Myeloid Expression of Adenosine A2A Receptor Suppresses T and NK Cell Responses in the Solid Tumor Microenvironment

Caglar Cekic1,4, Yuan-Ji Day2, Duygu Sag3, and Joel Linden1

Abstract

High concentrations of adenosine in tumor microenvironments inhibit antitumor cytotoxic lymphocyte responses. Although T cells express inhibitory adenosine A2A receptors (A2AR) that suppress their activation and inhibit immune killing of tumors, a role for myeloid cell A2ARs in suppressing the immune response to tumors has yet to be investigated. In this study, we show that the growth of transplanted syngeneic B16F10 melanoma or Lewis lung carcinoma cells is slowed in Adora2afl/fl-LysMCre+/− mice, which selectively lack myeloid A2ARs. Reduced melanoma growth is associated with significant increases in MHCII and IL12 expression in tumor-associated macrophages and with >90% reductions in IL10 expression in tumor-associated macrophages, dendritic cells (DC), and Ly6C− or Ly6G+ myeloid-derived suppressor cells (MDSC). Myeloid deletion of A2ARs significantly increases CD44 expression on tumor-associated T cells and natural killer (NK) cells. Depletion of CD8+ T cells or NK cells in tumor-bearing mice indicates that both cell types initially contribute to slowing melanoma growth in mice lacking myeloid A2AR receptors, but tumor suppression mediated by CD8+ T cells is more persistent. Myeloid-selective A2AR deletion significantly reduces lung metastasis of melanomas that express luciferase (for in vivo tracking) and ovalbumin (as a model antigen). Reduced metastasis is associated with increased numbers and activation of NK cells and antigen-specific CD8+ T cells in lung infiltrates. Overall, the findings indicate that myeloid cell A2ARs have direct myelosuppressive effects that indirectly contribute to the suppression of T cells and NK cells in primary and metastatic tumor microenvironments. The results indicate that tumor-associated myeloid cells, including macrophages, DCs, and MDSCs all express immunosuppressive A2ARs that are potential targets of adenosine receptor blockers to enhance immune killing of tumors. Cancer Res; 74(24); 7250–9. ©2014 AACR.

Introduction

Many elements of myeloid cell, T-cell, and natural killer (NK) cell activation in the tumor environment are shaped by their interaction, for example, antigen presentation and communication through cytokines (1). Immunosuppressive tumor microenvironments inhibit these interactions and facilitate immune system evasion by tumor cells. Tumor-associated macrophages and myeloid-derived suppressor cells (MDSC) are early responders to neoplastic growth. Hence, lymphocyte cytotoxicity and activation are shaped by the phenotypes of the macrophages they initially interact with. Tumor-associated macrophages are often polarized towards an anti-inflammatory/proangiogenic M2 phenotype rather than the tumoricidal M1 phenotype that produces high amounts of IL12 and MHCII to enhance antitumor T-cell responses (2). Macrophage polarization is influenced by location within the tumor. Normoxic tumor areas are more likely to contain M1 macrophages while proangiogenic M2/M2-like macrophages preferentially reside in hypoxic areas (3).

Solid tumor microenvironments are hypoxic, inflamed, and exhibit a high frequency of apoptotic cell death. Cells that are stressed by hypoxia or inflammation and apoptotic cells release ATP. Although extracellular ATP enhances immune cell chemotaxis and activation through engagement with P2 purinergic receptors, it is rapidly degraded to adenosine by ectonucleotidases. CD39, which converts ATP/ADP to AMP, is expressed on regulatory T cells and macrophages. CD73, which converts AMP to adenosine, is highly expressed by some tumors including ER+ breast tumors, endothelial cells, regulatory T cells, and most B cells (4, 5). Macrophages modulate their activation state by increasing the synthesis and secretion of ATP that in the tumor microenvironment is immunosuppressive due to its rapid catabolism into adenosine by CD39 and CD73 (6). Therefore, solid tumor microenvironments favor the production high concentrations of adenosine that impairs antitumor T-cell responses (7, 8).

