

Adenosine A_{2A} Receptors Intrinsically Regulate CD8⁺ T Cells in the Tumor Microenvironment

Caglar Cekic^{1,2} and Joel Linden¹

Abstract

Adenosine A_{2A} receptor (A_{2A}R) blockade enhances innate and adaptive immune responses. However, mouse genetic studies have shown that A_{2A}R 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 2 weeks of tumor inoculation. A_{2A}R 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 A_{2A}R. 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 antiapoptotic IL7Rα (CD127) expression on antigen-experienced cells. Intratumoral pharmacologic blockade similarly reduced CD8⁺ T-cell density within tumors in wild-type hosts. We found that A_{2A}R-proficient CD8⁺ T cells specific for melanoma cells displayed a relative survival advantage in tumors. Thus, abrogating A_{2A}R signaling appeared to reduce IL7R expression, survival, and differentiation of T cells in the tumor microenvironment. One implication of these results is that the antitumor effects of A_{2A}R 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 A_{2A}R inhibitors for cancer immunotherapy may require careful dose optimization to prevent activation-induced T-cell death in tumors. *Cancer Res*; 74(24); 7239–49. ©2014 AACR.

Introduction

Solid tumors produce high concentrations of adenosine in response to hypoxia, cell necrosis, and the rapid metabolism of extracellular adenine nucleotides by ecto-nucleotidases expressed on tumor cells, tumor cell exosomes, and T regulatory cells (1–4). Adenosine engages four adenosine receptor subtypes: A₁, A_{2A}, A_{2B}, and A₃. The adenosine A_{2A} receptor (A_{2A}R) is the predominant subtype found on T cells, and is induced upon cell activation (4, 5).

A_{2A}R signaling inhibits innate and adaptive immune responses (5, 6). Global deletion of A_{2A}Rs 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). A_{2A}R deletion also enhances lym-

phoma killing and the effectiveness of an antilymphoma tumor vaccine (8). Hence, adenosine has been viewed as an inhibitor of T-cell–mediated tumor surveillance (9, 10), and blockade of lymphocyte A_{2A}Rs has been advocated to facilitate tumor immunotherapy. Curiously, global deletion of A_{2A}Rs 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 A_{2A}R 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 A_{2A}R deletion on tumor growth are due to disinhibition of tumor macrophages, dendritic cells (DC), or natural killer (NK) cells.

In contrast with the failure of global A_{2A}R 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 antitumor adaptive immune responses (6, 14–17). This could be in part due to the involvement of A_{2B}Rs in tumor suppression (11) or to differential effects on immune cell function caused by moderately reducing A_{2A}R stimulation by deleting CD73 as opposed to eliminating A_{2A}R signaling by deleting receptors.

In the current study, we evaluated the effects on B16F10 melanoma growth and tumor-associated T-cell survival of: (i) global A_{2A}R deletion, (ii) LckCre-mediated T-cell selective

¹Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, California. ²Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Joel Linden, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037. Phone: 858-752-6603; Fax: 858-752-6985; E-mail: jlinden@liai.org

doi: 10.1158/0008-5472.CAN-13-3581

©2014 American Association for Cancer Research.

deletion of floxed A_{2A} Rs, and (iii) adoptive cotransfer of T cells to tumor-bearing mice with and without A_{2A} Rs. The results indicate that T-cell-specific A_{2A} R 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 A_{2A} R signaling is needed for maintenance and effector differentiation of tumor-associated T cells. Opposing effects of A_{2A} R deletion to enhance T-cell activation but to reduce effector cell numbers in solid tumors provide an explanation for why global deletion of the A_{2A} R causes inconsistent effects on tumor growth.

Materials and Methods

Cell lines, animals, and reagents

Animal experiments were approved by the ACUC of the La Jolla Institute (La Jolla, CA). B16F10 cells stably expressing luciferase were obtained from Caliper Life Sciences. MB49 bladder carcinoma cells were from Dr. Timothy Ratliff of Purdue University (West Lafayette, IN). MB49 Bladder carcinomas were characterized as indicated by Luo and colleagues (18) and further tested at the time of experimentation for adherence, freeze thaw viability, growth properties, and mouse MHC 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 (Bonn, Germany). 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 MHC expression before and after IFN γ treatment, cell surface expression of MHC/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 FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 U/mL penicillin, and 50 μ g/mL streptomycin). Tumor cells were injected into mice after reaching 60% to 80% confluence. A_{2A} R KO mice produced by Chen and colleagues (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 to 6 weeks. $LckCre^{+}$ mice (21) were obtained from Taconic (B6.Cg-Tg(Lck-cre)1Cwi N9) and used to create $Adora2a^{f/f}$ - $LckCre^{+/-}$ mice. Tail DNA from all mice was genotyped (Transnetx, Inc.) to detect the presence of Cre recombinase and to quantify by qPCR $LckCre$ -mediated excision of floxed $Adora2a$ DNA. Global versus 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 A_{2A} R mRNA in $LckCre/Adora2a^{f/f}$ mice is only deleted after thymocytes activate Lck (22). Yellow or aqua fluorescent reactive dyes were from Invitrogen. SIINFEKL-

loaded H2K^b tetramers with human β -2 microglobulin were provided by the NIH tetramer core facility. Fluorescent antibodies used in this study, their sources, and dilutions are listed in Supplementary Table S1.

Flow cytometry

Single-cell suspensions from indicated tissues were prepared by sequential pressing through 100 and 40 μ m cell strainers. Dead cells were removed from tumor samples by Ficoll gradient centrifugation at 2,000 rpm (900 g) for 20 minutes 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 (BeckmanCoulter). Cells ($3-5 \times 10^6$) were preincubated for 10 minutes in 100 μ L FACS buffer with antibody to block Fc receptors. Each sample tube received 100 μ L fluorescently labeled antibody cocktail and was incubated for 30 minutes at 4°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) was used to exclude dead cells before analysis. Flow-cytometric data were analyzed using FlowJo software (9.5.3 version, TreeStar Software Inc.).

Establishment and *in vivo* imaging of solid tumors

B16F10 or MB49 cells (10^5) were injected into the right flanks of mice. B16F10 melanoma cells expressing luciferase were injected into $Adora2a^{f/f}$ - $LckCre^{-/+}$ and used for *in vivo* imaging. Tumor volumes were measured using digital calipers and calculated as height \times width²/2. Luciferase activity was determined using an IVIS 200 Bioluminescence imager (Caliper Life Sciences) after intravenous injection of 1 mg D-Luciferin (Caliper Life Sciences) in 100 μ L PBS to validate that tumor size differences were not due to infiltration of host cells. To measure tumor metastasis, 3×10^5 B16F10 melanoma cells expressing luciferase were injected intravenously into mouse tail veins and luciferase activity was measured in the lungs 1 and 2 weeks later. After measuring luciferase activity, lungs were removed, photographed, and weighted to validate that luciferase activity correlated with tumor mass.

