Adenosine A2A receptors intrinsically regulate CD8+ T cells in the tumor microenvironment

Caglar Cekic1 and Joel Linden1*

1Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, 92037

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*Correspondence to:
Joel Linden, Ph.D.
9420 Athena Circle
La Jolla, CA, 92037
Phone: 858-752-6603
jlinden@liai.org
ABSTRACT

Adenosine A2A receptor (A2AR) blockade enhances innate and adaptive immune responses. However, mouse genetic studies have shown that A2AR deletion does not inhibit the growth of all tumor types. In the current study, we showed that growth rates for ectopic melanoma and bladder tumors are increased in Adora2a-/- mice within two weeks of tumor inoculation. A2AR deletion in the host reduced numbers of CD8+ T cells and effector-memory differentiation of all T cells. To examine intrinsic functions in T cells, we generated mice harboring a T cell-specific deletion of A2AR. In this host strain, tumor-bearing mice displayed increased growth of ectopic melanomas, decreased numbers of tumor-associated T cells, reduced effector-memory differentiation and reduced anti-apoptotic IL-7Rα (CD127) expression on antigen-experienced cells. Intratumoral pharmacological blockade similarly reduced CD8+ T cell density within tumors in wild-type hosts. We found that A2AR-proficient CD8+ T cells specific for melanoma cells displayed a relative survival advantage in tumors. Thus, abrogating A2AR signaling appeared to reduce IL-7R expression, survival and differentiation of T cells in the tumor microenvironment. One implication of these results is that the anti-tumor effects of A2AR blockade that can be mediated by activation of cytotoxic T cells may be overcome in some tumor microenvironments as a result of impaired T cell maintenance and effector/memory differentiation. Thus, our findings imply that the efficacious application of A2AR inhibitors for cancer immunotherapy may require careful dose optimization to prevent activation-induced T cell death in tumors.
INTRODUCTION

Solid tumors produce high concentrations of adenosine in response to hypoxia, cell necrosis and the rapid metabolism of extracellular adenine nucleotides by ectonucleotidases expressed on tumor cells, tumor cell exosomes and T regulatory cells (1-4). Adenosine engages four adenosine receptor subtypes: A₁, A₂A, A₂B and A₃. The A₂AR is the predominant subtype found on T cells, and is induced upon cell activation (4, 5).

A₂AR signaling inhibits innate and adaptive immune responses (5, 6). Global deletion of A₂ARs facilitates activation of CD8+ T cells and enhances rejection of certain tumors that were genetically engineered to be highly sensitive to cytotoxic T cell killing due to overexpression on tumor cells of MHC-I molecules (7). A₂AR deletion also enhances lymphoma killing and the effectiveness of an anti-lymphoma tumor vaccine (8). Hence, adenosine has been viewed as an inhibitor of T-cell mediated tumor surveillance (9, 10) and blockade of lymphocyte A₂ARs has been advocated to facilitate tumor immunotherapy. Curiously, global deletion of A₂ARs did not affect the growth of B16F10 melanomas or MB49 bladder carcinomas that were not modified by genetic engineering (7, 11), despite the fact that these tumors produce immune cell activation (12). This might occur because adenosine levels are high in solid tumors, and A₂AR signaling can inhibit activation-induced death of T cells and thus facilitate their survival (13). It is also possible that some of the effects of global A₂AR deletion on tumor growth are due to disinhibition of tumor macrophages, DC’s or NK cells.
In contrast to the failure of global A2AR deletion to inhibit B16F10 growth, reduced adenosine production due to deletion of CD73, an ecto-enzyme that converts AMP to adenosine, was found to consistently enhance anti-tumor adaptive immune responses (6, 14-17). This could be due in part to the involvement of A2B Rs in tumor suppression (11) or to differential effects on immune cell function caused by moderately reducing A2AR stimulation by deleting CD73 as opposed to eliminating A2AR signaling by deleting receptors.

In the current study we evaluated the effects on B16F10 melanoma growth and tumor-associated T cell survival of: 1) global A2AR deletion; 2) LckCre-mediated T-cell selective deletion of floxed A2ARs; and 3) adoptive co-transfer of T cells to tumor-bearing mice with and without A2ARs. The results indicate that T-cell specific A2AR deletion does activate T cells, but can also lead to reduced numbers of tumor-associated T cells and an increase over time in the growth rate of large solid tumors. Hence, some degree of A2AR signaling is needed for maintenance and effector differentiation of tumor-associated T cells. Opposing effects of A2AR deletion to enhance T cell activation but to reduce effector cell numbers in solid tumors provides an explanation for why global deletion of the A2AR causes inconsistent effects on tumor growth.