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Adenosine exerts its effects by engaging four subtypes of P1 purinergic or adenosine receptors: A1, A2A, A2B, and A3. A2AR and A3R mRNA expression increases in activated macrophages (9-13), and signaling through A3Rs inhibits the activation of macrophages by inflammatory stimuli and promotes remodeling to an M2-like phenotype (9, 14). Prolonged A2AR stimulation facilitates tissue-healing responses by stimulating the production of factors such as VEGF and IL6 that promote angiogenesis and fibrosis (9, 14, 15). These findings suggest that adenosine plays an important dynamic role in shaping macrophage responses during acute and chronic injury.

There is growing evidence that even syngeneic tumors can evoke immune responses that can suppress or sometimes arrest tumor growth. Depletion of T cells prevented the rejection of certain highly immunogenic melanomas in A3R-deficient mice (7). Increased metastasis due to high expression of CD73, which elevates adenosine, is prevented by blockade of CD73 in mice (7). Increased metastasis due to high expression of CD73, which elevates adenosine, is prevented by blockade of CD73 in mice (7). Therefore, blockade of A3Rs on NK cell and T cells (18, 19) has been viewed as an attractive strategy for enhancing immune-mediated inhibition of antitumor immune responses. However, studies of the effects on tumor growth of adenosine receptor–targeted deletion, particularly on myeloid cells, have not yet been undertaken. In this study, we focused on dissecting the effects on tumor growth of A3AR signaling by cells that express A3Rs or A2Rs, which increase NK cell activity (16, 17). Therefore, blockade of A3Rs on NK cell and T cells (18, 19) has been viewed as an attractive strategy for enhancing immune-mediated inhibition of antitumor immune responses. However, studies of the effects on tumor growth of adenosine receptor–targeted deletion, particularly on myeloid cells, have not yet been undertaken. In this study, we focused on dissecting the effects on tumor growth of A3ar signaling by cells that express CD73, which elevates adenosine, is prevented by blockade of CD73 in mice (7).

Materials and Methods

Cell lines, animals, and reagents

Animal experiments were approved by the Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology (La Jolla, CA). B16F10 cells stably expressing luciferase were obtained from Caliper Life Sciences and immunized for use in luciferase experiments. Animal experiments were approved by the Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology (La Jolla, CA). B16F10 cells stably expressing luciferase were obtained from Caliper Life Sciences and immunized for use in luciferase experiments. B16F10 cells stably expressing luciferase were obtained from Caliper Life Sciences and immunized for use in luciferase experiments. Brieﬂy, ﬁemurs and tieiae from 8- to 12-week-old mice were collected and ﬂushed twice with sterile Hank balanced salt solution. Bone marrow cells were cultured overnight in standard tissue culture plates in the presence of 10 ng/mL macrophage colony stimulating factor (M-CSF). Nonadherent cells from this initial culture were then transferred to low-attachment 6-well plates (Corning Life Sciences) in 4 mL R5F containing 30% L929 conditioned medium and 10 ng/mL M-CSF for 7 days, adding 1.5 mL fresh medium on days 3 and 5. Resulting macrophages were either prestimulated with 100 ng lipopolysaccharide (LPS; Invitrogen) or kept unstimulated for 24 hours before coculturing with B16F10 tumor cells (1:20 target to effector ratio) in 96-well round-bottom culture plates in the presence or absence of the A2AR agonist CGS 21680 (1 μmol/L). 7AAD staining and CD45 staining was used to identify dead cells and myeloid cells, respectively.

Ex vivo tumor cell killing by macrophages

Bone marrow–derived macrophages were prepared according to a protocol modiﬁed from Cekic and colleagues (22). Brieﬂy, ﬁemurs and tieiae from 8- to 12-week-old mice were collected and ﬂushed twice with sterile Hank balanced salt solution. Bone marrow cells were cultured overnight in standard tissue culture plates in the presence of 10 ng/mL macrophage colony stimulating factor (M-CSF). Nonadherent cells from this initial culture were then transferred to low-attachment 6-well plates (Corning Life Sciences) in 4 mL R5F containing 30% L929 conditioned medium and 10 ng/mL M-CSF for 7 days, adding 1.5 mL fresh medium on days 3 and 5. Resulting macrophages were either prestimulated with 100 ng lipopolysaccharide (LPS; Invitrogen) or kept unstimulated for 24 hours before coculturing with B16F10 tumor cells (1:20 target to effector ratio) in 96-well round-bottom culture plates in the presence or absence of the A2AR agonist CGS 21680 (1 μmol/L). 7AAD staining and CD45 staining was used to identify dead cells and myeloid cells, respectively.
Flow cytometry

