. Stagg and M.J. Smyth declare a current sponsored research agreement with Medimmune LLC. No potential conflicts of interest were disclosed by the other authors.

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