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Enhancement of tumor immunotherapy by deletion of the A_{2A} adenosine receptor

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Abstract

The A_{2A} adenosine receptor plays a critical and non-redundant role in suppressing inflammation at sites of hypoxia and tissue damage. The tumor microenvironment has high levels of adenosine as a result of hypoxia and ectopic expression of enzymes responsible for the generation of extracellular adenosine. Thus, we sought to determine the ability of A_{2A} receptor null mice to immunologically reject tumors. We observed that mice lacking the A_{2A} adenosine receptor showed significantly delayed growth of lymphoma cells when compared to WT mice. Furthermore, when immunized with a low dose of tumor or with an irradiated GM-CSF-secreting tumor vaccine, A_{2A} receptor null mice showed significantly enhanced protection from a subsequent high-dose challenge from both immunogenic and poorly immunogenic tumor lines. This increase in protection was accompanied by an increase in the number of tumor-antigen-specific CD8 T cells at the vaccine-site draining lymph node. Finally, we found that A_{2A} receptor null mice displayed more robust anti-tumor responses than WT mice when they were treated with a soluble B7-DC/Fc fusion protein designed to antagonize B7-H1-mediated co-inhibition. This combinatorial immunotherapy strategy could also be recapitulated with pharmacological A_{2A} receptor blockade paired with B7-DC/Fc administration. In light of these data, we believe that blockade of the A_{2A} adenosine receptor is an attractive target for tumor immunotherapy that synergizes with other immunomodulatory approaches currently in clinical trials.

Keywords

A2a Adenosine receptor; Tumor; T cell; Co-inhibition; B7-DC; Vaccine

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Conflict of interest Jonathan Powell is a Scientific Founder of Amplimmune, Inc.

Introduction

Extracellular adenosine is a primordial signaling molecule that serves as a potent regulator of inflammation [1, 2]. This ubiquitous purine nucleoside is normally only found at nanomolar levels in the extracellular environment [3] due to the activity of the enzyme Adenosine Deaminase which rapidly catalyses the generation of inosine. However, the concentration of extracellular adenosine can increase dramatically at sites of trauma, hypoxia and inflammation [4]. Damaged cells with compromised membrane integrity in these environments release into the surrounding interstitial space not only adenosine, but also AMP, ADP, ATP and other purine catabolites [5, 6]. These nucleotides are quickly broken down to form adenosine by the extracellular ectonucleotidase CD39 and CD73 [7, 8]. There are four known adenosine receptors, A_1 , A_{2A} , A_{2B} and A_3 , all of which modulate the activity of adenylyl cyclase to influence the generation of intracellular cAMP [9–11]. The A_1 and A_3 receptors are G_i protein linked and inhibit the generation of cAMP when stimulated, while the A_{2A} and A_{2B} receptors are G_s linked and activate the production of cAMP [12]. These receptors are expressed widely throughout the body, including the heart, brain and lung [13], as well as epithelial cells [14] and lymphocytes [15].

It is becoming clear that this extracellular adenosine is not only the product of inflammation but can profoundly regulate inflammation. Seminal studies by the Sitkovsky group demonstrated the critical role of the adenosine A_{2A} receptor in regulating inflammation [16]. In spite of the fact that there are 4 adenosine receptors, knocking out the A_{2A} receptor led to exaggerated and lethal inflammation in response to normally moderate stimuli. The A_{2A} receptor is found on cells of hematopoietic origin and has been shown to inhibit inflammatory gene expression from activated neutrophils, macrophages, dendritic cells, NK cells and lymphocytes [17–20]. Indeed, acutely, A_{2A} receptor activation is a potent inhibitor of TCR-induced cytokine production [21]. Further, our group has shown that signaling via the A_{2A} adenosine receptor can induce anergy in CD4 T cells despite strong TCR engagement and co-stimulation [22]. In addition, adenosine has been shown to play a pivotal role in regulating the generation and functionality of regulatory CD4 T cells [22, 23] and the expression of inhibitory molecules such as LAG3 on activated T cells [22]. Treatment of mice with agonists to the A_{2A} adenosine receptor has been shown to decrease the severity of several models of autoimmunity, such as Inflammatory Bowel Disease (IBD) [24].