Single-cell suspensions from indicated tissues were prepared by sequentially pressing cells through 100 μm and 40 μm cell strainers. After RBC lysis (Biologend) cells were washed and resuspended in R10F, and counted in a Z2-Coulter particle counter (Beckman Coulter). Most dead cells were removed from tumor samples by Ficoll gradient centrifugation at 2,000 rpm (900 × g) for 20 minutes at room temperature. Cells (3–5 × 10^6) were preincubated for 10 minutes 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 minutes at 4°C in the dark. Cells were analyzed using an LSRIII equipped with four lasers or an LSR Fortessa with five lasers and FACS Diva software (BD Biosciences). Live/dead fixable yellow, blue, or aqua fluorescent reactive dyes (Invitrogen) were used to exclude dead cells before analysis. Flow cytometry data were analyzed using FlowJo software (version 9.6.4, TreeStar Software Inc.).

Intracellular staining

For intracellular cytokine staining of T cells, single-cell suspensions of tumors in 7 mL R5F medium were layered on 2 mL Ficoll and centrifuged for 20 minutes at 2,000 rpm at room temperature. Spleen and Ficoll-enriched cell suspensions from tumors were restimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 100 ng/mL ionomycin (Sigma) or OVA257-264 (SIINFEKL) peptide (Genescript USA) in the presence of Golgi Plug (eBioscience) for 5 hours at 37°C. Cells were fixed and permeabilized after surface staining and incubated for 25 minutes at 4°C in 100 μL permeabilization/washing buffer containing 1:100 fluorescently labeled anti-IFNγ. After a subsequent wash, cells were resuspended in 350 μL FACS buffer. For intracellular cytokine staining of myeloid cells, cell suspensions from tumors were resuspended in FACS buffer containing Golgi Plug and kept in Golgi Plug throughout the surface staining procedure before fixation and permeabilization.

Tumor growth and metastasis

We injected 2 × 10^5 LLC or 10^5 B16F10 melanoma cells expressing luciferase into the right flanks of Adora2a^Cre^−/−/LysMCre^−/−^ mice. Tumor volumes were measured using digital calipers and calculated as height × width^2/2. We also measured luciferase activity by using an IVIS 200 BIoluminescence Imager (Caliper Life Sciences) after intravenous injection of 1 mg n-luciferin (Caliper Life Sciences) in 100 μL PBS. This method was used to demonstrate that tumor size differences are not due to infiltration of host cells into the tumor mass. For metastasis analysis, 3 × 10^5 B16F10 melanoma cells expressing luciferase and ovalbumin antigens were injected intravenously into the tail vain and luciferase activity was measured one and two weeks after the injection of cancer cells. After measuring luciferase activity, lungs were removed, photographed, and weighted to validate luciferase activity correlates with lung tumor mass. For in vivo depletion of CD8^+ T cells or NK cells, respectively, 200 μg anti-CD8α (clone 53–6.72) or anti-NK1.1 (PK136) antibodies were injected intraperitoneally four times at 5-day intervals, beginning a day before the subcutaneous injection of tumors. Cell depletion from spleen was verified by flow cytometry.

Results

Myeloid deletion of Adora2a inhibits solid tumor growth

To investigate cell-intrinsic effects of myeloid Adora2a expression on APC function and antitumor immune responses we generated mice with floxed Adora2a (21) and crossed these to mice that express Cre recombinase under control of the LysM promoter to create Adora2a^Cre^−/−/LysMCre^−/−^ mice with myeloid-selective A2AR deletion. These mice and Cre− littermate controls were injected with syngeneic tumors. Myeloid deletion of Adora2a significantly reduced the growth rates of B16F10 melanomas and LLCs (Fig. 1A). In the case of B16F10 melanomas, tumor growth measured with calipers (Fig. 1A) was confirmed by luciferase activity (Fig. 1B).