METHODS

Cell Lines, Animals and Reagents: Animal experiments were approved by the ACUC of the La Jolla Institute. B16F10 cells stably expressing luciferase were obtained from Caliper Life Sciences. MB49 bladder carcinoma cells were from Dr. Timothy Ratliff of Purdue
University. MB49 Bladder carcinomas were characterized as indicated by Luo et al (18) and further tested at the time of experimentation for adherence, freeze thaw viability, growth properties and mouse MHCI expression, without further authentication. Ovalbumin-expressing B16F10 cells produced as described previously (19) were a gift of Dr. Stephen Schoenberger of the La Jolla Institute. Ovalbumin and luciferase expressing B16F10 cells were obtained from Dr. Andreas Limmer of the University of Bonn. Both ovalbumin expressing melanoma cell lines were received within 6 months of experimentation and evaluated at the time of experimentation by morphology, adherence, freeze thaw viability, growth properties, mouse MHCI expression before and after IFNγ treatment, cell surface expression of MHCI/Ova peptide complexes, and antigen specific recognition of TRP2 or OVA peptides by respective transgenic T cells. B16F10 cells were cultured in R5F (RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin). Tumor cells were injected into mice after reaching 60-80% confluence. A2aR KO mice produced by Chen et al. (20) on a mixed genetic background were backcrossed onto C57BL/6. Six-week-old C57BL/6J, EGFP+ and OT-I Rag-/- mice were purchased from Jackson Laboratories, crossed with Adora2a-/- mice, and used for experiments after being acclimated for 2-6 weeks. LckCre+ mice (21) were obtained from Taconic (B6.Cg-Tg(Lck-cre)1Cwi N9) and used to create Adora2afl/+LckCre+/- mice. Tail DNA from all mice was genotyped (Transnetyx, Inc.) to detect the presence of Cre recombinase and to quantify by qPCR lckCre-mediated excision of floxed Adora2a DNA. Global vs lck-mediated Cre expression was found to increase the amount of excision by > 20-fold in tail DNA. Hence qPCR was used to exclude from experiments occasional mice with non-lymphoid deletion.
As further evidence of lymphoid-selective deletion, we have shown previously by qPCR that thymocyte expression of A2AR mRNA in \textit{lckCre/Adora2a\textsubscript{f/f}} mice is only deleted after thymocytes activate lck (22). Yellow or Aqua fluorescent reactive dyes were from Invitrogen. SIINFEKL-loaded H2K\textsubscript{b} tetramers with human beta-2 microglobilin were provided by the NIH tetramer core facility. Fluorescent antibodies used in this study, their sources and dilutions are listed in supplementary table 1.

**Flow cytometry:** Single cell suspensions from indicated tissues were prepared by sequential pressing through 100µm and 40µm cell strainers. Dead cells were removed from tumor samples by Ficoll gradient centrifugation at 2000 rpm (900g) for 20 min at room temperature. After RBC lysis (Biolegend) of spleen samples, remaining cells were washed and resuspended in R10F, and counted in a Z2-Coulter particle counter (Beckman Coulter). Cells (3-5x10\textsuperscript{6}) were pre-incubated for 10 min in 100 µL FACS buffer with antibody to block Fc receptors. Each sample tube received 100 µL fluorescently labeled antibody cocktail and was incubated for 30 min at 4\textdegree C in the dark. Cells were analyzed using an LSRII equipped with 4 lasers or a LSR Fortessa equipped with 5 lasers and FACS Diva software (BD-Biosciences). Live/dead fixable yellow, aqua or blue (invitrogen) were used to exclude dead cells before analysis. Flow cytometry data were analyzed using FlowJo software (9.5.3 version, TreeStar Software Inc.).