Adoptive transfer and cotransfer 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 3×10^6 OT-1 $Rag^{-/-}$ and 7×10^6 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 adoptive transfer experiments, 10^7 OT-1 $Rag^{-/-}$ or OT-1 $Rag^{-/-}$ - $Adora2a^{-/-}$ cells were injected intraperitoneally into the mice bearing B16F10-OVA tumors established for 2 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 A_{2A}Rs 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 A_{2A}R deletion failed to decrease the rate of growth of either tumor; in fact, the growth rates of both tumors were significantly increased at days 14 to 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 whether 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 (Fig. 1B) but not the frequencies of CD4⁺ T cells (Fig. 1B), myeloid cells, or CD11b^{dim}CD11c⁺ cells (Fig. 1C). *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. Figure 1D shows that a large reduction in CD8⁺ T-cell density is associated with an increase in NK cell density in tumors. Local intratumoral injection of an irreversible A_{2A}R blocker, 5-amino-7-[2-(4-fluorosulfonyl)phenylethyl]-2-(2-furyl)-pyrazolo-[4,3-*e*]-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 (Fig. 1E) and these effects are not dependent on elevated

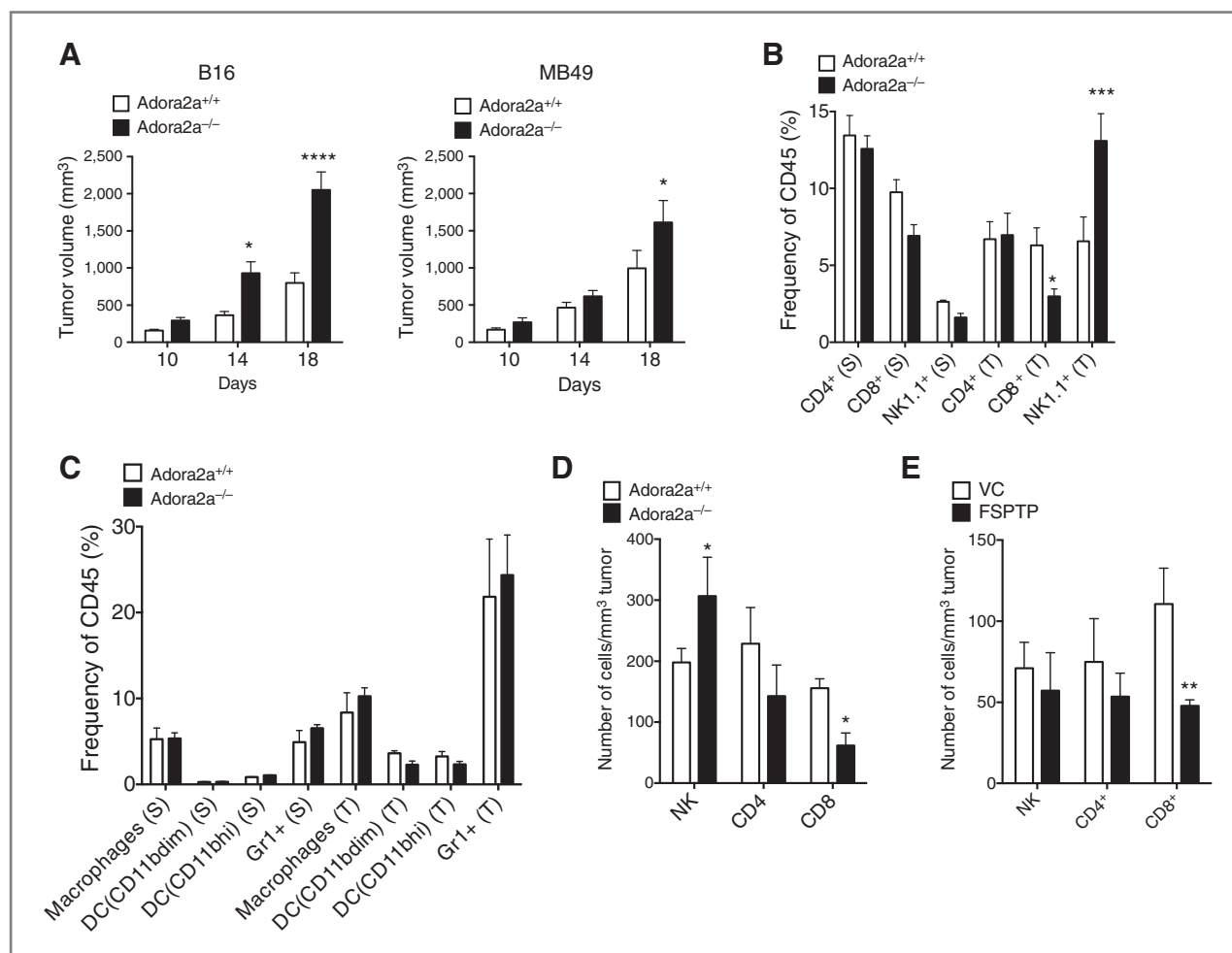


Figure 1. Global deletion of *Adora2a* fails to reduce the growth rate of syngeneic tumors. A, growth of B16F10 melanoma ($N = 9/\text{group}$) or MB49 bladder carcinoma ($n = 5/\text{group}$) cells in *Adora2a*^{+/+} and *Adora2a*^{-/-} C57BL/6 mice after subcutaneous inoculations of 10^5 cells. B, frequencies of CD4⁺ and CD8⁺ T cells and NK cells. C, frequencies of myeloid cell populations and CD11b^{dim}CD11c⁺ cells in B16F10 melanomas isolated from *Adora2a*^{+/+} versus *Adora2a*^{-/-} mice 18 days after inoculation. D and E, cell density (log of cell number per mm³ tumor) was calculated in solid tumors grown in *Adora2a*^{+/+} versus *Adora2a*^{-/-} mice (D) or in mice receiving intratumoral injections of 100 μL , 1 $\mu\text{mol/L}$ FSPTP, or vehicle control (VC; $n = 4/\text{group}$, from two independent experiments; E). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, by two-way ANOVA and Bonferroni *post hoc* analyses for A and Student *t* test for B–E.