Myeloid deletion of Adora2a increases macrophage activation and effector function in tumors

LysMCre excises floxed target genes in granulocytes including macrophages and to a lesser extent in myeloid DCs. We measured the number and activation states of myeloid cell populations in tumors using the gating strategy shown in Fig. 2A (left). Deletion of Adora2a did not significantly change myeloid cell density in tumors (Fig. 2A, bottom right) measured by dividing the total myeloid cell number by tumor volume. We performed quantitative PCR analysis to measure A2AR mRNA in myeloid cells. In cells from Adora2a^Cre^−/−/LysMCre^−/−^ mice, A2AR mRNA was not detected in Gr1^+^ cells. A2AR deletion from myeloid cells increased the cell surface expression of MHCII and the production of IL12 in tumor-associated macrophages (Fig. 2C). A2AR deletion did not significantly change expression of MHCII or IL12 in macrophages and dendritic cells was reduced by 65% and 45%, respectively (Fig. 2B). A2AR mRNA was not detected in Gr1^+^ cells. A2AR deletion from myeloid cells increased the cell surface expression of MHCII and the production of IL12 in tumor-associated macrophages (Fig. 2C). A2AR deletion did not significantly modify this low expression in Gr1^+^ cells, in tumors generally have a phenotype similar to M2 "alternatively activated" macrophages; therefore, they have very low expression of MHCII and IL12. Adora2a deletion did not significantly modify this low expression in Gr1^+^ cells (Fig. 2C). Although myeloid DCs displayed somewhat increased IL12 and MHCII expression in response to LysMCre-mediated Adora2a^Cre^−/−^ deletion, this did not reach statistical significance, possibly owing to relatively low deletion efficiency in these cells. Activated macrophages can kill tumors through secretion of effector molecules or by cell–cell interactions. To determine whether A2AR deletion influences the overall cytotoxicity of macrophages, we isolated bone marrow from Adora2a^Cre^−/−/LysMCre^−/−^ animals or Cre− littermate controls and differentiated them into macrophages. Although coincubation of macrophages and tumor cells with LPS increased tumor killing, A2AR deletion or addition of the selective A2AR agonist CGS 21680 did not significantly affect the overall cytotoxic activity of macrophages (Fig. 2D). These
findings suggest that increased cytotoxic activity of NK cells or T cells is important for increasing tumor killing upon A2A-R blockade/deletion.

We next incubated single-cell suspensions of cells derived from tumors or spleens from mice with myeloid-selective A2A-R deletion and littermate controls for 5 hours in the presence of Golgi plug and Golgi stop, without further stimulation and measured intracellular IL10 as a marker for M2/tolerogenic differentiation of APCs. Figure 3 shows IL10 expression in APCs from myeloid cells lacking A2A-Rs is reduced by more than 90% in tumors, but is not significantly reduced in splenic myeloid cells. Interestingly, monocytic (Ly6C+) rather than granulocytic (Ly6G+) MDSCs are the main producers of A2A-R-dependent IL10 within tumors. IL10 mRNA is also reduced in sorted tumor-associated macrophages and DCs lacking A2A-Rs (Fig. 3D). Overall, these data indicate that myeloid-selective deletion of A2A-Rs favors M1 polarization of macrophages and substantially reduces anti-inflammatory IL10 production by myeloid cell populations.

**Myeloid deletion of A2A-Rs increases the number and activation of cytotoxic lymphocytes**

A2A-R signaling promotes tumor growth by inhibiting the activation of T cells and NK cells (7, 16, 19). The contribution of myeloid cell A2A-R signaling to these processes is not known. LysMcCre-mediated deletion of Adora2afl/fl significantly increased the proportion (Fig. 4A) and density (Fig. 4B) of tumor-associated cytotoxic lymphocytes and their surface expression of CD44 (Fig. 4C and D), indicative of increased activation or effector differentiation (for NK cells, the geometric mean of CD44 was used as there are no distinct CD44hi vs. CD44lo populations). To better understand the involvement of transactivation of T cells and NK cells through APCs in antitumor immune responses in LysMcCre/Adora2afl/fl, mice we depleted these cells with antibodies and measured tumor growth. Depletion of CD8+ T cells almost completely reversed the inhibition of tumor growth after LysMcCre deletion of Adora2a starting from day 14 (Fig. 4E). Depletion of NK cells reversed tumor growth inhibition on day 10, but did not reverse tumor growth on day 14 or later (Fig. 4E). This suggests that A2A-Rs on myeloid cells act to indirectly suppress tumor killing by both NK cells and CD8+ T cells, but the effect on CD8+ T cells is most important.