**Establishment and \textit{in vivo} imaging of solid tumors:** B16F10 or MB49 cells (10\textsuperscript{5}) were injected into the right flanks of mice. B16F10 melanoma cells expressing luciferase were injected into \textit{Adora2a\textsubscript{f/f}}-\textit{lckCre/\textasciitilde} and used for \textit{in vivo} imaging. Tumor volumes were
measured using digital calipers and calculated as height x width$^2$/2. Luciferase activity was
determined using an IVIS 200 Bioluminescence imager (Caliper Life Sciences) after
intravenous injection of 1mg D-Luciferin (Caliper Life Sciences) in 100 μL PBS to validate
that tumor size differences were not due to infiltration of host cells. In order to measure
tumor metastasis, 3x 10$^5$ B16F10 melanoma cells expressing luciferase were injected i.v.
into mouse tail veins and luciferase activity was measured in the lungs one and two weeks
later. After measuring luciferase activity lungs were removed, photographed and weighted
to validate that luciferase activity correlated with tumor mass.

**Adoptive transfer and co-transfer of T cells:** B16F10 cells (10$^5$) expressing ovalbumin
(B16F10-OVA) were injected into mouse flanks and allowed to expand for 16 days.
Mixtures of 3X10$^6$ OT-1 $Rag^{-/-}$ and 7 x 10$^6$ OT-1 $Rag^{-/-}Adora2a^{-/-}$ cells were injected
intraperitoneally. Greater numbers of OT-1 $Adora2a^{-/-}$ cells were included in the mixture
because A$_{2A}$R deficiency substantially reduced their numbers. On days 3 or 5 tumors and
spleens were harvested and stained for analysis by flow cytometry. For adaptive transfer
experiments 10$^7$ OT-1 $Rag^{-/-}$ or OT-1 $Rag^{-/-}Adora2a^{-/-}$ cells were injected i.p. into the mice
bearing B16F10-OVA tumors established for two weeks. Tumor growth was measured
after T cell transfer and on day 21. Mice were sacrificed and single cell suspensions from
tumors and spleen were analyzed for Annexin V staining, cell surface CD44 and CD127
expression and cell number and density.

**RESULTS**
Global deletion of Adora2a increases solid tumor growth and impairs CD8+ T cell effector differentiation and accumulation in tumors

In prior studies, global deletion of A2ARs failed to slow the growth rate of B16F10 melanomas transplanted into syngeneic mice (7, 11). In the current study we performed similar experiments in mice inoculated with B16F10 melanoma or MB49 bladder carcinomas and confirmed that A2AR deletion failed to decrease the rate of growth of either tumor; in fact, the growth rates of both tumors were significantly increased at day 14-18 after inoculation as the tumors became large (Fig 1A). By preparing single cell suspensions of tumors grown for 18 days after tumor inoculation we next determined if increased B16F10 growth was associated with reduced accumulation and/or impaired function of particular immune cell types within the tumor. Adora2a deletion significantly reduced the frequencies of CD8+ T cells (Fig1B) but not the frequencies of CD4+ T cells (Fig1B), myeloid cells or CD11b^{dim}CD11c+ cells (Fig1C). Adora2a deletion also caused a significant increase in frequencies of NK1.1+TCRβ- cells (henceforth referred to as NK cells) (Fig 1C).

