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

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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 Adora2adrLysMCre+/− 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.
Adenosine exerts its effects by engaging four subtypes of P1 purinergic or adenosine receptors: A1, A2A, A2B, and A3. A2A-R and A3-R mRNA expression increases in activated macrophages (9–13), and signaling through A2A-R inhibits the activation of macrophages by inflammatory stimuli and promotes remodeling to an M2-like phenotype (9, 14). Prolonged A2A-R and A3-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 A3-R-deficient mice (7). Increased metastasis due to high expression of CD73, which elevates adenosine, is prevented by blockade of A2A-Rs or A2B-Rs, which increase NK cell activity (16, 17). CD73, which elevates adenosine, is prevented by blockade of A2B 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.

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 Cell Line Core Facility and tetramerized using streptavidin–phycoerythrin conjugates from Invitrogen according to the instructions on NIH tetramer core facility website. SIINFEKL-loaded H2Kb tetramers were used to detect ovalbumin-antigen-specific 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 CD8+ 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 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 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 A3-R 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 × 10^7) 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 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 × 10^6 LLC or 10^5 B16F10 melanoma cells expressing luciferase into the right flanks of Adora2a^−/−/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 α-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^−/−/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^−/−/LysMcRe^−/− mice, A2AR mRNA 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 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 LysMcRe-mediated Adora2a^−/− 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^−/−/LysMcRe^−/− animals or Cre− littermate controls and differentiated them into macrophages. Although coinubation 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).
The findings suggest that increased cytotoxic activity of NK cells or T cells is important for increasing tumor killing upon A2AR blockade/deletion.

We next incubated single-cell suspensions of cells derived from tumors or spleens from mice with myeloid-selective A2AR 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 A2ARs is reduced by more than 90% in tumors, but is not significantly reduced in splenic myeloid cells. Interestingly, monocyotic (Ly6C+) rather than granulocytic (Ly6G+) MDSCs are the main producers of A2AR-dependent IL10 within tumors. IL10 mRNA is also reduced in sorted tumor-associated macrophages and DCs lacking A2ARs (Fig. 3D). Overall, these data indicate that myeloid-selective deletion of A2AR 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

A2AR signaling promotes tumor growth by inhibiting the activation of T cells and NK cells (7, 16, 19). The contribution of myeloid cell A2AR signaling to these processes is not known. LysMCre-mediated deletion of Adora2af/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 CD44HI vs. CD44LO populations). To better understand the involvement of transactivation of T cells and NK cells through APCs in antitumor immune responses in LysMCre/Adora2af/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 LysMCre 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 A2ARs 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 A2ARs 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+ and Cre− animals (Fig. 5C). However, CD44 expression increased in tumor-infiltrating CD4+ T cells isolated from Adora2af/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 A2AR deletion. Therefore, we next determined whether increased CD44 expression due to LysMCre-mediated deletion of Adora2af/f is correlated with enhanced effector functionality in T cells. Tumor-associated, but not lymphoid or splenic T cells from Adora2af/f−LysMCre−/− animals produced significantly more IFNγ after restimulation as compared with T cells
from Adora2af/f–LysMcKO−/− 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 Adora2af/–LysMcKO−/− mice or Cre KO 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).
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 A2ARs 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 A2ARs 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 A2AR signaling in hematopoietic cells (14, 16, 28). The current study suggests that myeloid-selective blockade of 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...
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 A2AR 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 A3Rs to promote metastasis. Reduced A2AR activation was primarily responsible for decreased lung metastasis of breast tumor cells after CD73 blockade (17). Both A2AR and A3R signaling were shown to promote metastasis of tumors highly expressing CD73 (16, 17). A3R 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 A2AR 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 A2AR 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 antimacrophages and TCRαβ+CD44+ cells were prepared from tumors 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+/Adora2αf/f mice and littermate controls received depleting antibodies against CD8 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 ≥ 6 mice/group).
Myeloid Adenosine Receptors Control Cytotoxic Lymphocytes

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<sup>f/f</sup> 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 using 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<sup>3</sup> 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.

Myeloid Adenosine Receptors Control Cytotoxic Lymphocytes

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<sub>2A</sub>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...
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.

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Other [created floxed A2AR mice and myeloid-specific A2AAR KO mice (lysMCre-A2AAR KO)]; Y.-J. Day
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