NK cell density. Additional experiments will be required to determine whether the increase in NK cell density in tumors caused by global A_2A R deletion is due to a cell intrinsic effect of A_2A R deletion on NK cells. Cell surface expression of CD44, KLRG1, and PD-1 was significantly lower in tumor-associated CD8⁺ T cells isolated from A_2A R-deficient mice as compared with tumor-associated CD8⁺ T cells from control

animals (Fig. 2A). However, expression of CD25 tended to increase in A_2A 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_2A 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_2A R deletion,

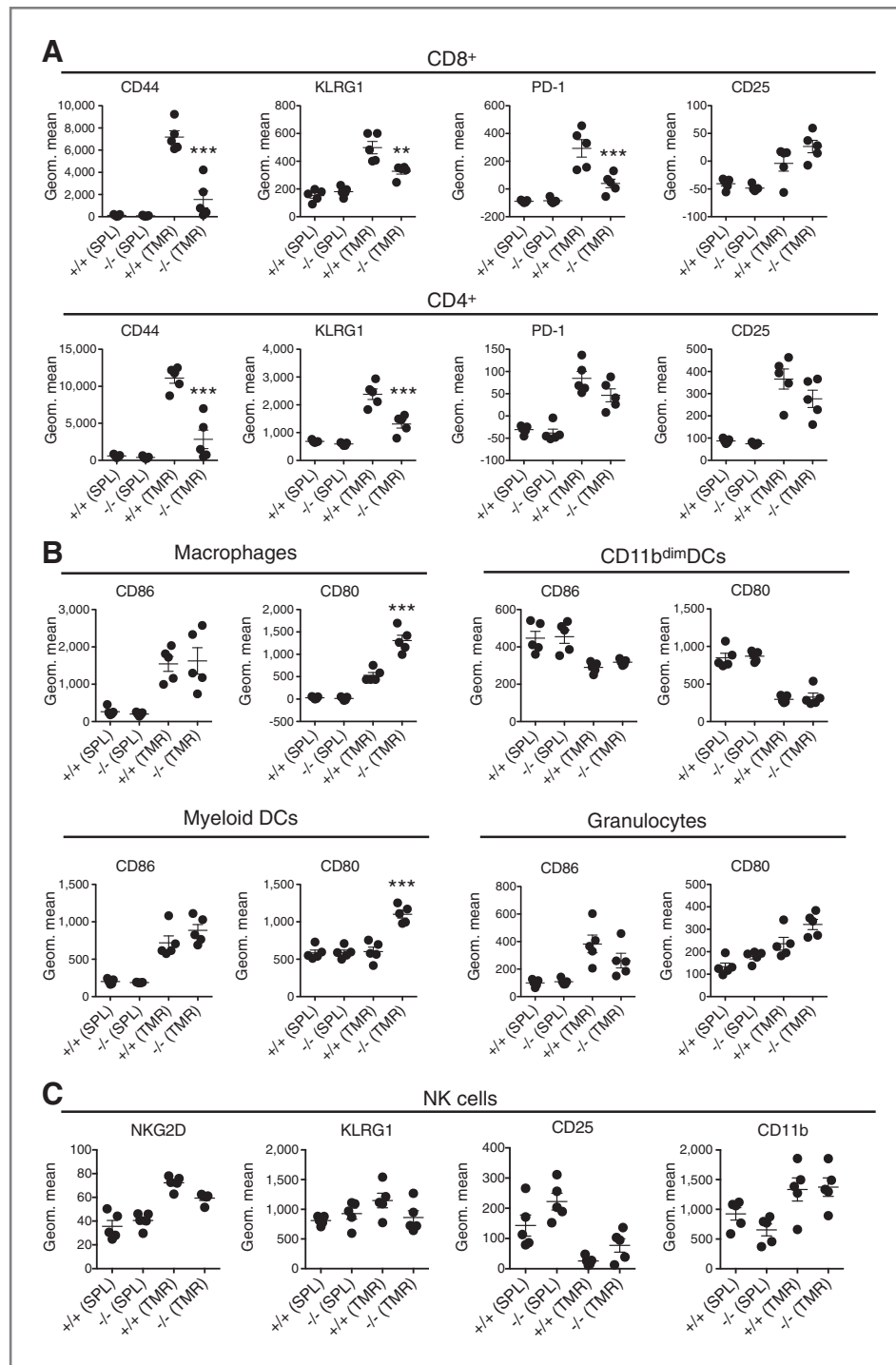


Figure 2. Global deletion of *Adora2a* inhibits effector-memory differentiation of tumor-associated T cells. Phenotypic analysis of lymphocyte (A), APC populations (B), and NK cells (C) isolated from spleen (SPL) and B16F10 tumors (TMR). Pooled data from two independent experiments, $n = 5/\text{group}$; **, $P < 0.01$; ***, $P < 0.001$, by two-way ANOVA and Tukey *post hoc* analyses.

CD80 expression on tumor-associated APCs increased, whereas CD86 expression and NK markers were unchanged (Fig. 2B and C). 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_{2A}R deficiency did not cause significant changes among any of these markers except for a reduction in cell surface PD-L1 expression (Supplementary Fig. S1). 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_{2A}R-deficient mice. Overall, these results suggest that decreased CD8⁺ T-cell infiltration and effector-memory differentiation in A_{2A}R^{-/-} mice are not due to APC inactivation. In fact, myeloid-selective deletion of A_{2A}Rs decreases melanoma growth and increases the number of tumor-associated T cells and NK cells (23).

As in mice with B16F10 tumors, in mice with solid MB49 carcinomas A_{2A}R deletion reduced CD8⁺ T-cell frequency and expression of the effector-memory marker CD44 (Fig. 3A and B). One possible explanation for the reduction in CD8⁺ T cells in the tumors of A_{2A}R^{-/-} mice is reduced expression of CXCR3, which is required for activated T cells

