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Myeloid Expression of Adenosine A_{2A} Receptor Suppresses T and NK Cell Responses in the Solid Tumor Microenvironment

Caglar Cekic[1,4], Yuan-Ji Day[2], Duygu Sag[3], and Joel Linden[1]

Abstract

High concentrations of adenosine in tumor microenvironments inhibit antitumor cytotoxic lymphocyte responses. Although T cells express inhibitory adenosine A_{2A} receptors ($A_{2A}R$) that suppress their activation and inhibit immune killing of tumors, a role for myeloid cell $A_{2A}Rs$ 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 *Adora2a*$^{f/f}$–LysM*Cre*$^{+/-}$ mice, which selectively lack myeloid $A_{2A}Rs$. 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 $A_{2A}Rs$ 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 A_{2A} receptors, but tumor suppression mediated by CD8$^+$ T cells is more persistent. Myeloid-selective $A_{2A}R$ 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 $A_{2A}Rs$ 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 $A_{2A}Rs$ 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 activated NK 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).

[1]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California. [2]Department of Anesthesiology, Chang Gung Memorial Hospital, Institute of Clinical Medical Science, Chang Gung University, Tauyuan, Taiwan. [3]Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, California. [4]Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey.

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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

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Adenosine exerts its effects by engaging four subtypes of P1 purinergic or adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3. A_{2A}R and A_{2B}R mRNA expression increases in activated macrophages (9–13), and signaling through A_{2A}Rs inhibits the activation of macrophages by inflammatory stimuli and promotes remodeling to an M2-like phenotype (9, 14). Prolonged A_{2A}R and A_{2B}R 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 A_{2A}R-deficient mice (7). Increased metastasis due to high expression of CD73, which elevates adenosine, is prevented by blockade of either A_{2A}Rs or A_{2B}Rs, which increase NK cell activity (16, 17). Therefore, blockade of A_{2A}Rs on NK cell and T cells (18, 19) has been viewed as an attractive strategy for enhancing immune-mediated tumor killing. Previous studies have demonstrated that T cells and NK cells are direct cellular targets for A_{2A}R-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 A_{2A}R signaling by cells that express lysozyme 2: monocytes, macrophages, and to a lesser extent, dendritic cells (DC). Our results indicate that myeloid-selective deletion of *Adora2a* strongly enhances macrophage activation, increases the number and activation of tumor-infiltrating T cells and NK cells, and inhibits tumor growth and metastasis. The results identify myeloid cell A_{2A}Rs as important targets for adenosine-mediated suppression of innate and adaptive immune responses.

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 Lewis lung carcinoma (LLC) cells were obtained from the ATCC and cultured in R5F (RPMI1640 medium containing 10% heat-inactivated FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin). The cell lines were tested and authenticated by the ATCC for post-freeze viability, growth properties, mycoplasma contamination, species contamination, and sterility. Cell lines from Caliper Life Sciences were tested for being pathogen free. Ovalbumin-expressing B16F10 cells were obtained and characterized as described in ref. 20 were provided by Dr. Stephen Schoenberger (La Jolla Institute for Allergy & Immunology, La Jolla, CA). Ovalbumin- and luciferase-expressing B16F10 cells were obtained from Dr. Andreas Limmer (University of Bonn, Bonn, Germany) and Dr. Natalio Garbi (University of Bonn) through Dr. Gerhard Wingender (La Jolla Institute for Allergy and Immunology). All these cell lines were maintained according to ATCC guidelines. Authentication of luciferase- and/or ovalbumin-expressing cell lines was based on morphology, freeze-thaw viability, adherence, 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 ovalbumin peptides by respective transgenic T cells. All cell lines were passaged less than 10 times after initial revival from frozen stocks. Cells were injected into mice after reaching 60% to 80% confluence. LysM*Cre* mice (B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J) were purchased from Jackson Laboratories. *Adora2a*$^{f/f}$ mice were generated as previously described (21) and crossed with LysM*Cre*$^{+/-}$ mice. Cells derived from these mice were characterized by quantifying Cre protein expression and A_{2A}R mRNA expression in thioglycolate-elicited peritoneal macrophages and neutrophils, and CD3$^+$ T cells were prepared using MACS columns (Miltenyi Biotec). Compared with littermate controls, *Adora2a*$^{f/f}$–LysM*Cre*$^{+/-}$ mice expressed Cre protein in most CD11b$^+$ macrophages and Ly6G$^+$ neutrophils, but not CD3$^+$ T cells (Supplementary Fig. S1A). In the same cell populations, A_{2A}R mRNA expression was reduced by 84% and 91% in macrophages and neutrophils, respectively (Supplementary Fig. S1B). Interestingly, A_{2A}R mRNA expression in peritoneal T cells was increased in mice with myeloid-selective A_{2A}R deletion, probably as a consequence of APC-mediated T-cell activation. SIINFEKL-loaded H2K^b tetramers with human β2-microglobulin were provided by NIH Tetramer Core Facility and tetramerized using streptavidin–phycoerythrin conjugates from Invitrogen according to the instructions on NIH tetramer core facility website. SIINFEKL-loaded H2K^b tetramers were used to detect ovalbumin-antigen–specific CD8$^+$ T cells. Yellow, blue, or aqua fluorescent reactive dyes were from Invitrogen. Fluorescent antibodies used in this study, their sources, and dilutions used are listed in Supplementary Table S1. Depleting CD8α and NK1.1 antibodies were purchased from BioXCell.