Therefore, we calculated the density in tumors of NK and T cells by dividing the absolute numbers of these cells by tumor volume. Fig 1D shows a large reduction in CD8+ T cell density is associated with an increase in NK cell density in tumors. Local intra-tumoral injection of an irreversible A2AR blocker, 5-amino-7-[2-(4-fluorosulfonyl)phenylethyl]-2-(2-furyl)-pyrazolo[4,3-ε]-1,2,4-triazolo[1,5-c]pyrimidine (FSPTP) also reduced CD8 T cell density but not CD4 T cell or NK density within tumors, suggesting that local effects rather than global effects of Adora2a deletion are responsible for reduced T cell numbers (Figure 1E) and these effects are not dependent on elevated NK cell density. Additional
experiments will be required to determine if the increase in NK cell density in tumors caused by global A2AR deletion is due to a cell intrinsic effect of A2AR deletion on NK cells. Cell surface expression of CD44, KLRG1 and PD-1 were significantly lower in tumor-associated CD8+ T cells isolated from A$_2$A R-deficient mice as compared to tumor-associated CD8+ T cells from control animals (Fig 2A). However, expression of CD25 tended to increase in A$_2$A R-deficient CD8+ T cells, suggesting that CD8+ T cells in tumors are activated but fail to become effector/memory cells in the absence of A$_2$A Rs (Fig 2A, top). CD4+ T cell effector differentiation (as measured by CD44 and KLRG1) was also significantly inhibited in the absence of Adora2a (Fig 2A, bottom). After global A$_2$A R deletion, CD80 expression on tumor-associated APCs increased while CD86 expression was unchanged (Fig 2B). We also evaluated phenotypic markers in tumor associated myeloid APCs such as MHCII, PD-1 and PD-L1, which regulate T cell activation and CD39, which mediates tolerogenic activity of DCs by converting immunostimulatory ATP to ADP and AMP. A$_2$A R deficiency did not cause significant changes among any of these markers except for a reduction in cell surface PD-L1 expression (Supplementary Fig 1). PD-L1, although inhibitory for T cell activation, can be upregulated by inflammatory signals. It is possible that a reduction in the production of inflammatory cytokines due to reduced T cell accumulation and activation contributes to reduced PD-L1 expression in A$_2$A R deficient mice. Overall these results suggest that decreased CD8+ T cell infiltration and effector/memory differentiation in A$_2$A R-/- mice are not due to APC inactivation. In fact myeloid-selective deletion of A$_2$A Rs decreases melanoma growth and increases the number of tumor associated T cells and NK cells (Cekic and Linden, unpublished data).
As in mice with B16F10 tumors, in mice with solid MB49 carcinomas A2AR deletion reduced CD8+ T cell frequency and expression of the effector/memory marker CD44 (Fig 3A,B). One possible explanation for the reduction in CD8+ T cells in the tumors of A2AR-/- mice is reduced expression of CXCR3, which is required for activated T cells to home to inflamed sites. We did not observe any reduction in CXCR3 expression after A2AR-deletion (Fig 3B). Furthermore, local inhibition by intra-tumoral injection of the A2AR antagonist FSPTP, but not by selective A2BR blocker ATL-801, also significantly reduced the frequency of tumor-associated CD8+ T cells (Fig 3C), suggesting that as in melanomas, A2AR signaling facilitates the accumulation CD8+ T cells within bladder tumors as well.

It is notable that A2AR blockade consistently increased the frequencies of tumor-associated NK cells (Fig 1B, 3A,C). This observation agrees with findings by Beavis et al. (2) who found that blockade or global deletion of A2ARs reduced lung metastasis of CD73 expressing tumors by increasing NK cell activity and numbers, presumably by blocking A2AR-mediated effects of high adenosine in the tumor. However, this effect could also be due in part to reduced CD8+ T cell rather than NK cell density.

_Lymphoid-selective deletion of Adora2a reduces the number and differentiation to effector-memory cells of tumor-associated T cells and markedly increases the growth rate of large solid tumors_

Because global A2AR deletion activates APCs (Fig 2B), we hypothesized that A2AR signaling helps to maintain T cell numbers in the solid tumor microenvironment in a T cell-intrinsic
manner. In order to evaluate the effects of cell-intrinsic A$_2$AR signaling on tumor growth and on T cell responses we crossed mice with a floxed Adora2a gene with mice expressing Cre recombinase under control of the Lck promoter. Adora2a$^{f/f}$–LckCre$^{+/-}$ mice have normal numbers of thymic T cell precursors in the absence (23) or presence (supplementary figure 2) of solid tumors, suggesting that Adora2a$^{f/f}$–LckCre$^{+/-}$ mice have normal T cell development compared to littermate controls. However, T-cell selective deletion of A$_2$ARs markedly accelerated the growth rate of tumors after they reached a volume > 500 mm$^3$ (Fig 4A,B; see Supplementary Fig 3 for results of individual experiments). These findings suggest that paradoxically, some degree of A$_2$AR signaling in T cells is required to mount an optimal anti-tumor immune response in large solid tumors. A$_2$AR signaling increases cAMP production (24). It has been suggested that a temporary increase in cAMP levels may be required for T cell activation (25). However, A$_2$AR deletion failed to affect the expression of CD69 in tumor associated T cells (supplementary figure 4). We reasoned that the absence of A$_2$AR signaling in the tumor microenvironment might cause T cells to polarize toward a regulatory phenotype. Figure 4C shows that T cell A$_2$AR deletion does not enhance regulatory T cell differentiation in the tumor or tumor draining lymph nodes. Therefore, we measured CD25 expression in antigen experienced T cell populations. The deletion of the A$_2$AR from lymphocytes increased CD25 expression in tumor-associated/antigen experienced $CD44^{hi}Foxp3^{-}$ CD4$^+$ T cells and did not affect CD8$^+$ T cells or lymph node $CD44^{hi}Foxp3^{-}$ CD4$^+$ T cells (Fig 5D). These data suggest that deletion of A$_2$AR signaling does not hamper T cell activation in tumors.
We next considered the possibility that A2AR signaling sustains normal numbers of tumor-associated T cells. We found previously that A2AR signaling, by activating PKA, reduces the activity of the PI3K/Akt pathway (26). This suppresses TCR-mediated down-regulation of anti-apoptotic CD127, which is upregulated in long-lived effector memory cells and required for their maintenance. LckCre-mediated deletion of A2ARs significantly reduced CD127 expression in antigen-experienced T cells in the tumor (Fig 5), and significantly reduced the frequencies of tumor-associated T cells (Fig 6A-B) but not NK cells (Fig 6C). T cell-selective A2AR-deletion also significantly reduced the frequency of CD44+ effector-memory T cells in tumors, but not lymph nodes (Fig 6D-E). Fig 6F shows a reduction in A2AR-/- T cell density in tumors. Hence, although A2AR activation during TCR stimulation inhibits T cell activation, the data suggest a role for adenosine in maintaining effector T cells within the tumor microenvironment. These opposing effects of A2AR deletion to enhance T cell activation but to reduce effector cell numbers provides an explanation for why global deletion of the A2AR causes small or inconsistent effects on tumor growth.