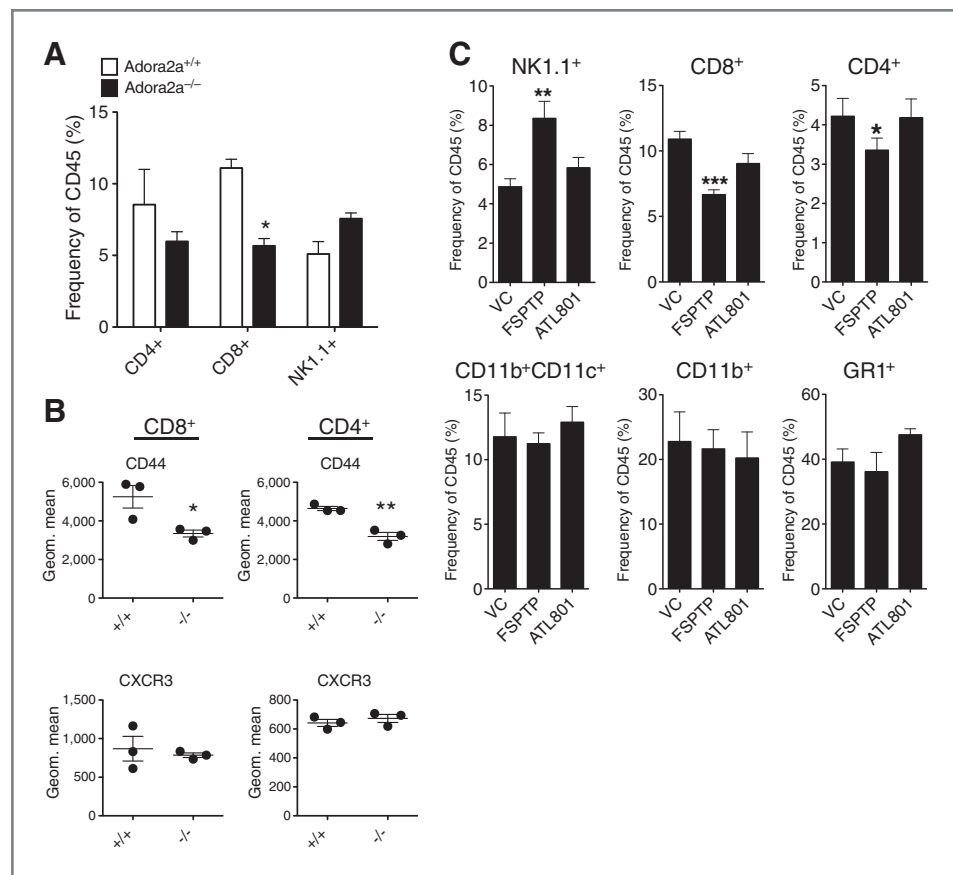
to home to inflamed sites. We did not observe any reduction in CXCR3 expression after A_{2A}R deletion (Fig. 3B). Furthermore, local inhibition by intratumoral injection of the A_{2A}R antagonist FSPTP, but not by selective A_{2B}R blocker ATL801, also significantly reduced the frequency of tumor-associated CD8⁺ T cells (Fig. 3C), suggesting that as in melanomas, A_{2A}R signaling facilitates the accumulation of CD8⁺ T cells within bladder tumors as well.

It is notable that A_{2A}R blockade consistently increased the frequencies of tumor-associated NK cells (Figs. 1B and 3A and C). This observation agrees with findings by Beavis and colleagues (2) who found that blockade or global deletion of A_{2A}Rs reduced lung metastasis of CD73-expressing tumors by increasing NK cell activity and numbers, presumably by blocking A_{2A}R-mediated effects of high adenosine in the tumor.

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 A_{2A}R deletion activates APCs (Fig. 2B), we hypothesized that A_{2A}R signaling helps to maintain T-cell numbers in the solid tumor microenvironment in a T-cell intrinsic manner. To evaluate the effects of cell-intrinsic A_{2A}R signaling on tumor growth and on T-cell

Figure 3. Global deletion or acute local blockade of A_{2A}Rs 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 Bonferroni *post hoc* analysis. **B**, flow-cytometry analysis of CD44 and CXCR3 expression in A_{2A}R^{-/-} and A_{2A}R^{+/+} T cells in MB49 tumors. *, $P < 0.05$; **, $P < 0.01$ by unpaired Student *t* test ($n = 3$). **C**, frequencies of major immune cell populations from MB49 tumors injected with 100 μ L, 1 μ mol/L FSPTP, 1 μ mol/L ATL801 or vehicle every 3 days after tumor inoculation. Results are pooled from independent experiments with similar results ($n \geq 5$; *, $P < 0.05$; **, $P < 0.01$; by two-way ANOVA and Bonferroni *post hoc* analyses.) **A–C**, all corresponding analyses were performed 3 weeks after tumor inoculation.