Ex vivo tumor cell killing by macrophages

Bone marrow–derived macrophages were prepared according to a protocol modified from Cekic and colleagues (22). Briefly, femurs and tibiae from 8- to 12-week-old mice were collected and flushed twice with sterile Hank balanced salt solution. Bone morrow 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 coincubating with B16F10 tumor cells (1:20 target to effector ratio) in 96-well round-bottom culture plates in the presence or absence of the A_{2A}R agonist CGS 21680 (1 μmol/L). 7AAD staining and CD45 staining was used to identify dead cells and myeloid cells, respectively.

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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 (Biolegend) 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 LSRII 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 $OVA_{257–264}$ (SIINFEKL) peptide (Genscript 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*$^{f/f}$–LysM*Cre*$^{-/+}$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 D-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*$^{f/f}$–LysM*Cre*$^{+/-}$ mice with myeloid-selective $A_{2A}R$ 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

LysM*Cre* 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 $A_{2A}R$ mRNA in myeloid cells. In cells from *Adora2a*$^{f/f}$–LysM*Cre*$^+$ mice, $A_{2A}R$ mRNA in macrophages and dendritic cells was reduced by 65% and 45%, respectively (Fig. 2B). $A_{2A}R$ mRNA was not detected in Gr1$^+$ cells. $A_{2A}R$ deletion from myeloid cells increased the cell surface expression of MHCII and the production of IL12 in tumor-associated macrophages (Fig. 2C). $A_{2A}R$ deletion did not significantly change expression of MHCII or IL12 in macrophages from spleen (data not shown), suggesting that locally produced adenosine within the tumor microenvironment contributes to the macrophage phenotype. 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 LysM*Cre*-mediated *Adora2a*$^{f/f}$ 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 $A_{2A}R$ deletion influences the overall cytotoxicity of macrophages, we isolated bone marrow from *Adora2a*$^{f/f}$–LysM*Cre*$^{+/-}$ animals or Cre$^-$ littermate controls and differentiated them into macrophages. Although coincubation of macrophages and tumor cells with LPS increased tumor killing, $A_{2A}R$ deletion or addition of the selective $A_{2A}R$ agonist CGS 21680 did not significantly affect the overall cytotoxic activity of macrophages (Fig. 2D). These