CD4+ T cells also can either promote or suppress tumor growth depending on their phenotype. Adenosine can directly promote differentiation of CD4+ T cells into the tumor-promoting regulatory phenotype. Contrary to effects on NK cells and CD8+ T cells, deletion of A2A-Rs from myeloid cells had little effect on numbers of CD4+ T cells (Fig. 5A and B). Proportions of CD4+ T cells with a regulatory phenotype in lymph nodes or tumors were also similar between LysMcCre−/− or Cre− animals (Fig. 5C). However, CD44 expression increased in tumor-infiltrating CD4+ T cells isolated from Adora2afl/fl−/−LysMcCre−/− animals (Fig. 5D), suggesting that along with CD8+ T cells, CD4+ T cells also can gain enhanced effector functions as a result of myeloid A2A-R deletion. Therefore, we next determined whether increased CD44 expression due to LysMcCre-mediated deletion of Adora2afl/fl is correlated with enhanced effector functionality in T cells. Tumor-associated, but not lymphoid or splenic T cells from Adora2afl/fl−/−LysMcCre−/− animals produced significantly more IFNγ after restimulation as compared with T cells
from *Adora2a*ff–*LysMCre*+/– littermate controls (Fig. 6). Overall, our results suggest that myeloid expression of *A2AR* is important in indirect adenosine-mediated suppression of T cells and NK cells.

**Myeloid deletion of Adora2a inhibits tumor metastasis**

Metastasis is a hallmark of late-stage tumors that is frequently responsible for cancer-associated deaths. Therefore, we determined whether the reduction in the growth of melanomas after myeloid deletion of *Adora2a* is associated with reduced lung metastases following intravenous transfer of B16F10 melanoma cells (expressing luciferase for *in vivo* imaging and ovalbumin as model antigen) into *Adora2a*ff–*LysMCre*+/– mice or *Cre*– littermate controls. Myeloid deletion of *Adora2a* reduced tumor-associated luciferase expression in the lungs over a two-week period by 10-to 30-fold (Fig. 7A).

Figure 2. Myeloid deletion of Adora2a increases macrophage activation and effector function in B16F10-ova tumors. Single-cell suspensions from tumors and lymph nodes isolated from the *LysMCre*+/–*Adora2a*ff mice and *Cre*– littermate controls were prepared 3 weeks after tumor inoculation. **A,** gating strategy (left) for selecting myeloid cell populations and cell density of myeloid populations (bottom right) from tumors. **B,** real-time PCR analysis of *A2AR* mRNA in tumor-associated DCs and macrophages. (**n = 3; *P < 0.05; ***P < 0.0001 by Student *t* test.** C, flow cytometry analysis of MHCII expression (top) and IL12 expression in myeloid populations in spleen and tumors. ***P < 0.0001 by two-way ANOVA and Bonferroni post hoc analyses (**n = 4**). D, LPS prestimulated or unstimulated bone marrow–derived macrophages from *LysMCre*+/–*Adora2a*ff mice and *Cre*– littermate controls were coincubated with B16 melanoma cells in the absence or presence of CGS 21680. Killing activity of macrophages was evaluated using 7AAD staining 48 hours after coincubation.
This was associated with reduced tumor mass in the lungs (Fig. 7B) and reduced weight (Fig. 7C). These data indicate that myeloid cells are important targets of \( \text{Adora}_2 \)R-mediated enhanced tumor metastasis. \( \text{Adora}_2 \)R and \( \text{Adora}_3 \)R blockade significantly reduced the metastasis of CD73-expressing tumors through enhanced NK cell activation (8, 16). Previous studies have focused on the role of CD8+ T cells in reducing metastasis upon \( \text{Adora}_2 \)R deletion (15, 16, 19). We observed a significant increase in the numbers of lung-associated NK cells but not CD8+ T cells in \( \text{Adora}_2 \)R/LysMCre+/- mice (Supplementary Fig. S2). However, numbers of antigen-specific CD8+ T cells were also increased (Supplementary Fig. S2). Both NK and CD8+ T cells had higher expression of CD44 in lung after tumor inoculation (Supplementary Fig. S2). These results suggest that both NK and CD8+ T cells may be important for reducing metastasis of tumors to the lung and their activity is strongly regulated by myeloid cell \( \text{Adora}_2 \)R expression. Overall, these data suggest that myeloid cell \( \text{Adora}_2 \)Rs contribute importantly to adenosine-mediated suppression of T cells and NK cells in tumors.