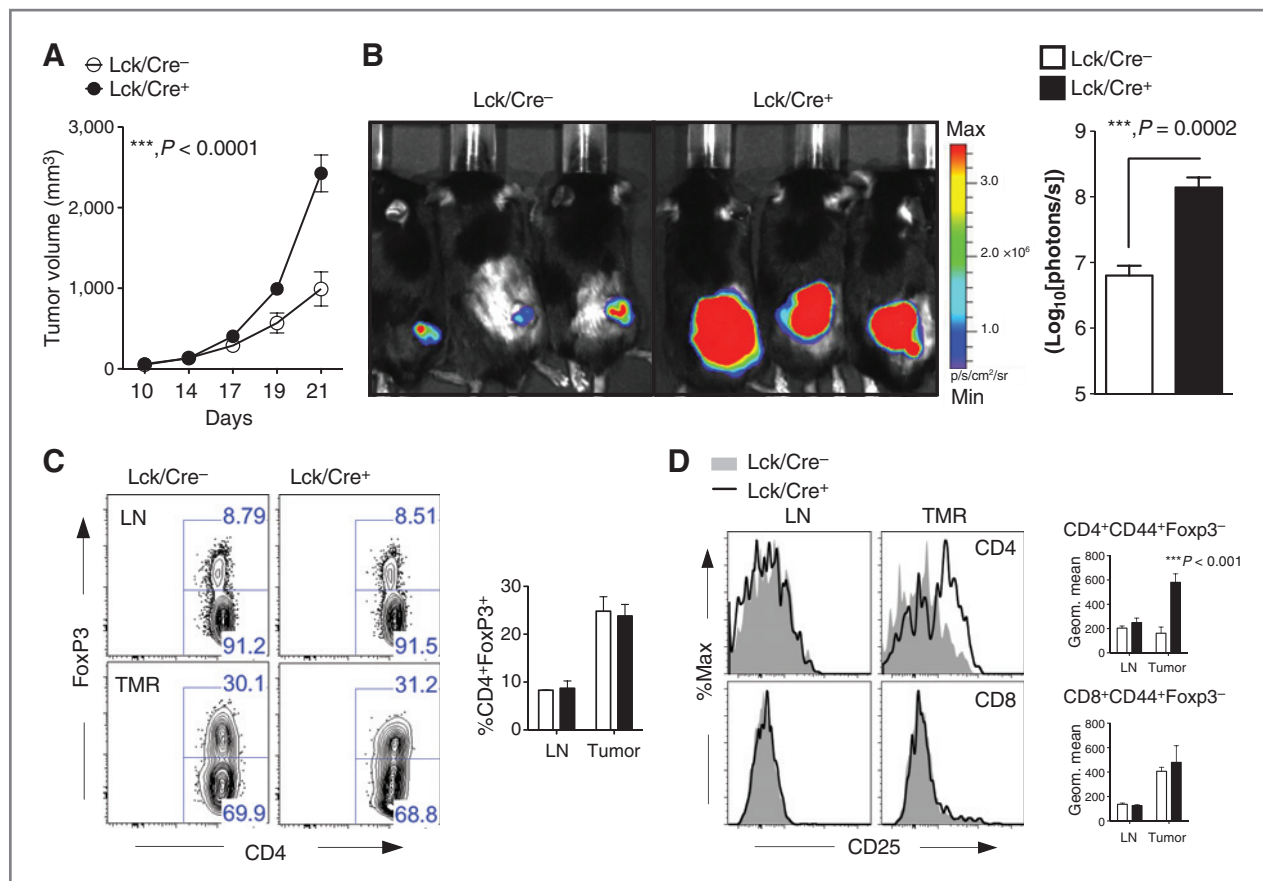


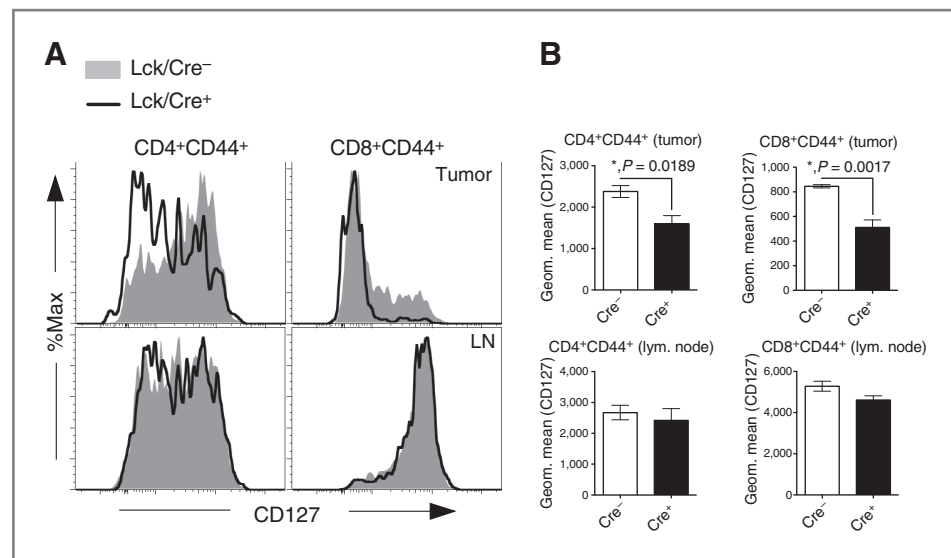
Figure 4. Lymphoid deletion of *Adora2a* promotes melanoma growth. **A**, growth of B16F10 melanoma cells in *Adora2a*^{fl/fl}-Cre⁺ and Cre⁻ littermates. Tumor sizes were measured by caliper ($n > 9$ from two independent experiments; ***, $P < 0.0001$, by two-way ANOVA and Bonferroni *post hoc* analysis). **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 *t* test. Single-cell suspensions from tumors and lymph nodes (LN) were isolated from *Adora2a*^{fl/fl}-LckCre^{+/+} or Cre^{-/-} littermate controls harvested 3 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 hoc* tests.

responses, we crossed mice with a floxed *Adora2a* gene with mice expressing Cre recombinase under control of the Lck promoter. *Adora2a*^{fl/fl}-LckCre^{+/+} mice have normal numbers of thymic T-cell precursors in the absence (22) or presence (Supplementary Fig. S2) of solid tumors, suggesting that *Adora2a*^{fl/fl}-LckCre^{+/+} mice have normal T-cell development compared with littermate controls. However, T-cell selective deletion of A_{2A}Rs markedly accelerated the growth rate of tumors after they reached a volume >500 mm³ (Fig. 4A and B; see Supplementary Fig. S3 for results of individual experiments). These findings suggest that paradoxically, some degree of A_{2A}R signaling in T cells is required to mount an optimal antitumor immune response in large solid tumors. A_{2A}R 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_{2A}R deletion failed to affect the expression of CD69 in tumor-associated T cells (Supplementary Fig. S4). We reasoned that the absence of A_{2A}R signaling in the tumor microenvironment might cause T

cells to polarize toward a regulatory phenotype. Figure 4C shows that T-cell A_{2A}R 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_{2A}R 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. 4D). These data suggest that deletion of A_{2A}R signaling does not hamper T-cell activation in tumors.

We next considered the possibility that A_{2A}R signaling sustains normal numbers of tumor-associated T cells. We found previously that A_{2A}R signaling, by activating PKA, reduces the activity of the PI3K/Akt pathway (22). This suppresses TCR-mediated downregulation of antiapoptotic CD127, which is upregulated in long-lived effector-memory cells and required for their maintenance. LckCre-mediated deletion of A_{2A}Rs significantly reduced CD127 expression in antigen-experienced T cells in the tumor (Fig. 5), and

Figure 5. Reduced expression of CD127 among effector T cells lacking A_{2A}Rs in tumors. A and B, flow-cytometric analysis of CD127 expression (A) and geometric means of corresponding populations (B) are shown. Data are from one of two independent experiments with similar results analyzed using Student *t* tests (*n* = 4).



significantly reduced the frequencies of tumor-associated T cells (Fig. 6A and B) but not NK cells (Fig. 6C). T-cell-selective A_{2A}R-deletion also significantly reduced the frequency of CD44⁺ effector-memory T cells in tumors, but not lymph nodes (Fig. 6D and E). Figure 6F shows a reduction in A_{2A}R^{-/-} T-cell density in tumors. Hence, although A_{2A}R 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 A_{2A}R deletion to enhance T-cell activation but to reduce effector cell numbers provide an explanation for why global deletion of the A_{2A}R causes small or inconsistent effects on tumor growth.

A_{2A}R signaling prolongs the maintenance of T cells in tumor-bearing hosts

Global A_{2A}R deletion significantly reduces the development and peripheral maintenance of naïve T cells (22). Although Lck-mediated *Adora2a*^{fl/fl} 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*^{fl/fl}. Also, reduced naïve T-cell numbers in mice lacking T-cell A_{2A}Rs could be a consequence of high tumor burden rather than to an intrinsic effect of A_{2A}R signaling. 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 cotransfer experiments. When cotransferred into the same host-bearing B16F10-OVA tumors, the proportion of *Adora2a*^{-/-} OT-I T cells was markedly decreased in the tumor relative to *Adora2a*^{+/+} OT-I T cells (Fig. 7A). A_{2A}R deletion also reduced cell surface expression of PD-1 (Fig. 7B), whereas 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 2 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 Fig. S5). 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 Fig. S5). Overall, these data show that in the tumor environment, A_{2A}R-deficient T cells have a survival disadvantage as compared with A_{2A}R-proficient T cells.