Inasmuch as adenosine is released into the inflammatory milieu, it is thought that signaling via the A_{2A} receptor serves as a negative feedback loop preventing collateral tissue destruction by overzealous immune responses [16]. However, many tumors have subverted the normally beneficial function of this signaling pathway to evade immune destruction [17, 25]. As a result of the highly hypoxic and/or inflammatory conditions, the tumor microenvironment has been shown to have high levels of adenosine which is an ideal situation for A_{2A} receptor activation [2]. In addition to being intrinsically hypoxic due to a high rate of metabolism and insufficient vascular development, many tumors ectopically express the ectonucleotidase responsible for the generation of extracellular adenosine [26, 27].

In this study, we show that mice lacking the A_{2A} adenosine receptor have significantly decreased rates of tumor growth relative to their WT counterparts. Furthermore, A_{2A} receptor null mice show increased responses to both lymphoma and melanoma tumor vaccines, resulting in a greater expansion of tumor-specific CD8 T cells and increased survival of tumor-bearing hosts. Finally, the combination of mitigating A_{2A} receptor signaling and blocking checkpoint-mediated inhibition of anti-tumor immunity with a B7-DC/Fc fusion protein resulted in significantly increased survival in tumor-bearing mice.

These results suggest that signaling via the A_{2A} adenosine receptor plays a critical role in regulating tumor growth in vivo and that the inhibition of A_{2A} receptor signaling, when combined with blockade of other inhibitory signals, offers an exciting possibility for enhancing the potency of tumor immunotherapy.

Materials and methods

Mice

A_{2A} receptor KO and C57Bl/6 WT mice were housed and bred in pathogen-free conditions prior to treatment on the Johns Hopkins School of Medicine East Baltimore campus. All mouse work was done in accordance with the Animal Care and Use Committee guidelines for Johns Hopkins University School of Medicine.

Cell lines and culture

B16 and EL4 cell lines were maintained in RPMI supplemented with 10% fetal bovine serum and 50 µg/mL penicillin, 50 µg/mL streptomycin. Luciferase expressing EL4 cells were generated by retrovirally transfecting EL4 cells with luciferase construct (a generous gift of the Levitsky lab), followed by the enrichment by FACS sorting and purification by limiting dilution.

In vivo luciferase imaging

EL4-luciferase bearing mice were anesthetized by an i.p injection of room temperature ketamine (80 µg/g of mouse) and xylene (17.5 µg/g of mouse). Once unresponsive, the tumor inoculation site was shaved and the mice injected with 1.5 mg of luciferin-potassium salt dissolved in PBS (Caliper LifeSciences). After 15 min to allow complete diffusion of the substrate, mice were imaged using an IVIS system (Caliper LifeSciences).

Tetramer staining and flow cytometry

Superficial inguinal lymph nodes from immunized mice were harvested and passed through a 60µm strainer to create a single-cell suspension. Cells were then stained at 21°C with MHC class I OVA tetramer (Beckman Coulter), diluted in FACS buffer (PBS + 10% FBS) followed by surface staining for CD8 (BD). Cells were then analyzed on a FACSCalibur (BD), and post-collection analysis performed using FlowJo (TreeStar).

Low-dose EL4 priming

During initial tumor dose-finding experiments, it was discovered that both WT and A_{2A} receptor KO mice completely rejected a subcutaneously (s.c.) dose of 10³ live EL4 cells. In light of these findings, WT and A_{2A} receptor null mice were primed with 10³ live EL4 cells and rested for 60 days, whereupon they were challenged with 10⁶ live EL4 cells along with age-matched naïve controls. This high tumor dose was previously determined to be lethal in both WT and A_{2A} KO mice, albeit with a slight delay in the A_{2A} receptor null mice. Tumor growth was monitored by palpation and caliper measurement. Mice were killed once tumor volume exceeded 100 mm³.

B16 melanoma model

WT and A_{2A} receptor KO mice were immunized with 10⁶ irradiated (10,000 rad) GM-CSF-secreting B16 melanoma cells, producing ~300 ng GM-CSF in a 24 h period. After 27 days, immunized mice, along with naïve age-matched controls, were injected i.v. via the lateral tail vein with 10⁶ B16 cells. After 15 days, all mice were killed by CO₂ inhalation and their lungs harvested, weighed and placed in Fekete's Solution (62% EtOH, 3.3% Formaldehyde,

1.5% Glacial Acetic Acid). After a week of fixation, lungs were removed from solution and visible B16 nodules were counted under a dissecting microscope.