A2AR signaling prolongs the maintenance of T cells in tumor bearing hosts

Global A2AR deletion significantly reduces the development and peripheral maintenance of naïve T cells (23). Although Lck-mediated Adora2a+/− deletion did not affect thymic progression of T cells, it did cause a decrease in the number of naïve T cells in the periphery. This decrease in the naïve T cell population may contribute to reducing numbers of T cells in tumors after global or LckCre-mediated deletion of Adora2a+/−. Also, reduced naïve T cell numbers in mice lacking T cell A2ARs could be a consequence of high tumor
burden rather than to an intrinsic effect of A$_{2A}$R signaling. In order to evaluate in vivo competition and phenotypic differentiation of antigen specific T cells lacking or expressing A$_{2A}$Rs in the same tumor microenvironment, we performed adoptive co-transfer experiments. When co-transferred into the same host bearing B16F10-OVA tumors, the proportion of Adora2a$^{-/-}$ OT-1 T cells was markedly decreased in the tumor relative to Adora2a$^{+/+}$ OT-1 T cells (Fig 7A). A$_{2A}$R deletion also reduced cell surface expression of PD-1 (Fig 7B) while CD25 expression was increased (Fig 7C), a phenotype similar to what was observed after global deletion of the A$_{2A}$R (Fig 2A). Figure 7D shows that A$_{2A}$R deletion caused a significant decrease in CD127 expression in both spleen and tumor-associated OT-I T cells.

To directly test the effects of Adora2a deletion on T cell survival and effector/memory differentiation we adoptively transferred Adora2a$^{+/+}$ and Adora2a$^{-/-}$ OT-I T cells two weeks after establishment of B16F10-OVA tumors in congenic hosts. One week after adoptive transfer we prepared single cell suspensions from tumor tissue by ficoll gradient and measured cell surface staining of Annexin V as an apoptosis marker, CD44 as marker for effector memory differentiation and CD127 as mediator of T cell survival. Tumor-associated but not splenic Adora2a$^{-/-}$ OT-I T cells expressed significantly more Annexin V than Adora2a$^{+/+}$ OT-I T cells and this was associated with decreased expression of CD44 and CD127 within tumors (Supplementary figure 5). Transfer of either Adora2a$^{+/+}$ or Adora2a$^{-/-}$ OT-I cells induced a transient decrease in tumor growth suggesting that Adora2a$^{-/-}$ cells are initially functional, but immunostimulatory effects of Adora2a deletion appear to be counteracted by reduced survival/effector-memory differentiation.
Supplementary figure 5). Overall, these data show that in the tumor environment, A2AR deficient T cells have a survival disadvantage as compared to A2AR-proficeint T cells.