Discussion

Cancer immunotherapy is emerging as a treatment option for patients with late-stage tumors (23, 24). Modulating tumor microenvironments by antagonizing tumor-associated negative immune regulators such as PD-1, TGFβ, or adenosine has been viewed as an attractive treatment strategy (25, 26). In the current study, we found that myeloid-selective deletion of \( \text{Adora}_2 \)a slowed tumor growth and significantly increased activation markers. IL12 and MHCII, on macrophages without affecting \textit{ex vivo} cytotoxicity. Myeloid-selective deletion of the \( \text{A2AR} \) also decreased >90% IL10 production by tumor-associated macrophages, DCs, and MDSCs. This was associated with increased NK and CD8+ T-cell numbers, CD44 expression, and T-cell IFNγ production in the tumor.

Deletion of \( \text{A2AR} \)s from T cells causes T-cell activation, but reduces T-cell survival and memory cell differentiation in the solid tumor environment (27). Consequently, selective deletion of T-cell \( \text{Adora}_2 \)a sometimes reduces T-cell numbers and enhances the growth rate of large solid tumors. Here, we show that sparing \( \text{A2AR} \)s on T cells while depleting them from myeloid cells indirectly enhances tumor killing by increasing T-cell and NK cell activation in tumors. In previous studies, limiting adenosine production through inhibition or deletion of CD73 also enhanced solid tumor killing through activation of adaptive immune responses and through reduction in \( \text{A2AR} \) signaling in hematopoietic cells (14, 16, 28–30). The current study suggests that myeloid-selective blockade of \( \text{A2AR} \) signaling may be preferential to global or T-cell–selective blockade that can trigger T cell apoptosis in tumors. Moreover, enhanced APC activity likely mediates some of the effects of CD73 deletion. CD73 deletion limits, but does not abolish, adenosine production in tumor

Figure 3. Myeloid expression of \( \text{Adora}_2 \)a significantly increased IL10 expression in B16F10-ova tumor-associated APCs and MDSCs. Single-cell suspensions from tumors or spleen were isolated from the LysMCre+/– mice and littermate controls were prepared 3 weeks after tumor inoculation. CD45+/– cells were enriched from these suspensions at 4°C and defined by flow cytometry as macrophages (F4/80+), myeloid DCs (F4/80+/CD11c+), mononuclear MDSCs (Ly6Clow/Ly6Ghigh), or granulocytic MDSCs (Ly6Chigh/Ly6Glow). A–C intracellular cytokine staining for IL10 (A) and corresponding bar graphs of frequencies of IL10–producing cells in tumor (B) or in spleen (C) samples after incubating single-cell suspensions for 5 hours at 37°C in the presence of Golgi plug and Golgi stop. ***, \( P < 0.001 \) by two-way ANOVA and Bonferroni post hoc analyses (\( n = 5 \)/group). D, sorted tumor-associated DCs (TADC) and tumor-associated macrophages (TAM) were analyzed for IL10 mRNA by real-time PCR.
Microenvironments; thereby possibly sparing T cells from apoptosis or impaired memory differentiation that results from T-cell A2AR deletion. Our studies and others also suggest that cell-targeted A2AR blockade, the use of competitive A2AR antagonists, and/or the use of A3AR antagonists may be more effective for combating tumor growth than strong T-cell A2AR blockade or deletion.