B7-DC/Fc fusion protein administration and pharmacological A_{2A} receptor blockade

A B7-DC/Fc fusion protein was provided by Amplimmune (Rockville, MD). For experiments using WT and A_{2A} receptor null mice, EL4 tumor-bearing mice were treated with 100 µg B7-DC/Fc or with 100 µg control mouse IgG, by i.p. injection on day 1 post-tumor inoculation and every 3 days following for the duration of the experiment. Pharmacological A_{2A} receptor blockade was achieved by daily i.p. injections of 100 µl of the A_{2A} antagonist ZM-241385 (Tocris) diluted in a 1:1:4 emulsion of DMSO, Cremophor and ddH₂O at a concentration of 3 mg/ml. This treatment was supplemented with either 25 µg of control mouse IgG or 25 µg B7-DC/Fc.

Statistic

Unless otherwise stated error bars were generated using SEM, and *P* values by a Mann–Whitney *t* test. Curves were compared using a 2-way ANOVA. All analysis was performed by Prism 5.0c (Graphpad).

Results

A_{2A} receptor KO mice reject tumors more efficiently than WT controls

Mice lacking the A_{2A} adenosine receptor (A_{2A} KO) have previously been described [28] and show no gross baseline immunologic phenotype compared to WT C57Bl/6 mice. However, in light of the increased severity of inflammation observed in the A_{2A} KO mice in several models of autoimmunity, we decided to determine whether any difference could be detected in their ability to reject a syngeneic tumor challenge. To this end, A_{2A} KO and WT control mice were injected s.c. with 10⁶ luciferase expressing cells from the EL4 thymoma line. Tumor growth was monitored twice weekly by i.p. injection of luciferase substrate, followed by whole-body imaging. WT mice showed steady tumor growth, as measured by photon flux, during the 26 day course of the experiment. All mice in the WT group were killed on day 26 due to unacceptably large tumor size. In contrast, mice lacking the A_{2A} adenosine receptor showed a much-reduced rate of tumor growth compared to the WT controls, with several mice showing near complete rejection (Fig. 1a). The difference between the two groups was most evident on day 26 of the experiment, where there was almost a 100-fold difference in the luminescent output (Fig. 1b). However, there was a significant difference in the rate of tumor growth even at early timepoints of the experiment (Fig. 1c) **P* < 0.05. These data suggest that absence of the A_{2A} adenosine receptor can increase anti-tumor responses.

Enhanced tumor vaccine efficacy observed in A_{2A} KO mice

During the course of the initial tumor challenge experiments utilizing the non-luciferase expressing EL4 line, several doses of the tumor were used to determine the necessary number of cells required for successful tumor implantation. A dose of 10⁶ live EL4 cells was found to be universally lethal in both WT and A_{2A} receptor null mice, albeit with a slight increase in survival time in the A_{2A} KO mice, while a dose of 10⁵ cells proved lethal to the majority of WT mice but not to A_{2A} receptor KO mice. However, mice from both genotypes easily rejected tumor doses of 10⁴ and 10³ cells (data not shown). We wondered whether these low-level tumor doses could act as a tumor vaccine, providing protection against a later high-dose challenge.

WT and A_{2A} KO mice were initially primed with a non-lethal dose of 10³ live EL4 cells, which both genotypes easily rejected. Sixty days post-priming, both groups of mice, along

with naive controls, were challenged with 10^6 EL4 cells. Both naive WT and A_{2A} KO mice showed a relatively fast rate of tumor growth, but there was a slightly higher rate of survival in the naive A_{2A} KO group compared to WT controls despite the high tumor dose (Fig. 2a). Both primed WT and A_{2A} KO mice showed greater overall survival compared to their naive counterparts. However, primed A_{2A} KO mice showed the highest rate of overall survival of all experimental groups ($P < 0.05$) and had a significantly slower rate of tumor volume growth (Fig. 2b). These observations suggested that elimination of the anti-inflammatory A_{2A} adenosine receptor signaling not only enhanced the ability of mice to initially reject tumor but also led to enhanced recall responses.