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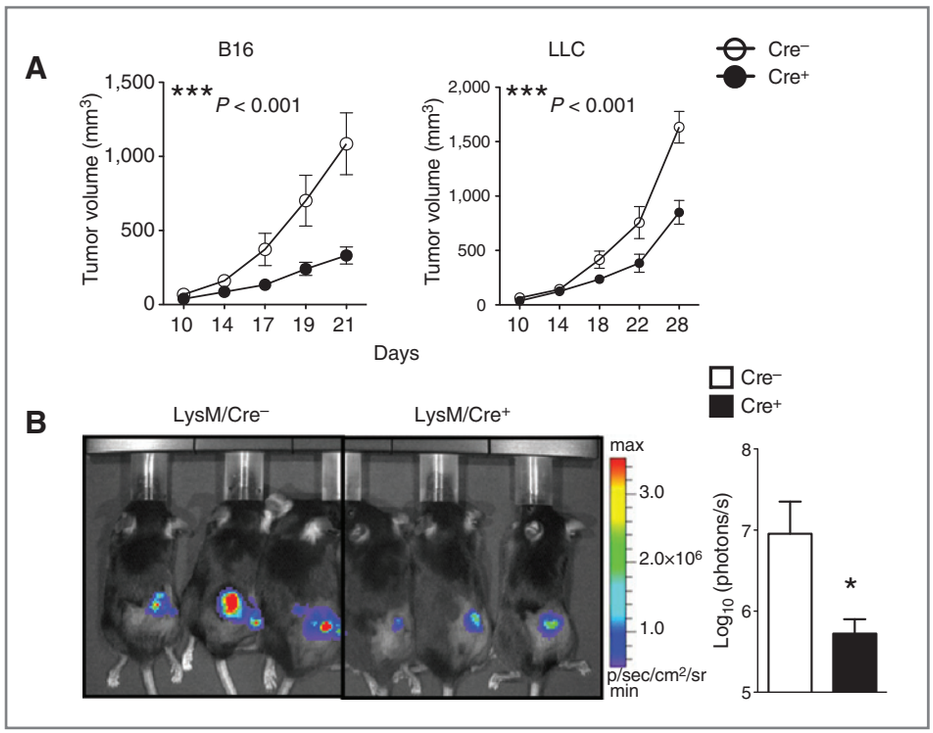

Figure 1. Myeloid deletion of *Adora2a* inhibits tumor growth. A, growth of LLC cells and B16F10 melanoma cells in mice with myeloid deletion of *Adora2a* driven by LysM/Cre$^+$ and in Cre$^-$ littermates. Tumor sizes were measured with calipers ($N > 9$ from two independent experiments; ***, $P < 0.001$ by two-way ANOVA and Bonferroni *post hoc* analyses). B, luminescence from B16F10 melanoma cells expressing luciferase 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.

findings suggest that increased cytotoxic activity of NK cells or T cells is important for increasing tumor killing upon $A_{2A}R$ blockade/deletion.

We next incubated single-cell suspensions of cells derived from tumors or spleens from mice with myeloid-selective $A_{2A}R$ 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 $A_{2A}Rs$ 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 $A_{2A}R$-dependent IL10 within tumors. IL10 mRNA is also reduced in sorted tumor-associated macrophages and DCs lacking $A_{2A}Rs$ (Fig. 3D). Overall, these data indicate that myeloid-selective deletion of A_{2A} Rs favors M1 polarization of macrophages and substantially reduces anti-inflammatory IL10 production by myeloid cell populations.

Myeloid deletion of A2ARs increases the number and activation of cytotoxic lymphocytes

$A_{2A}R$ signaling promotes tumor growth by inhibiting the activation of T cells and NK cells (7, 16, 19). The contribution of myeloid cell $A_{2A}R$ signaling to these processes is not known. LysM*Cre*-mediated deletion of *Adora2a*$^{f/f}$ 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 CD44^{hi} vs. CD44^{low} populations). To better understand the involvement of transactivation of T cells and NK cells through APCs in antitumor immune responses in LysM*Cre*/*Adora2a*$^{f/f}$, 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 LysM*Cre* 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 A_{2A} 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 $A_{2A}Rs$ 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 LysMCre$^+$ or Cre$^-$ animals (Fig. 5C). However, CD44 expression increased in tumor-infiltrating CD4$^+$ T cells isolated from *Adora2a*$^{f/f}$ *–LysMCre*$^{+/-}$ animals (Fig. 5D), suggesting that along with CD8$^+$ T cells, CD4$^+$ T cells also gain enhanced effector functions as a result of myeloid $A_{2A}R$ deletion. Therefore, we next determined whether increased CD44 expression due to LysMCre-mediated deletion of *Adora2a*$^{f/f}$ is correlated with enhanced effector functionality in T cells. Tumor-associated, but not lymphoid or splenic T cells from *Adora2a*$^{f/f}$*–LysMCre*$^{+/-}$ animals produced significantly more IFNγ after restimulation as compared with T cells