Discussion

Adenosine accumulates to high levels in solid tumors (1–4). A_{2A}Rs on antigen-presenting cells and T cells, and A_{2B} receptors on antigen-presenting immune cells are primarily responsible for immunosuppression by adenosine (26, 27). Global deletion or intratumoral blockade of A_{2B}Rs delays the growth of lung and bladder carcinoma and breast cancers, consistent with the immunosuppressive roles of these receptors (11). Curiously, global A_{2A}R 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

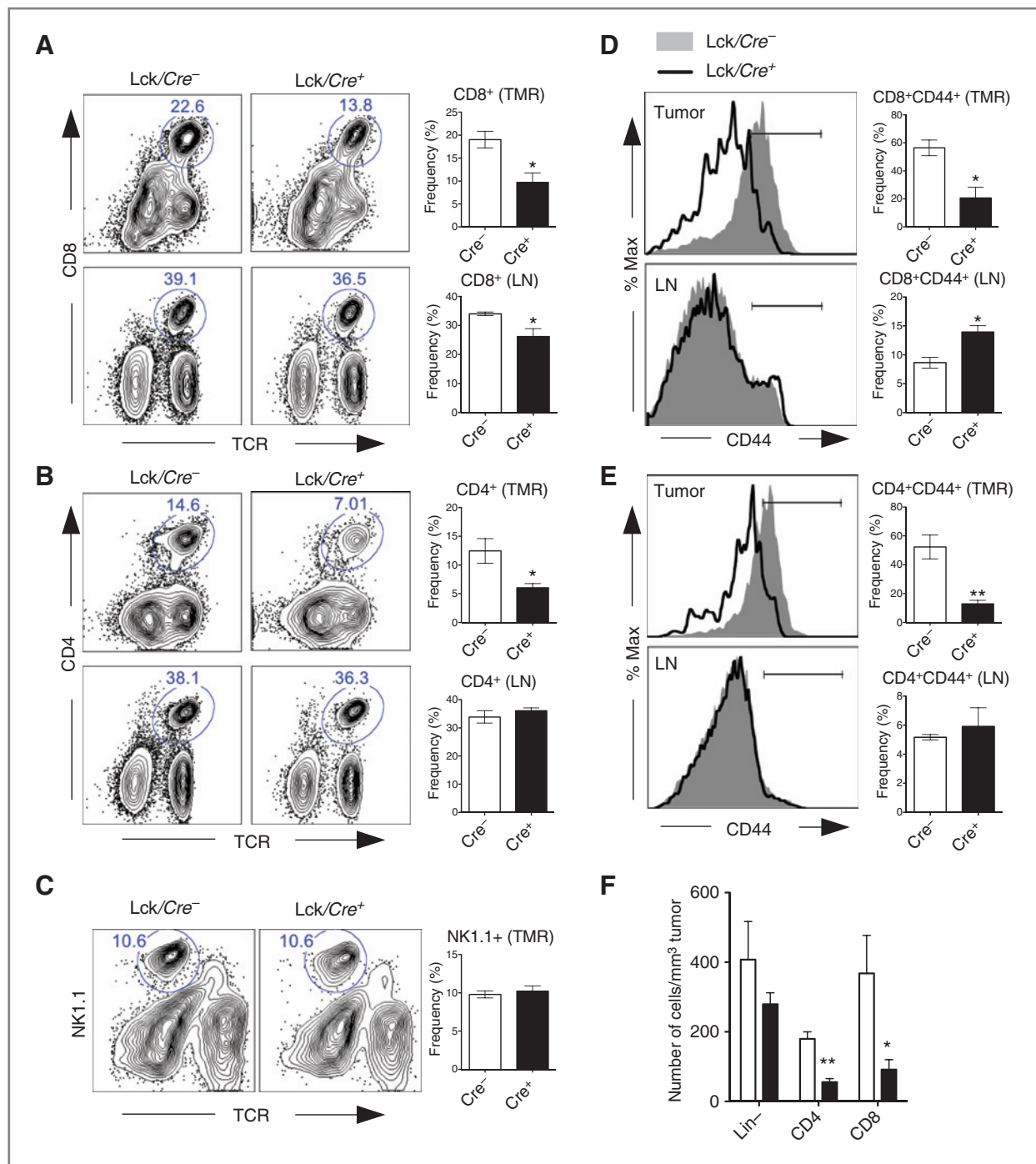


Figure 6. Reduction in numbers and memory-effector differentiation of tumor-associated T cells after lymphoid deletion of A2ARs. Single-cell suspensions of tumors and lymph nodes isolated from the *Adora2a*^{fl/fl}-LckCre^{+/+} or Cre^{-/-} littermate controls were prepared 3 weeks after tumor inoculation. Frequencies of CD8⁺ T cells (A), CD4⁺ T cells (B), and NK cells (C). D and E, CD44 expression is shown for CD8⁺ (D) and CD4⁺ (E) 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 experiments with similar results. Data were analyzed using Student *t* tests.

by transfection with H-2K^b (7). These findings, and the results of the current study suggest that A_{2A}R blockade, as a strategy to treat cancer, is more complex than previously thought (9, 10). Although T cells are acutely activated by A_{2A}R