DISCUSSION

Adenosine accumulates to high levels in solid tumors (1-4). A2ARs on antigen presenting cells and T cells, and A2B receptors on antigen presenting immune cells are primarily responsible for immunosuppression by adenosine (27, 28). Global deletion or intra-tumoral blockade of A2BRs delays the growth of lung and bladder carcinoma and breast cancers, consistent with the immunosuppressive roles of these receptors (11). Curiously, global A2AR deletion failed to slow the growth of bladder carcinomas and B16BL6 melanomas (7, 11), but was found to enhance the rejection of CL8-1 cells that were genetically engineered to be highly immunogenic by transfection with H-2Kb (7). These findings, and the results of the current study suggest that A2AR blockade, as a strategy to treat cancer, is more complex than previously thought (9, 10). Although T cells are acutely activated by A2AR deletion, long-term T-cell mediated solid tumor rejection is compromised, likely as a result of impaired maintenance of T cells and reduced effector/memory differentiation in the tumor. It is important to point out however that the effect of A2AR deletion on T cell functions are not necessarily indicative of the effects of A2AR blocking drugs that lessen, but do not eliminate A2AR signaling. In this respect, a more clinically relevant reversible A2AR antagonist rather than an irreversible antagonist may have different properties.

Ohta et al. previously showed that silencing of A2A and A2B adenosine receptors by siRNA in
adoptively transferred, tumor-specific T cells significantly reduced lung metastasis of H2-Kb expressing RMA T cell lymphoma cells and improved the survival of tumor bearing mice (7). The current study shows that Adora2a deficient OT-I T cells do not slow the growth of solid B16F10 cells expressing ovalbumin, which can be recognized by OT-I T cells. The data suggest that: 1) anti-tumor effects of Adora2a deletion vary among different types of tumors; 2) A2aR signaling in tumor-associated T cells may contribute to adenosine suppression of T cell activity; 3) solid vs. metastatic tumor growth may be differentially influenced by A2aR deletion; 4) highly immunogenic tumors may preferentially elicit anti-tumor effects of Adora2a deletion; and 5) siRNA silencing of Adora2a expression or pharmacological inhibition may result in A2aR residual signaling may contribute to tumor killing by sustaining T cells in the tumor microenvironment.

T cells go through an expansion phase after activation but many fail to survive either due to excess activation of inhibitory signals, or due to the absence of co-stimulation by certain cytokines or homing signals in the tumor (9). Interestingly, the absence of T cell-intrinsic inhibitory A2aR signaling reduces numbers of tumor associated T cells after 2-3 weeks of solid tumor expansion. Our recent findings indicate that A2aR signaling can prevent TCR-induced down-regulation of anti-apoptotic CD127 (23). A2aR deficiency significantly reduced the development and peripheral maintenance of naïve T cells. This decrease in the naïve T cell population may contribute to reduced numbers of T cells in tumors after Lck-mediated deletion of Adora2afl/fl. However, the irreversible A2aR antagonist, FSPTP, injected directly into solid tumors, also reduced tumor-associated T cell numbers in wild type recipients. Also, the ratio of antigen specific A2aR-deficient/A2aR-proficient T cells
decreased within the same tumor after adoptive co-transfer, suggesting a T cell survival
defect due to either deletion or irreversible blockade of A2AR signaling in the tumor
environment.

We show that CD127 expression by effector/memory cells in tumors is significantly
reduced in T cells lacking A2ARs while CD25 expression is largely intact or even increased.
This supports the notion that although A2AR signaling acutely inhibits initial T cell
activation, A2AR dependent control of CD127 expression may be necessary for the
maintenance of T cells after they differentiate into long-lived effector/memory cells.
Consistent with this idea, we noted a significant impairment in the ex vivo survival of A2AR-
/- T cells in response to IL-7 (23). The pattern of tumor T cell responses caused by deletion
of the A2AR is very similar to the effect of IL-2 in tumor immunotherapy: although IL-2
increases the activation and early expansion of T cells, it causes increased activation-
induced death (29). Therefore, it is possible that CD127-deficiency and increased IL-2
signaling due to increased CD25 expression both impair T cell survival in the tumor
microenvironment.