Adenosine may have multiple cellular targets and engage both A2ARs and A3ARs to promote metastasis. Reduced A3AR activation was primarily responsible for decreased lung metastasis of breast tumor cells after CD73 blockade (17). Both A2AR and A3AR signaling were shown to promote metastasis of tumors highly expressing CD73 (16, 17). A3AR stimulation in tumor cells promotes metastasis by reducing cell-to-cell contact (31, 32), and influences endothelial cells and APCs to further promote metastasis (16, 31, 33, 34). A2AR signaling in NK cells and T cells is thought to promote tumor growth and metastasis by directly inhibiting their cytotoxic activity (16, 17, 31, 33). To our knowledge, the current study is the first to show that myeloid A3AR signaling strongly suppress NK and T-cell responses in lungs and promotes lung metastasis of B16 melanoma cells. Therefore, some of the effects of adenosine-mediated immune suppression on NK cells and T cells described in previous studies can be attributed to indirect effects of A3AR signaling in myeloid cells.

Macrophages can be polarized to different phenotypes that have opposing functions. Endogenous TLR ligands released from dead cells and cytokines such as IFNγ produced by NK cells and T cells remodel macrophages into antitumor effectors (2). These effector macrophages produce IL12 to enhance T-cell and NK cell activation and proliferation in tumors (35, 36). Effector macrophages can also cross present tumor-associated antigens to CD8+ T cells (37–39). Adenosine polarizes macrophages into a tissue healing/tumor-promoting

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Figure 4. Myeloid deletion of Adora2a increases numbers and activation of cytotoxic lymphocytes in B16F10-ova tumors. Single-cell suspensions were prepared from tumors and lymph nodes isolated from the LysMCre/Adora2a–/– mice and littermate controls shown in Fig. 1B. Frequencies of NK cells (A), CD8+ T cells (B), and CD44 expression on NK cells (C) and CD8+ T cells (D) were measured by flow cytometry. *P < 0.05; **P < 0.01; ***P < 0.001; n = 4 from one of two independent experiments with similar results. Data were analyzed using Student’s t-tests. (N > 9 from two independent experiments; ***, P < 0.001 by two-way ANOVA and Bonferroni post hoc analyses.) E, LysMCre/Adora2a–/– mice and littermate controls received depleting antibodies against CD8e or NK1.1 before and during tumor growth. Tumor sizes were measured with calipers. Results are graphed as relative tumor size in percentages for each time point and each time point was analyzed by Student’s t test. *, P < 0.05; ***, P < 0.01; ****, P < 0.001 (n ≥ 6 mice/group).
phenotype. These M2-like macrophages produce anti-inflammatory IL10 to suppress immune cells and VEGF to support angiogenesis (9, 40). Therefore, potential therapies targeting macrophages may improve our arsenal of antitumor agents.

The current study provides evidence indicating that macrophage A2ARs have great potential as therapeutic targets. It is important to note that Adora2a is expressed not only by macrophages, but also by other immune cell types, such as...
NK cells and DCs, which have only low levels of LysM promoter activity. It is well established that A2AR stimulation on DCs and NK cells regulate their activation (14). Therefore, additional studies are needed to elucidate how NK or DC-specific deletions of Adora2a will affect antitumor immune responses. Our findings demonstrate clearly that myeloid activation by targeted A2AR deletion is sufficient to strongly inhibit tumor growth and metastasis. These findings suggest that myeloid cell activation by targeted adenosine receptor blockade or by other means may be useful approaches for enhancing tumor killing by immunotherapy.

Disclosure of Potential Conflicts of Interest
J. Linden received commercial research grants from Lewis and Clark Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C. Cekic, Y.-J. Day, D. Sag, J. Linden
Development of methodology: C. Cekic, Y.-J. Day, D. Sag
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Cekic, Y.-J. Day, D. Sag
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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: C. Cekic, J. Linden
Other [created floxed A2AR mice and myeloid-specific A2AR KO mice (lysMCre-A2AAR KO)]: Y.-J. Day
Other (performed the primary phenotyping of the mice with myeloid-specific ablation of A2AAR): Y.-J. Day

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