A_{2A} KO mice show increased responsiveness to tumor vaccines compared to WT mice

Given our findings concerning the enhanced rejection of tumor in the A_{2A} receptor knockout mice, we wanted to demonstrate that this was associated with increased anti-tumor T-cell responses. Indeed, our findings concerning the enhanced protection upon rechallenge suggested that A_{2A} receptor null mice would demonstrate robust responses to tumor vaccines. Irradiated GM-CSF-secreting tumor vaccines initiate potent immune responses against a wide range of tumor antigens without the need to immunize with individual, isolated peptides [29]. While this immunization technique is most often used with a single irradiated transformed cell line secreting GM-CSF, it is also possible to induce a bystander response by mixing the irradiated GM-CSF-secreting line with a second, non-cytokine secreting, irradiated target line [30]. To this end, we immunized both WT and A_{2A} KO mice s.c. with a 1:1 mixture of irradiated ovalbumin (OVA) expressing EL4 cells and a GM-CSF-secreting B16 melanoma line. Generation of an OVA-specific CD8 T-cell response was monitored using MHC class I tetramer staining. Flow cytometric analysis of the injection site draining lymph nodes of immunized mice (Fig. 3a) 7 days post-immunization revealed significantly more OVA-specific CD8⁺ T cells in the A_{2A} KO mice compared to WT mice (Fig. 3b) $*P < 0.05$. There was no significant increase in the percentage of tetramer-positive CD8 T cell in the draining LN of the immunized A_{2A} receptor null mice compared to their WT counterparts, but the significantly larger size of the A_{2A} KO draining lymph node resulted in the significant increase in the overall number of antigen-specific CD8 T cells. Thus, while both the WT and the A_{2A} receptor null mice demonstrated a robust CD8⁺T-cell response, the A_{2A} KO mice were able to generate a markedly increased response to the tumor vaccine.

A_{2A} KO mice demonstrate increased responsiveness to GM-CSF-secreting melanoma vaccine

To further elucidate the potential for tumor vaccine enhancement by the loss the A_{2A} receptor, we decided to again utilize an irradiated GM-CSF-secreting tumor vaccine, this time in the context of a systemic tumor model. To this end, we immunized WT and A_{2A} KO mice with 10^6 irradiated GM-CSF-secreting B16 cells and rested the mice for 60 days. Immunized mice, along with naive controls, were injected i.v. with 10^6 B16 melanoma cells. After 17 days, the mice were harvested and the number of lung nodules counted and lungs weighed.

Naive WT mice showed extremely heavy tumor burden at the time of harvest, both in terms of the number of countable metastases and the wet-weight of the lungs. As would be predicted by our previous data, the naive A_{2A} KO mice showed a significantly lower level of tumor growth than the naive WT mice (Fig. 4a, b). Thus, in addition to lymphoma, the A_{2A} receptor null mice demonstrate an increased ability to reject melanoma, even in the absence of vaccine. Lungs from WT mice that were previously immunized with the irradiated GM-CSF-secreting B16 line showed a lower tumor burden than either naive group demonstrating the efficacy of the GM-CSF-secreting tumor vaccine in this model. As we

would have predicted, the lungs from previously immunized A_{2A} KO mice showed near complete protection from the growth of the transferred melanoma. Overall these findings demonstrate the ability of A_{2A} receptor null mice to respond more vigorously to GM-CSF-secreting whole cell tumor vaccines than their WT counterparts. The significance of these data is compounded by the fact that the B16 melanoma line is considered poorly immunogenic [31], suggesting that even non-ideal vaccine strategies can be enhanced by elimination of A_{2A} receptor signaling.

Genetic deletion of the A_{2A} adenosine receptor synergizes with administration of a soluble B7-DC/Fc fusion protein to enhance tumor rejection