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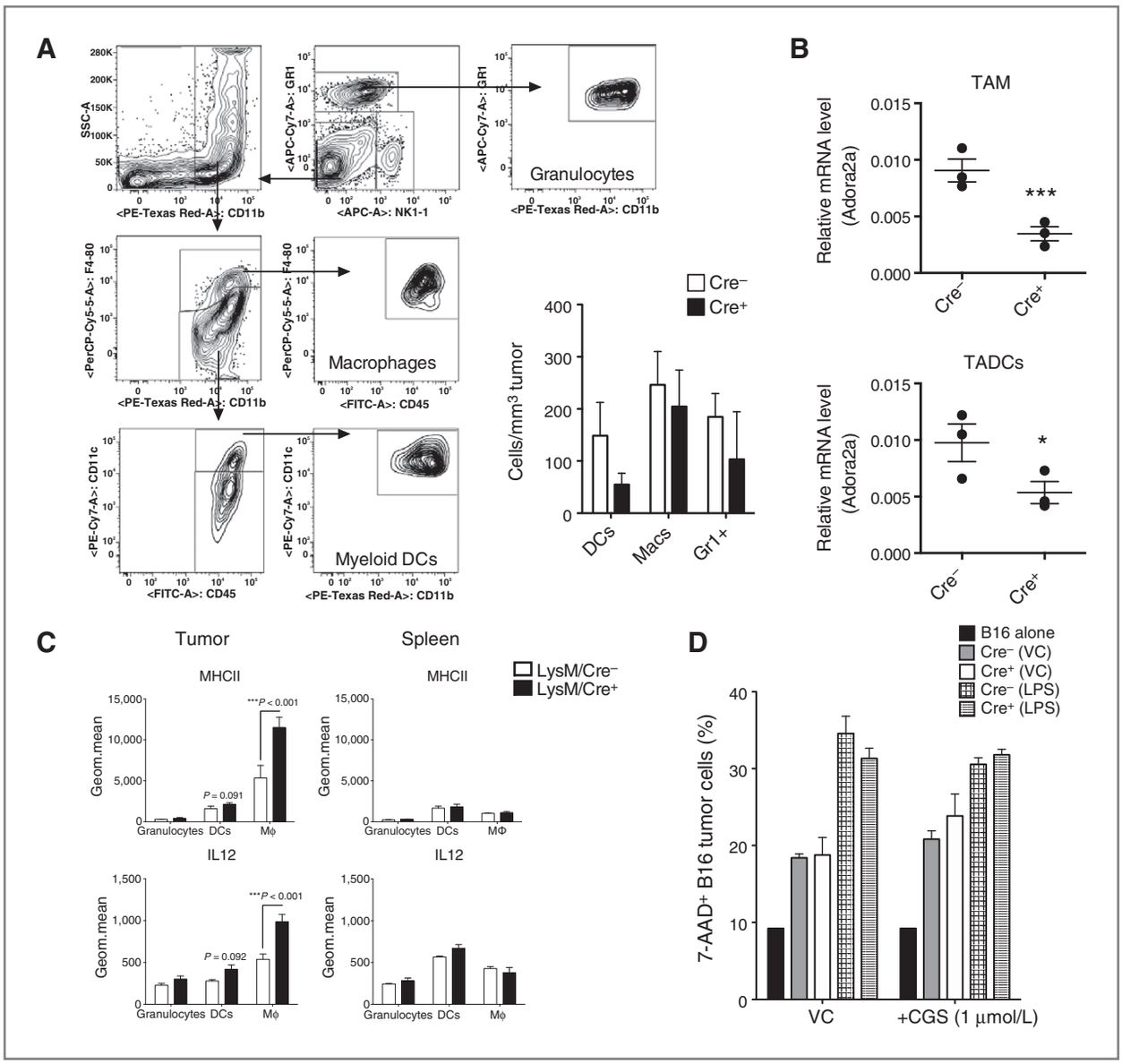

Figure 2. Myeloid deletion of *Adora2a* increases macrophage activation and effector function in B16F10-ova tumors. Single-cell suspensions from tumors and lymph nodes isolated from the LysM*Cre*$^{+}$/*Adora2a*$^{f/f}$ mice and *Cre*$^{-}$ littermate controls were prepared 3 weeks after tumor inoculation. 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 cell populations in spleen and tumors. ***, $P < 0.001$ by two-way ANOVA and Bonferroni *post hoc* analyses ($n = 4$). D, LPS prestimulated or unstimulated bone marrow–derived macrophages from LysM*Cre*$^{+}$/*Adora2a*$^{f/f}$ *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.

from *Adora2a*$^{f/f}$–*LysMCre*$^{-/-}$ littermate controls (Fig. 6). Overall, our results suggest that myeloid expression of $A_{2A}R$ 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*$^{f/f}$–*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).