R 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

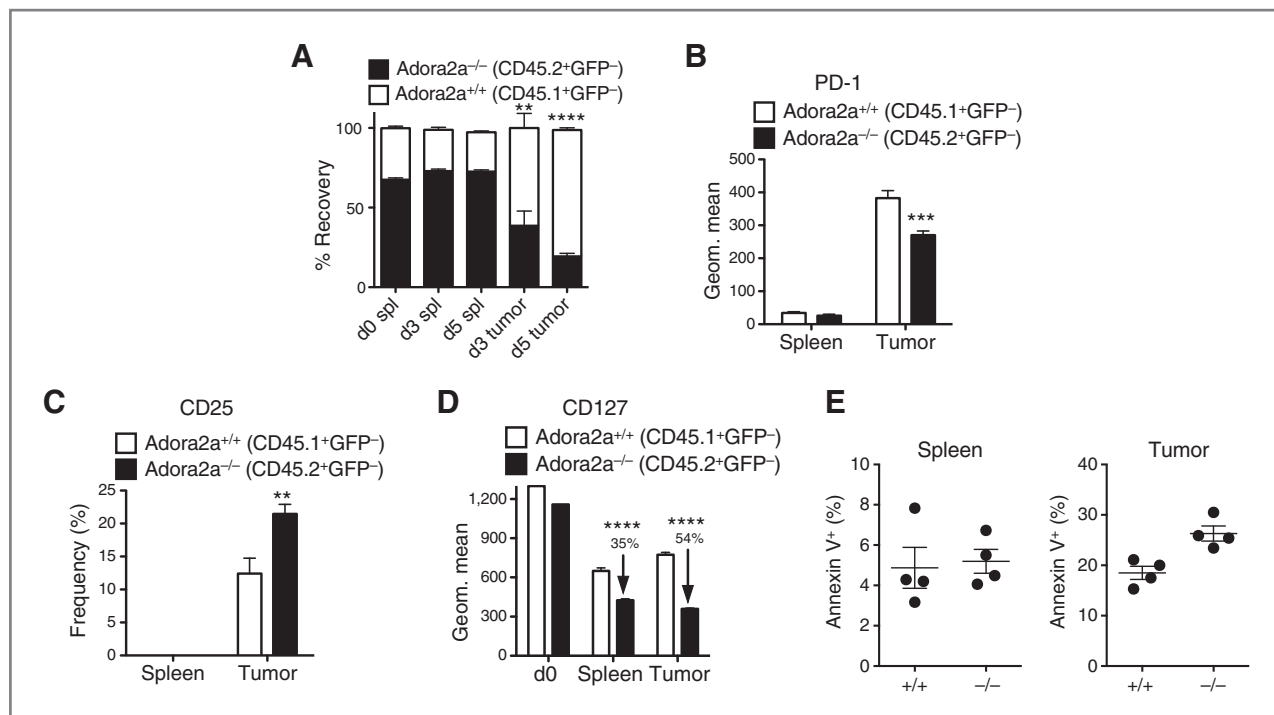


Figure 7. A_{2A}R signaling is required for T-cell maintenance in solid tumors. A, recovery of *Adora2a*^{+/+} (CD45.1) and *Adora2a*^{-/-} (CD45.2) OT-1 Rag^{-/-} T cells 3 and 5 days after the cotransfer of 5×10^5 cells into GFP⁺ mice bearing Ova expressing B16F10 tumors >300 mm³ in size produced following s.c. injection of 10^5 tumor cells. Expression on day 5 of PD-1 (B), CD25 (C), and CD127 (D) 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). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, by two-way ANOVA and Bonferroni *post hoc* analysis.

effect of A_{2A}R deletion on T-cell functions is not necessarily indicative of the effects of A_{2A}R blocking drugs that lessen, but do not eliminate A_{2A}R signaling. In this respect, a more clinically relevant reversible A_{2A}R antagonist rather than an irreversible antagonist may have different properties.

Ohta and colleagues previously showed that silencing of A_{2A} and A_{2B} adenosine receptors by siRNA in adoptively transferred, tumor-specific T cells significantly reduced lung metastasis of H2-K^b-expressing RMA T-cell lymphoma cells and improved the survival of tumor-bearing mice (7). The current study shows that *Adora2a*-deficient OT-1 T cells do not slow the growth of solid B16F10 cells expressing ovalbumin, which can be recognized by OT-1 T cells. The data suggest that: (i) antitumor effects of *Adora2a* deletion vary among different types of tumors; (ii) A_{2B}R signaling in tumor-associated T cells may contribute to adenosine suppression of T-cell activity; (iii) solid versus metastatic tumor growth may be differentially influenced by A_{2A}R deletion; (iv) highly immunogenic tumors may preferentially elicit antitumor effects of *Adora2a* deletion; and (v) siRNA silencing of *Adora2a* expression or pharmacologic inhibition may result in A_{2A}R residual signaling and 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 costimulation by certain cytokines or homing signals in the tumor (9). Inter-

estingly, the absence of T-cell intrinsic inhibitory A_{2A}R signaling reduces numbers of tumor-associated T cells after 2 to 3 weeks of solid tumor expansion. Our recent findings indicate that A_{2A}R signaling can prevent TCR-induced downregulation of antiapoptotic CD127 (22). A_{2A}R 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 *Adora2a*^{fl/fl}. However, the irreversible A_{2A}R antagonist, FSPTP, injected directly into solid tumors, also reduced tumor-associated T-cell numbers in wild-type recipients. Also, the ratio of antigen-specific A_{2A}R-deficient/A_{2A}R-proficient T cells decreased within the same tumor after adoptive cotransfer, suggesting a T-cell survival defect due to either deletion or irreversible blockade of A_{2A}R signaling in the tumor environment.

We show that CD127 expression by effector-memory cells in tumors is significantly reduced in T cells lacking A_{2A}Rs, whereas CD25 expression is largely intact or even increased. This supports the notion that although A_{2A}R signaling acutely inhibits initial T-cell activation, A_{2A}R-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 A_{2A}R^{-/-} T cells in response to IL7 (22). The pattern of tumor T-cell responses

caused by deletion of the A_{2A}R is very similar to the effect of IL2 in tumor immunotherapy: although IL2 increases the activation and early expansion of T cells, it causes increased activation-induced death (28). Therefore, it is possible that CD127 deficiency and increased IL2 signaling due to increased CD25 expression impair T-cell survival in the tumor microenvironment.

A_{2A}R signaling may sustain tumor-associated T cells by inhibiting the PI3K/Akt pathway. As with A_{2A}R 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 (29). Expansion of tumor-associated effector-memory T cells might account for the observations that despite their immunosuppressive properties, adenosine (this study) and rapamycin (30) have antiapoptotic effects on T cells in some tumors.

The A_{2A}R is upregulated in multiple immune cell types upon activation (5, 31, 32). Blockade or deletion of A_{2A}Rs in NK cells (2) or myeloid cells (23) significantly inhibits tumor growth and metastasis and is associated with transactivation of cytotoxic lymphocytes. Consistent with these findings, the current study suggests that to avoid apoptosis of tumor-associated T cells, myeloid or NK cell targeted therapies should be considered as preferable targets for A_{2A}R deletion/blockade as a strategy for tumor immunotherapy. Alternatively, to optimize the beneficial effects of T-cell activation by A_{2A}R 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Cekic, J. Linden

Development of methodology: C. Cekic

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Cekic, J. Linden

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Cekic, J. Linden

Writing, review, and/or revision of the manuscript: C. Cekic, J. Linden

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Cekic

Study supervision: J. Linden

Acknowledgments

The authors thank Drs. Stephen Schoenberger and Michael Croft of the La Jolla Institute for Allergy and Immunology for helpful discussions, Dr. Jiang-Fan Chen of Boston University for A_{2A}R^{-/-} mice, Dr. Timothy Ratliff of Purdue University for MB49 bladder carcinoma, Dr. Andreas Limmer of the University of Bonn for ovalbumin-expressing B16F10 cells, and Dr. Stephen Schoenberger of the La Jolla Institute for B16F10 cells expressing ovalbumin and luciferase.