Extracellular adenosine is just one of many inhibitory signals that tumors use to evade the immune system. It is clear that tumors have developed mechanisms to activate co-inhibitory receptors on T cells to effectively shut down anti-tumor responses. For example, expression of the inhibitory receptor Programed Death (PD)-1 on activated T cells is a negative feedback signal that under normal conditions serves to limit proliferation and effector function following the resolution of an immune response, but is frequently subverted by tumors to inhibit infiltrating lymphocytes [32]. The canonical ligand for PD1, B7-H1, is normally found on activated antigen presenting cells, including B cells and macrophages [33]. B7-DC is a recently discovered, dendritic cell restricted PD-1 ligand that provides potent co-stimulation to T cells [34]. The exact mechanism by which B7-DC enhances T-cell stimulation is still unknown, but it has been proposed to antagonize the inhibitory PD1/B7-H1 interaction, or interacts with an as-of-yet undiscovered receptor [35]. Many tumors take advantage of the ability of B7-H1 expression to limit the proliferation and cytokine production of PD1 expressing T cells by ectopically expressing the ligand [36]. We wondered whether we could further enhance an anti-tumor immune response by simultaneously mitigating A_{2A} signaling and providing additional co-stimulation signal via B7-DC. To this end, we utilized a B7-DC/Fc fusion protein in the context of an EL4 thymoma challenge in both WT and A_{2A} receptor null mice.

WT and A_{2A} KO mice were injected with 10⁶ EL4 thymoma cells s.c. and treated i.p. with 100 µg of either control IgG or B7-DC/FC on day 1 of the experiment and every 3 days following. IgG-treated WT mice demonstrated steady tumor growth, first showing tumor development by day 8 post-inoculation (Fig. 5a) and reaching the cutoff size for killing by day 37 (Fig. 5b). Also, at this dose of tumor, A_{2A} KO mice showed steady tumor growth during the course of the experiment. A slight increase in overall survival was observed in IgG-treated A_{2A} KO mice compared to similarly treated WT controls, but no difference was observed in the duration of tumor-free survival. WT mice receiving B7-DC/Fc treatment show a marked increase in the duration of tumor-free survival compared to IgG-treated groups ($P < 0.001$). Interestingly, the B7-DC/Fc-treated A_{2A} KO mice demonstrated a significant increase in the duration of both tumor-free survival and overall survival compared to all other treatment groups, even the B7-DC/Fc-treated WT mice ($P = 0.05$). In addition, combining pharmacological A_{2A} receptor blockade with B7-DC/Fc treatment resulted in a significant reduction in tumor growth (Fig. 5c). However, this combinatorial effect of pharmacological A_{2A} receptor blockade and B7-DC/Fc administration was only observed during the first week of therapy, after which all groups receiving B7-DC/Fc showed similar reduced tumor growth. These data suggest that combination therapy attacking A_{2A} signaling and co-inhibitory molecules might represent a novel avenue of enhancing tumor immunotherapy.

Discussion

In this report, we demonstrate the ability of A_{2A} receptor null mice to readily reject syngeneic tumors. In doing so, our data support the findings of Ohta et al. [25] This group,

by demonstrating the ability of A_{2A} KO mice to immunologically reject tumors, unequivocally established a role for adenosine in the tumor microenvironment as contributing to the ability of tumors to evade immune responses. Herein we reproduce and additionally extend these findings to demonstrate that mitigating A_{2A} receptor signaling can enhance the efficacy of irradiated GM-CSF-secreting vaccines and additionally can synergize with agents which target co-inhibitory pathways. As such, our findings further support the development of A_{2A} receptor antagonists as a means of enhancing anti-tumor immunity. Interestingly, A_{2A} receptor antagonists have been safely tested in Phase III clinical trials for Parkinson's disease paving the way for the initiation of clinical trials in cancer [37].

Extracellular adenosine is emerging as a potent regulator of inflammation at sites of extensive tissue damage and hypoxia. In cancer, the hypoxic environment of tumors leads to an increase in the local concentrations of extracellular adenosine [2]. In addition, it is becoming clear that tumors upregulate enzymes that lead to the generation of adenosine [26, 27]. For example, it has recently been shown that the ecto-5'-nucleotidase CD73 that catalyzes AMP breakdown to adenosine is upregulated on the cell surface of many tumor cell lines. Indeed, knocking down the expression of CD73 on tumors led to enhanced anti-tumor responses in vivo [27]. This increase in adenosine not only serves to inhibit anti-tumor immunity in terms of T-cell responses but also down modulates the activity of macrophages, neutrophils, dendritic cells and NK cells [17–20]. It is important to keep in mind that the extracellular adenosine found in hypoxic tumor environments can not only influence the host immune response to the tumor, but also the ability of a tumor to proliferate and survive. Several studies have suggested that extracellular adenosine signaling via the A_{2A} adenosine receptor can negatively impact the viability of several tumor lines [56]. These observations highlight the balance a tumor must strike between generating a suppressive microenvironment that can inhibit the ability of the immune system initiate tumor rejection, while still permitting the tumor itself to proliferate.