A2AR signaling may sustain tumor-associated T cells by inhibiting the PI3K/Akt pathway. As
with A2AR agonists, PI3K is also inhibited by rapamycin, which has been shown to increase
numbers of long-lived effector/memory T cells in virally infected mice (30). Expansion of
tumor-associated effector memory T cells might account for the observations that despite
their immunosuppressive properties, adenosine (this study) and rapamycin (31) have anti-
apoptotic effects on T cells in some tumors.
The A2AR is upregulated in multiple immune cell types upon activation (5, 32, 33). Blockade or deletion of A2ARs in NK cells (2) or myeloid cells (Cekic et al. unpublished data) significantly inhibits tumor growth and metastasis and is associated with transactivation of cytotoxic lymphocytes. Consistent with these findings, the current study suggests that in order to avoid apoptosis of tumor-associated T cells, myeloid or NK-cell targeted therapies should be considered as preferable targets for A2AR deletion/blockade as a strategy for tumor immunotherapy. Alternatively, in order to optimize the beneficial effects of T cell activation by A2AR blockade for solid tumor killing, it may be necessary to find complementary strategies to enhance T cell survival and effector/memory differentiation to counteract activation-induced cell death. These concepts apply not only to the potential use of adenosine receptor blockade or deletion to treat cancer, but also more broadly to the general use of T cell activators.

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REFERENCES


**Figure Legends**
Figure 1. Global deletion of Adora2a fails to reduce the growth rate of syngeneic tumors. A. Growth of B16F10 melanoma (N=9/group) or MB49 bladder carcinoma (n=5/group) cells in Adora2a+/+ and Adora2a−/− C57BL/6 mice after subcutaneous inoculations of 10^5 cells. B. Frequencies of CD4+ and CD8+ T cells and NK cells; and C. frequencies of myeloid cell populations and CD11b-dim CD11c+ cells in B16F10 melanomas isolated from Adora2a+/+ vs. Adora2a−/− mice 18 days after inoculation. Cell density (log of cell number per mm^3 tumor), were calculated in solid tumors grown in D. Adora2a+/+ vs. Adora2a−/− mice or E. in mice receiving intratumor injections of 100µL, 1µM FSPTP or vehicle control (VC) (n=4/group, from two independent experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by two way anova and Benferoni post-hoc analyses for A and Student’s t test for B-E.)

Figure 2. Global-deletion of Adora2a inhibits effector/memory differentiation of tumor-associated T cells. Phenotypic analysis of A. Lymphocyte, and B. APC populations isolated from spleen (SPL) and B16F10 tumors (TMR). (Pooled data from two-independent experiments, n=5/group, *p<0.05, *p<0.01, ***p<0.001, by two way anova and Tukey’s post-hoc analyses.

Figure 3. Global deletion or acute local blockade of A2ARs reduces the frequency of CD8+ T cells in MB49 bladder carcinoma. A. Frequencies of tumor-associated lymphocytes from MB49 tumors, n=3 from two independent experiments, *p<0.05, by two way anova and Benferoni post-hoc analysis. B. Flow cytometry analysis of CD44 and CXCR3 expression in
A2AR-/- and A2AR+/+ T cells in MB49 tumors, *p<0.05, **p<0.01 by unpaired Student’s t test (n=3). C. Frequencies of major immune cell populations from MB49 tumors injected with 100µL, 1µM FSPTP, 1 µM ATL801 or vehicle every three days after tumor inoculation. Results are pooled from independent experiments with similar results (n≥5, *p<0.05, *p<0.01, ***p<0.001, ****p<0.0001 by two way anova and Benferoni post-hoc analyses.) A-C all corresponding analysis were performed three weeks after tumor inoculation.

**Figure 4.** Lymphoid deletion of Adora2a promotes melanoma growth. A. Growth of B16F10 melanoma cells in Adora2a+/f-Cre+ and Cre- littermates. Tumor sizes were measured by caliper, (n>9 from two independent experiments, ***p<0.0001 by two way anova and Benferoni post-hoc analysis). B. Luciferase luminescence was measured after injecting 1 mg/mouse of luciferin into tumor bearing mice (n>4 from one of two independent experiments). Data were analyzed by the Student’s t test. Single cell suspensions from tumors and lymph nodes (LN) were isolated from Adora2a+/f-LckCre+ or Cre+/− littermate controls harvested three weeks after tumor inoculation. C. Intracellular staining for Foxp3; D. surface staining for CD25 was performed, n=4 from one of two independent experiments with similar results. Data were analyzed using two-way Anova and Bonferroni post-doc tests.