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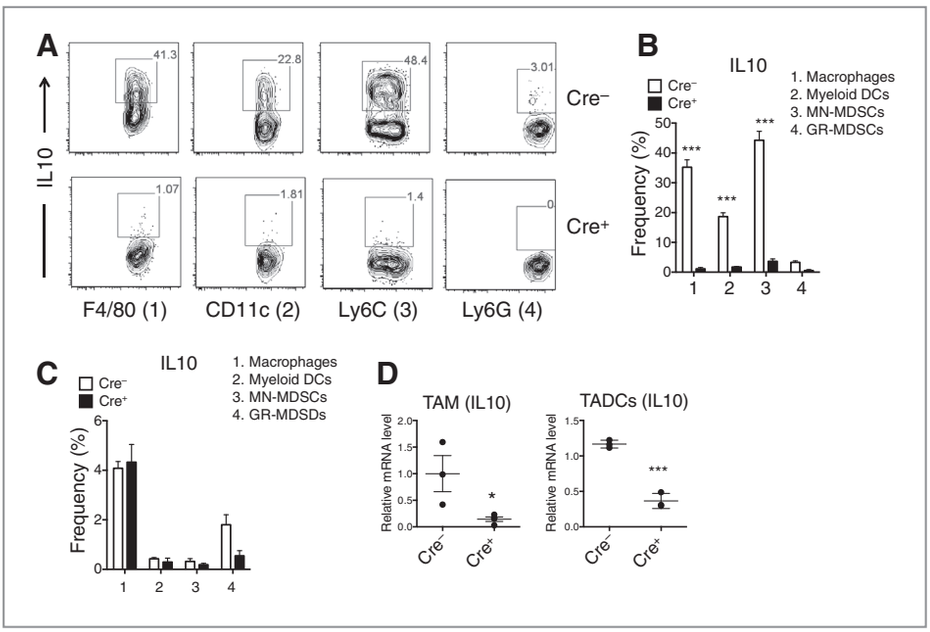

Figure 3. Myeloid expression of *Adora2a* significantly increased IL10 expression in B16F10-ova tumor-associated APCs and MDSCs. Single-cell suspensions from tumors or spleen were isolated from the LysM*Cre*$^{+}$/*Adora2a*$^{f/f}$ mice and littermate controls were prepared 3 weeks after tumor inoculation. 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 (Ly6C^{+}), or granulocytic MDSCs (Ly6G^{+}). 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.

This was associated with reduced tumor mass in the lungs (Fig. 7B) and reduced lung weight (Fig. 7C). These data indicate that myeloid cells are important targets of A_{2A}R-mediated enhanced tumor metastasis.

A_{2A}R and A_{2B}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 A_{2A}R deletion (15, 16, 19). We observed a significant increase in the numbers of lung-associated NK cells but not CD8^{+} T cells in *Adora2a*$^{f/f}$*–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 A_{2A}R expression. Overall, these data suggest that myeloid cell A_{2A}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 *Adora2a* slowed tumor growth and significantly increased activation markers, IL12 and MHCII, on macrophages without affecting *ex vivo* cytotoxicity. Myeloid-selective deletion of the 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 A_{2A}Rs 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 *Adora2a* sometimes reduces T-cell numbers and enhances the growth rate of large solid tumors. Here, we show that sparing A_{2A}Rs 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 A_{2A}R signaling in hematopoietic cells (14, 16, 28–30). The current study suggests that myeloid-selective blockade of A_{2A}R 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

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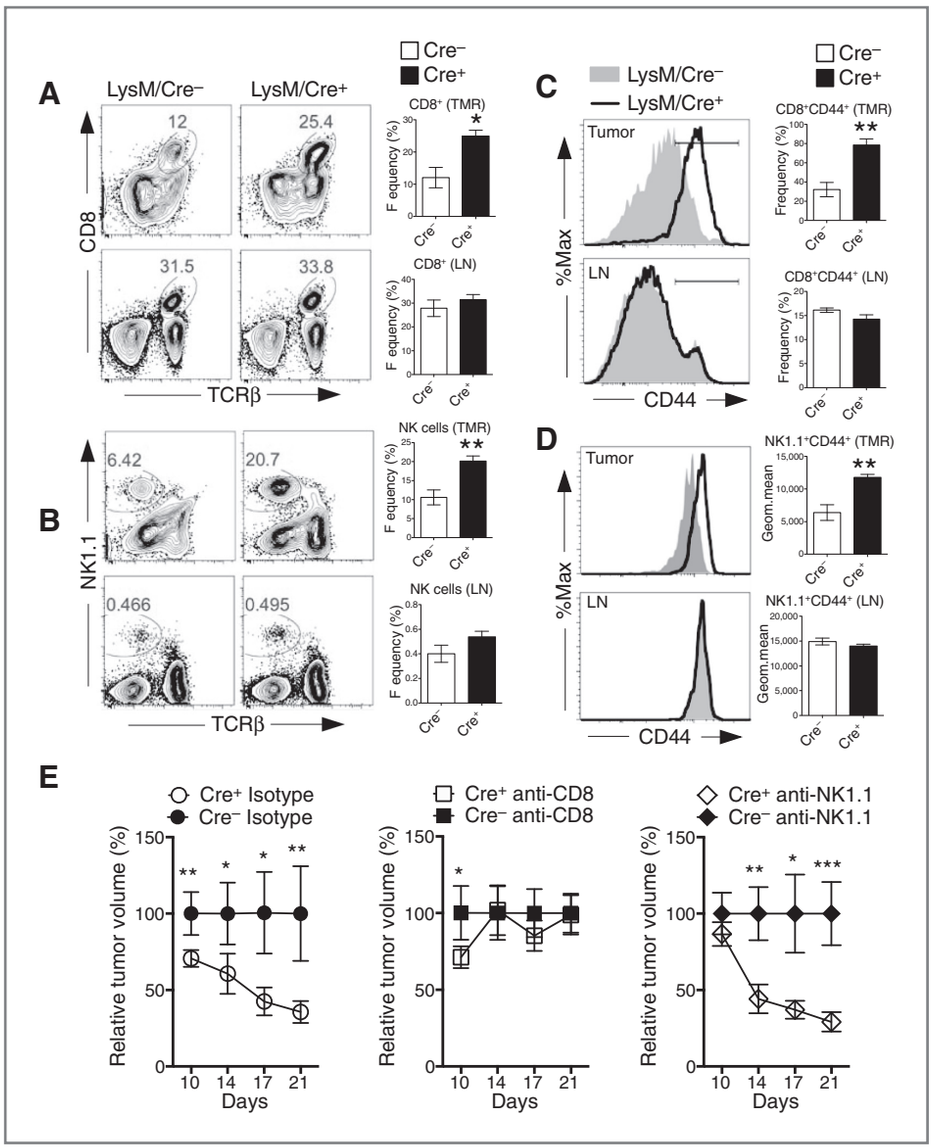

Figure 4. Myeloid deletion of *Adora2a* increases numbers and activation of cytotoxic lymphocytes in B16F10-ova tumors. Single-cell suspensions were prepared from tumors and lymph nodes isolated from the LysM*Cre/Adora2a*$^{f/f}$ mice and littermate controls shown in Fig. 1B. Frequencies of NK cells (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 *t* tests. ($N > 9$ from two independent experiments; ***, $P < 0.001$ by two-way ANOVA and Bonferroni *post hoc* analyses.) E, LysM*Cre/Adora2a*$^{f/f}$ mice and littermate controls received depleting antibodies against CD8α 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 *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n \geq 6$ mice/group).

microenvironments; thereby possibly sparing T cells from apoptosis or impaired memory differentiation that results from T-cell $A_{2A}R$ deletion. Our studies and others also suggest that cell-targeted $A_{2A}R$ blockade, the use of competitive $A_{2A}R$ antagonists, and/or the use of $A_{2B}R$ antagonists may be more effective for combating tumor growth than strong T-cell $A_{2A}R$ blockade or deletion.

Adenosine may have multiple cellular targets and engage both $A_{2A}R$s and $A_{2B}R$s to promote metastasis. Reduced $A_{2B}R$ activation was primarily responsible for decreased lung metastasis of breast tumor cells after CD73 blockade (17). Both $A_{2A}R$ and $A_{2B}R$ signaling were shown to promote metastasis of tumors highly expressing CD73 (16, 17). $A_{2B}R$ 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). $A_{2A}R$ 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 $A_{2A}R$ 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 $A_{2A}R$ 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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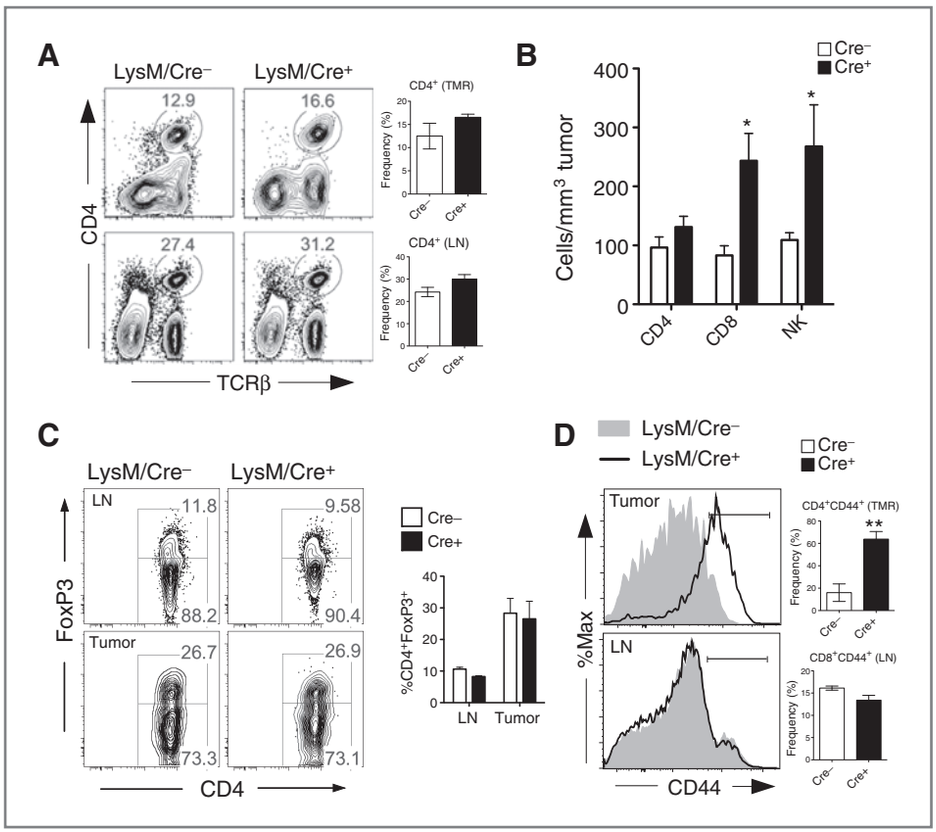

Figure 5. Myeloid deletion of *Adora2a* increases numbers and activation of T cells in B16F10-ova tumors. Single-cell suspensions from tumors and lymph nodes were isolated from the LysM/Cre-*Adora2a*$^{f/f}$ mice and littermate controls shown in Fig. 1B. Frequencies of T cells (A) and CD44 expression (B), as an indication of effector/memory differentiation, were measured by flow cytometry; $n = 4$ from one of two independent experiments with similar results. Data were analyzed using Student *t* tests. C, absolute numbers of myeloid cells, T cells, and NK cells per mm^3 of tumor were calculated using counting beads. *, $P < 0.05$; $n = 4$ from one of two independent experiments with similar results. Data were analyzed using two-way ANOVA and *post hoc* Bonferroni testing. D, intracellular staining for Foxp3 was performed to test differentiation into regulatory T cells; $n = 4$ from one of two independent experiments with similar results. Data were analyzed using two-way ANOVA and Bonferroni *post hoc* analyses.

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 A_{2A}Rs 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

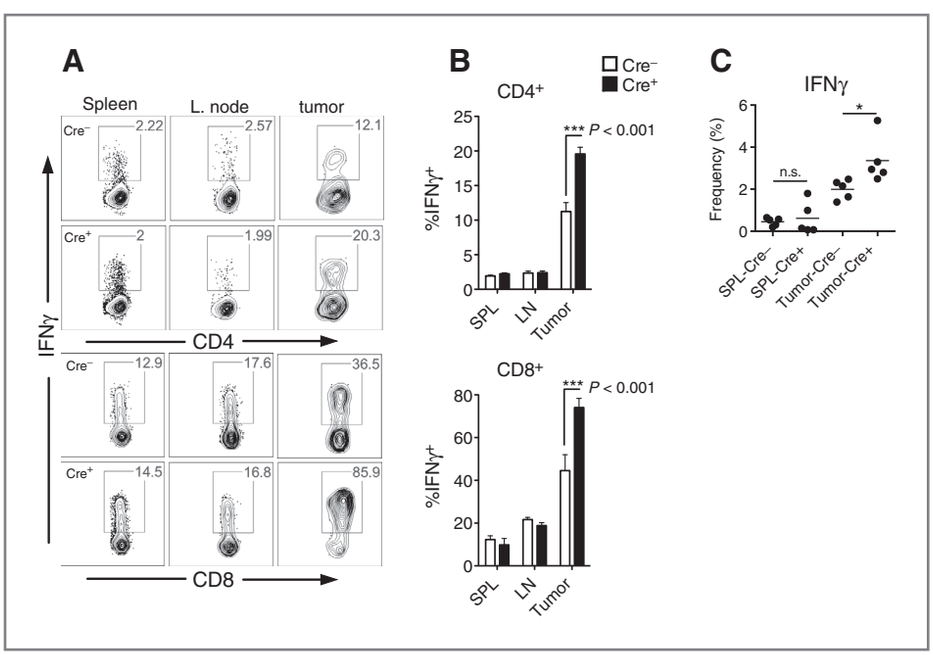

Figure 6. Myeloid deletion of *Adora2a* promotes T-cell differentiation into IFNγ-producing cells. Single-cell suspensions of cells from tumors, lymph nodes, and spleen were isolated from the LysM/Cre-*Adora2a*$^{f/f}$ mice and littermate controls shown in Fig. 1B. Cells were restimulated with PMA/ionomycin in the presence of Golgi plug for 5 hours at 37°C. A, flow cytometry analysis of intracellular staining, and bar graph and statistical analysis of IFNγ production by corresponding cell populations (B); $n = 4$; ***, $P < 0.001$ by two-way ANOVA and *post hoc* Bonferroni analyses.

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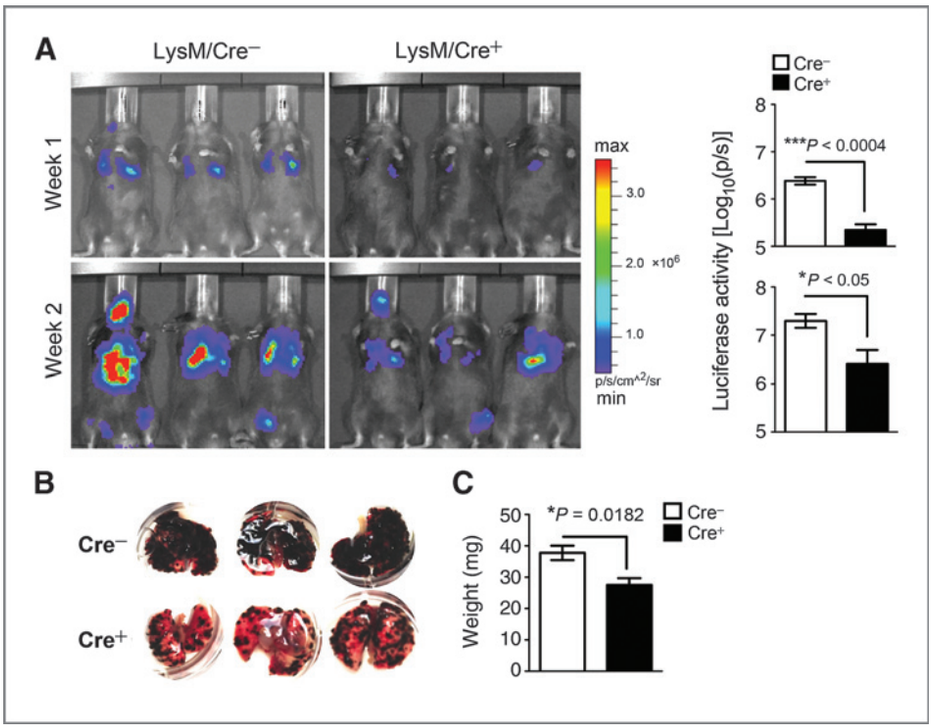

Figure 7. Myeloid deletion of *Adora2a* inhibits melanoma lung metastasis A, luciferase activity measured 1 and 2 weeks after intravenous injection of B16F10-ova-Luc cells to LysM/Cre^{+}-*Adora2a*$^{f/f}$ mice and Cre^{-} littermate controls. B, photograph of lungs from mice shown in A. C, weights of lungs isolated 2 weeks after intravenous melanoma injection ($n = 4$) from one of two independent experiments with similar results. Data were analyzed using the Student *t* test.

NK cells and DCs, which have only low levels of LysM promoter activity. It is well established that $A_{2A}R$ 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
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Cekic, D. Sag, 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: C. Cekic, J. Linden
Other [created floxed A2AAR mice and myeloid-specific A2AAR 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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