A_{2A} adenosine receptor exhibits a similar expression pattern and shares some functional redundancy with the A_{2B} adenosine receptor. Recent studies have shown that mice lacking the A_{2B} adenosine receptor show an increased resistance to tumor growth, which is attributable to a decrease in VEGF production by tumor infiltrating lymphocytes [57]. Our current study adds to the numerous previous reports demonstrating a central non-redundant role for the adenosine A_{2A} receptor in mediating the immune-inhibitory effects of adenosine.

While there is a plethora of studies demonstrating the ability of tumor vaccines to both prevent and treat cancer in a wide variety of preclinical models, such findings have yet to be robustly translated in Phase III clinical trials. Of note, however, recently the FDA granted approval to the first therapeutic cancer vaccine. Sipuleucel-T is an autologous dendritic cell vaccine loaded with prostate-specific peptides which has been shown to be efficacious in extending survival in patients with metastatic castration-resistant prostate cancer [38]. Our data suggest that inhibiting A_{2A} receptor signaling has the ability to enhance GM-CSF-secreting whole cell vaccines. Recent Phase III trials using this tumor vaccine strategy failed to demonstrate efficacy in prostate cancer [39]. However, there are a number of other clinical trials demonstrating safety and in some cases hints of efficacy using this vaccine platform [40]. Of note, several studies suggest that the efficacy of irradiated GM-CSF-secreting tumor vaccines can be markedly enhanced when it is combined with agents which block co-inhibition. Specifically, in preclinical models, there is evidence to suggest that blocking PD-1 and CTLA-4 can increase anti-tumor responses in irradiated GM-CSF-secreting tumor vaccines treated mice [41]. Our current studies suggest that blocking A_{2A} receptor signaling represents an additional avenue to enhance vaccine efficacy. Our current

data specifically employs GM-CSF-secreting “whole cell” vaccines. Based on these studies, we predict that A_{2A} receptor blockade would also enhance other “whole cell” vaccines such as Sipuleucel-T. Of note, preliminary studies in our laboratory suggest that in addition to “whole cell” vaccines, A_{2A} receptor blockade can also enhance the generation of effector cells in response to viral vaccines (data not shown).

Many tumor-associated antigens have been identified, and indeed various cancer vaccine strategies have been able to induce immunity to these antigens [42, 43]. What is clear, however, is that the tumor microenvironment can inhibit the ability of tumor-specific recognition to lead to immune-mediated tumor destruction. The tumor microenvironment is replete with inhibitory cytokines such as TGF- β , IL-10 and IL-13 [44–46]. Such cytokines not only inhibit anti-tumor Th1-mediated immunity, but also promote the generation of regulatory T cells and monocytic-derived cells [47, 48]. Accumulating data, including our current study, suggest that adenosine in the tumor micro-environment also represents a potent means by which tumors convert anti-tumor recognition by T cells into tumor-antigen-specific tolerance [25].

A_{2A} receptor activation inhibits both CD4+ and CD8+ T-cell proinflammatory cytokine expression [15, 22, 49–51]. Alternatively, A_{2A} receptor signaling does not appear to inhibit anti-inflammatory cytokine expression [50]. In addition, adenosine acting via the A_{2A} receptor has previously been shown to promote T-cell anergy even in the presence of costimulation [22]. Further, A_{2A} signaling has been shown to play a role in not only the generation of Foxp3 and Lag-3+ regulatory cells, but also in mediating their inhibitory effects [22, 23]. Thus, in the setting of infection, adenosine acts as an inhibitory metabokine [52] here preventing self-destruction by effectively down modulating immune responses. In the tumor microenvironment, cancer has usurped the adenosine-induced negative feedback loop to inhibit the anti-tumor immune response.

Co-inhibitory molecules are emerging as potentially potent adjuvants to cancer immunotherapy [53, 54]. Specifically, targeting CTLA-4- and PD-1-mediated negative regulatory pathways has shown promise in a number of clinical trials [55]. Our current data demonstrate that administering B7-DC/FC fusion protein to A_{2A} receptor null mice led to marked enhancement of anti-tumor immunity. In these experiments, mice were challenged with doses of tumor that led to death in the A_{2A} receptor null mice and in the WT mice treated with B7-DC/FC. However, when we treated A_{2A} receptor null mice with B7-DC/FC, there was a significant decrease in mortality. Such data suggest that simultaneously blocking the A_{2A} receptor- and B7-H1-mediated co-inhibition can act additively/synergistically to enhance anti-tumor immune responses. Experiments are underway to determine whether A_{2A} receptor blockade in combination with anti-CTLA4 or anti-PD1 also leads to enhanced responses.

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Fig. 1.

Decreased rate of tumor growth observed in mice lacking the A_{2A} adenosine receptor. C57Bl/6 WT and A_{2A} KO mice were injected s.c. with 10⁶ luciferase expressing EL4 cells. Growth of the tumor was monitored by anesthetization, followed by i.p. injection with 1.5 mg of luciferin-K + salt and imaging with a IVIS system. **a** A_{2A} KO mice showed significantly reduced rate of tumor growth compared to WT controls. **b** Detailed view of day 26 post-inoculation. **c** Time course of EL4-luciferase tumor growth. **P* < 0.05

Fig. 2.

A_{2A} KO mice are protected from lethal tumor challenge by previous low-dose tumor. Inoculation WT and A_{2A} KO mice were immunized s.c. with 10³ EL4 cells, a dose that both genotypes easily rejected, and were allowed to rest for 60 days. After this period, all immunized mice, along with age-matched naive controls, were challenged with a normally lethal s.c. dose of 10⁶ EL4 cells. The duration of tumor-free survival was recorded, along with the average tumor volume once growth was detected. **a** A_{2A} KO mice receiving a low-dose tumor immunization showed increased rates of overall survival compared to either immunized WT mice or naive controls of either genotype, and **b** decreased rate of tumor growth

Fig. 3. A_{2A} KO mice show an increased response to a tumor vaccine compared to WT mice. C57Bl/6 WT and A_{2A} KO mice were immunized with a s.c. injection of 5×10^5 irradiated OVA-expressing EL4 cells mixed with 5×10^5 irradiated GM-CSF producing B16 cells. Eight days after immunization, the draining lymph nodes from the injection site were harvested and stained with a MHC Class I OVA tetramer and anti-CD8 antibody to enumerate the number of OVA-specific CD8 T cells. Significantly more OVA-specific CD8 T cells were found in the draining LNs of A_{2A} KO mice, compared to WT controls. * $P < 0.05$

Fig. 4.

Immunization with an irradiated GM-CSF-secreting melanoma line shows increased efficacy in mice lacking the A_{2A} adenosine receptor. WT and A_{2A} KO mice were immunized with 10⁶ GM-CSF-secreting B16 melanoma cells s.c. and rested for 50 days. Immunized mice and naive age-matched controls were injected i.v. with 10⁶ B16 cells and their lungs harvested after 16 days. After fixation in Fekete's Solution, B16 nodules were counted under a dissecting microscope. **a** Images of dissected lungs following fixation. **b** Immunized A_{2A} KO mice had significantly lower loads of countable B16 metastases than immunized WT mice, or either naive control. **c** weights of harvested lungs. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001

Fig. 5.

Treatment with soluble B7-DC/Fc fusion protein synergizes with the loss of A_{2A} adenosine receptor signaling to enhance tumor rejection. WT and A_{2A} KO mice were injected s.c. with 10⁶ EL4 cells and injected i.p. on day 1, and every 3 days following for the duration of the experiment, with 100 μg B7-DC/FC or control IgG. **a** A_{2A} KO mice receiving PD1 blockade by B7-DC/FC treatment showed significantly enhanced duration of tumor-free survival following inoculation compared to B7-DC/FC treat WT mice, or either IgG-treated control. **b** B7-DC/FC-treated A_{2A} KO mice had a longer rate of overall survival compared to B7-DC/FC-treated WT mice, or either IgG-treated controls. **c** Combination of pharmacological A_{2A} receptor blockade and B7-DC/Fc treatment results in reduced tumor growth. **P* < 0.05 unpaired *t* test