**Figure 5.** Reduced expression of CD127 among effector T cells lacking A2ARs in tumors. A. Flow cytometry analysis of CD127 expression, and B. geometric means of corresponding populations are shown. Data are from one of two independent experiments with similar results analyzed using Student’s t tests (n=4).
**Figure 6.** Reduction in numbers and memory/effector differentiation of tumor-associated T cells after lymphoid deletion of A2ARs. Single cell suspensions of tumors and lymph nodes isolated from the *Adora2a*<sup>+/f</sup>-LckCre<sup>+</sup> or Cre<sup>-</sup> littermate controls were prepared 3 weeks after tumor inoculation. Frequencies of A. CD8+ T cells; B. CD4+ T cells; and C. NK cells. CD44 expression is shown in D. CD8+; and E. CD4+ T cells, as an indication of effector/memory differentiation. F. Densities of CD4, CD8 T cells and NK cells as calculated by absolute numbers of cells divided by tumor volume,*p<0.05, **p<0.01, n=4 from one of two independent experiment with similar results. Data were analyzed using Student's t tests.

**Figure 7.** A<sub>2A</sub>R signaling is required for T cell maintenance in solid tumors. A. Recovery of Adora2a<sup>+/+</sup> (CD45.1) and Adora2a<sup>-/-</sup> (CD45.2) OT-1 Rag<sup>-/-</sup> T cells 3 and 5 days after the co-transfer of 5X10<sup>5</sup> cells into GFP+ mice bearing Ova expressing B16F10 tumors >300 mm<sup>3</sup> in size produced following s.c. injection of 10<sup>5</sup> tumor cells. Expression on day 5 of B. PD-1; C. CD25; and D. CD127 in transferred OT-1 cells. Host cells were excluded by GFP fluorescence. T cells were gated by tetramer, CD8, CD45.1 and CD45.2 staining, n=4 for day 5 (A-D) and n=3 for day 3 (A), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by two way anova and Bonferroni post-hoc analysis.
Figure 1

A. B16

Adora2a+/+  Adora2a-/

Tumor volume (mm³) vs. Days

B. MB49

Adora2a+/+  Adora2a-/

Tumor volume (mm³) vs. Days

B. MB49

Adora2a+/+  Adora2a-/

Frequency of CD45 (%) vs. Days

C. Adora2a+/+  Adora2a-/

Frequency of CD45 (%) vs. Macrophages (S), DC (CD11b+/hi), DC (CD11b–/low), Gr1+ (T)

D. Adora2a+/+  Adora2a-/

Frequency of CD45 (%) vs. NK, CD4, CD8

E. VC, FPSTP

Frequency of CD45 (%) vs. NK, CD4, CD8
Figure 2
Figure 3

A. Frequency of CD45 (%)

- Adora2a+/+
- Adora2a-/

B. CD8+

- CD44
- CD44

- CXCR3
- CXCR3

C. NK1.1+

- CD8+
- CD4+

- CD11b+CD11c+
- CD11b+
- GR1+
Figure 4

A. ⊕ Lck/Cre-  • Lck/Cre+

***p<0.0001

Tumor volume (mm³)

Days

0 10 14 17 19 21

B. Lck/Cre-  Lck/Cre+

***p=0.0002

Tumor volume (mm³)

C. Lck/Cre-  Lck/Cre+

***p<0.0001

D. Lck/Cre-  Lck/Cre+

***p=0.0002

CD4+CD44+Foxp3-
**A.**

- **Lck/Cre-**
- **Lck/Cre+**

<table>
<thead>
<tr>
<th>CD4+CD44+</th>
<th>CD8+CD44+</th>
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<td>%Max</td>
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**B.**

- **CD4+CD44+ (Tumor)**
  - *p=0.0189*
- **CD8+CD44+ (Tumor)**
  - *p=0.0017*

- **CD4+CD44+ (Lym. Node)**
- **CD8+CD44+ (Lym. Node)**
Figure 7

A. **Adora2a-/- (CD45.2+GFP-)**

B. **PD-1**

C. **CD25**

D. **CD127**

E. **SPLEEN**

**TUMOR**
Adenosine A2A receptors intrinsically regulate CD8+ T cells in the tumor microenvironment

Caglar Cekic and Joel Linden

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