Grant Support

This work was supported by NIH grant P01 HL073371 (J. Linden) and by American Heart Association post-doctoral fellowship (C. Cekic).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 12, 2013; revised September 23, 2014; accepted October 10, 2014; published OnlineFirst October 23, 2014.

References

- Allard B, Turcotte M, Stagg J. CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth. *J Biomed Biotechnol* 2012;2012:485156.
- Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, et al. Blockade of A_{2A} receptors potently suppresses the metastasis of CD73⁺ tumors. *Proc Natl Acad Sci U S A* 2013;110:14711–6.
- Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J Immunol* 2011;187:676–83.
- Deaglio S, Dwyer KM, Gao W, Friedman D, Ushuva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007;204:1257–65.
- Lappas CM, Rieger JM, Linden J. A_{2A} adenosine receptor induction inhibits IFN- γ production in murine CD4⁺ T cells. *J Immunol* 2005;174:1073–80.
- Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 2010;29:5346–58.
- Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, et al. A_{2A} adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci U S A* 2006;103:13132–7.
- Waickman AT, Alme A, Senaldi L, Zarek PE, Horton M, Powell JD. Enhancement of tumor immunotherapy by deletion of the A_{2A} adenosine receptor. *Cancer Immunol Immunother* 2012;61:917–26.
- Gao J, Bernatchez C, Sharma P, Radvanyi LG, Hwu P. Advances in the development of cancer immunotherapies. *Trends Immunol* 2013;34:90–8.
- Sitkovsky MV, Kjaergaard J, Lukashev D, Ohta A. Hypoxia-adenosine immunosuppression: tumor protection by T regulatory cells and cancerous tissue hypoxia. *Clin Cancer Res* 2008;14:5947–52.
- Cekic C, Sag D, Li Y, Theodorescu D, Strieter R, Linden J. Adenosine A_{2B} receptor blockade slows growth of bladder and breast tumors. *J Immunol* 2012;188:198–205.
- Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J Exp Med* 2009;206:1457–64.
- Himer L, Csoka B, Selmecezy Z, Kosco B, Pocza T, Pacher P, et al. Adenosine A_{2A} receptor activation protects CD4⁺ T lymphocytes against activation-induced cell death. *FASEB J* 2010;24:2631–40.
- Stagg J, Beavis PA, Divisekera U, Liu MC, Moller A, Darcy PK, et al. CD73-deficient mice are resistant to carcinogenesis. *Cancer Res* 2012;72:2190–6.
- Stagg J, Divisekera U, Duret H, Sparwasser T, Teng MW, Darcy PK, et al. CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res* 2011;71:2892–900.
- Wang L, Fan J, Thompson LF, Zhang Y, Shin T, Curiel TJ, et al. CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. *J Clin Invest* 2011;121:2371–82.
- Yegutkin GG, Marttila-Ichihara F, Karikoski M, Niemela J, Laurila JP, Elima K, et al. Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. *Eur J Immunol* 2011;41:1231–41.
- Luo Y, Han R, Evanoff DP, Chen X. Interleukin-10 inhibits Mycobacterium bovis bacillus Calmette-Guerin (BCG)-induced macrophage

- cytotoxicity against bladder cancer cells. *Clin Exp Immunol* 2010; 160:359–68.
19. Faló LD Jr, Kovacsóvics-Bankowski M, Thompson K, Rock KL. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat Med* 1995;1:649–53.
 20. Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, et al. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* 1999;19:9192–200.
 21. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 2001;15:763–74.
 22. Cekic C, Sag D, Day YJ, Linden J. Extracellular adenosine regulates naive T cell development and peripheral maintenance. *J Exp Med* 2013;210:2693–706.
 23. Cekic C, Day YJ, Sag D, Linden J. Myeloid expression of Adenosine A_{2A} receptor suppresses T and NK cell responses in the solid tumor microenvironment. *Cancer Res* 2014;74:7250–59.
 24. Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A_{2A} extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* 1997;90:1600–10.
 25. Conche C, Boulla G, Trautmann A, Randriamampita C. T cell adhesion primes antigen receptor-induced calcium responses through a transient rise in adenosine 3',5'-cyclic monophosphate. *Immunity* 2009;30:33–43.
 26. Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 2008;7:759–70.
 27. Wilson JM, Ross WG, Agbai ON, Frazier R, Figler RA, Rieger J, et al. The A_{2B} adenosine receptor impairs the maturation and immunogenicity of dendritic cells. *J Immunol* 2009;182:4616–23.
 28. Shrikant P, Mescher MF. Opposing effects of IL-2 in tumor immunotherapy: promoting CD8 T cell growth and inducing apoptosis. *J Immunol* 2002;169:1753–9.
 29. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature* 2009;460:108–12.
 30. Svirshchevskaya EV, Mariotti J, Wright MH, Viskova NY, Telford W, Fowler DH, et al. Rapamycin delays growth of Wnt-1 tumors in spite of suppression of host immunity. *BMC Cancer* 2008; 8:176.
 31. Murphree LJ, Sullivan GW, Marshall MA, Linden J. Lipopolysaccharide rapidly modifies adenosine receptor transcripts in murine and human macrophages: role of NF- κ B in A(2A) adenosine receptor induction. *Biochem J* 2005;391:575–80.
 32. Lin G, Field JJ, Yu JC, Ken R, Neuberg D, Nathan DG, et al. NF- κ B is activated in CD4 iNKT cells by sickle cell disease and mediates rapid induction of adenosine A receptors. *PLoS ONE* 2013;8:e74664.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Adenosine A_{2A} Receptors Intrinsically Regulate CD8⁺ T Cells in the Tumor Microenvironment

Caglar Cekic and Joel Linden

Cancer Res 2014;74:7239-7249. Published OnlineFirst October 23, 2014.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-13-3581
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/10/23/0008-5472.CAN-13-3581.DC1

Cited articles	This article cites 32 articles, 17 of which you can access for free at: http://cancerres.aacrjournals.org/content/74/24/7239.full#ref-list-1
Citing articles	This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/74/24/7239.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .