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Adenosine A2B Receptor Blockade Slows Growth of Bladder and Breast Tumors

Caglar Cekic,* Duygu Sag,* Yuesheng Li, † Dan Theodorescu, ‡ Robert M. Strieter, † and Joel Linden*

The accumulation of high levels of adenosine in tumors activates A2A and A2B receptors on immune cells and inhibits their ability to suppress tumor growth. Deletion of adenosine A2A receptors (A2AARs) has been reported to activate antitumor T cells, stimulate dendritic cell (DC) function, and inhibit angiogenesis. In this study, we evaluated the effects of intermittent intratumor injection of a nonselective adenosine receptor antagonist, aminophylline (AMO; theophylline ethylenediamine) and, for the first time to our knowledge, a selective A2BAR antagonist, ATL801. AMO and ATL801 slowed the growth of MB49 bladder and 4T1 breast tumors in syngeneic mice and reduced by 85% metastasizes of breast cancer cells from mammary fat to lung. Based on experiments with A2AR−/− or adenosine A2B receptor−/− mice, the effect of AMO injection was unexpectedly attributed to A2BAR and not to A2AAR blockade. AMO and ATL801 significantly increased tumor levels of IFN-γ and the IFN-inducible chemokine CXCL10, which is a ligand for CXCR3. This was associated with an increase in activated tumor-infiltrating CXCR3+ T cells and a decrease in endothelial cell precursors within tumors. Tumor growth inhibition by AMO or ATL801 was eliminated in CXCR3−/− mice and this effect was attributed to A2BAR and not to A2AAR expression on bone marrow cells is required for the antitumor effects of AMO. The data suggest that blockade of A2BARs enhances DC activation and CXCR3-dependent antitumor responses. The Journal of Immunology, 2012, 188: 198–205.

Production of adenosine is one mechanism that tumors employ to suppress immune surveillance (1). Exosomes from diverse cancer cell types exhibit potent ATP- and 5′-AMP phosphohydrolytic activity, partly attributed to exosomally expressed CD39 and CD73 that together increase extracellular adenosine production (2). Adenosine binds to four G protein-coupled receptors, A1, A2A, A2B, and A3, which are expressed in most tissues and cancer cells (3–5). Activation of host adenosine A2AAR or A2BAR receptors has been found to inhibit rejection of solid tumors and promote metastasis (5–7). Deletion of the adenosine A2AAR receptor (A2AAR) has been shown to enhance immune killing of certain immunogenic tumors in association with activation of CD8+ T cells that express inhibitory A2AARs (6). Adenosine A2B receptor (A2BAR) activation is thought to support tumor growth by stimulating the release of angiogenic factors from vascular smooth muscle, endothelial cells, and host immune cells (7–9). The growth of Lewis lung tumors was slowed in mice lacking A2BARs, and this effect was attributed to A2BAR-dependent secretion of vascular endothelial growth factor from host immune cells (7).

We evaluated aminophylline (AMO; theophylline ethylenediamine) as a nonselective adenosine receptor antagonist because it is available for clinical use (10, 11). We also evaluated the nonclinical compound ATL801 [N-(5-(1-cyclopropyl-2,6-dioxo-3-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)pyrindin-2-yl)-N-ethyl-6-nicotinamide], because it is a selective and bioavailable A2BAR antagonist. The most potent biological actions of AMO are nonselective blockade of adenosine receptors. Ki values of AMO for binding to recombinant human adenosine receptor subtypes are (μM): A1, 6.2; A2A, 4.2; A2B, 9.2; and A3, 52.3 (12). Hence, AMO blocks A1, A2A, and A2B receptors with similar affinities. We show that infrequent (three times weekly) intratumor injection of AMO or ATL801 block A2BAR to inhibit the growth of bladder cancer cells and the growth and metastasis from mammary fat to lung of breast cancer cells in syngeneic mice. A2BAR blockade inhibits MB49 growth by a process associated with the activation of CD11b+ dendritic cells (DCs) and requiring T cells and CXCR3. The data suggest that A2BAR blockade amplifies DC activation resulting in increased production of the IFN-γ-inducible CXCR3 chemokine CXCL10 that is chemotactic and activating to lymphocytes and elicits angiostatic effects within tumors (13).

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 and Immunology. MB49 bladder carcinoma cells were derived from C57BL/6 mice and obtained from American Type Culture Collection. Metastatic breast cancer cells (4T1-12B) derived from BALB/c mice and modified to express firefly luciferase from Tao et al. (14) were purchased from Tufts University (Boston, MA). Cancer cells were cultured in R10F (RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin). Cells were injected into mice after reaching 60–80% confluency. Six-week-old C57BL/6 or BALB/c
mice were purchased from The Jackson Laboratory and used for experiments after being acclimated for 2–6 wk. A2AAR−/−, A2BAR−/−, mice lacking both A2A and A2B receptors (A2A_AR−/−, CXCR3−/−, and RAG1−/−; mice were bred in the La Jolla Institute for Allergy and Immunology Vivarium. AMO was from Sigma-Aldrich. The selective A2BAR antagonist ATL801 (15) was a gift from Dogwood Pharmaceuticals, a wholly owned subsidiary of Forest Labs. Fluorescent Abs were purchased from eBioscience except for PE-AF610–labeled CD4 Ab, which was from Invitrogen, and Pacific Blue-labeled CD45, which was from BioLegend. Cytofix/Cytoperm intracellular staining kit was from BD Biosciences.

Establishment of solid tumors

We injected 10^4 4T1-12B breast cancer cells into the mammary fat pads of female wild-type (WT) BALB/c mice or 10^5 MB49 bladder carcinoma cells s.c. into the right flanks of female C57BL/6 WT mice. Intratumor injections of 200 μM AMO or 1 μM ATL801 in 100 μl PBS were performed twice over 1 wk. Compounds were injected s.c. into the same site where tumors were inoculated. PBS alone was used as vehicle control for AMO. PBS containing 0.01% DMSO was used as vehicle control for ATL801. AMO had no effect on the growth of tumor cells in vitro. For AMO, PBS containing 0.01% DMSO was used as vehicle control for AMO. For AMO. PBS containing 0.01% DMSO was used as vehicle control for ATL801. AMO had no effect on the growth of tumor cells in vitro. For AMO. PBS containing 0.01% DMSO was used as vehicle control for ATL801. AMO had no effect on the growth of tumor cells in vitro. For AMO.

Bone marrow transplantation

C57BL/6 recipient mice (6 wk old) were fasted for 24 h and then lethally irradiated (10^7 cGy). After the second radiation exposure, 5 × 10^5 bone marrow (BM) cells from donor C57BL/6 mice (WT, A2AAR−/−, or CXCR3−/−) were injected i.v. Mice were treated with antibiotics from 3 d before until 2 wk after radiation. Tumor cells were injected 7 to 8 wk after BM transplantation.

Flow cytometry

Tumors were removed and used to prepare single-cell suspensions by sequential pressing through 100- 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). Cells (3–5 × 10^6) were preincubated for 10 min in 100 μl FACS buffer with Ab to block Fc receptors. Each sample tube received 100 μl fluorescein labeled Ab mixture and was incubated for 30 min at 4°C in the dark. For intracellular cytokine staining, cells were restimulated with 10 ng/ml PMA, 100 ng/ml ionomycin (Sigma-Aldrich), and Golgi Plug (eBioscience) for 5 h at 37°C. Cells were fixed and permeabilized after surface staining and incubated for 25 min at 4°C in 100 μl permeabilization/washing buffer containing 1:100 PerCP-Cy5.5-labeled anti–IFN-γ. After a subsequent wash, cells were resuspended in 350 μl FACS buffer. Cells were analyzed using an LSR II equipped with four lasers and FACS Diva software (BD Biosciences). Live/dead fixable yellow (Invitrogen) was used to exclude dead cells before analysis. Flow cytometry data were analyzed using FlowJo software (9.0.1 version; Tree Star).

Quantitative real-time PCR

Tumors were processed as described above, and equal numbers of cells were lysed in guanidine thiocyanate buffer containing 10 μl/ml 2-ME. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the iScript cDNA SuperMix (Quanta). Quantitative RT-PCR (qPCR) was performed using the PerfeCta SYBR-Green SuperMix for iQ (Quanta) and quantiTec primers (Qiagen) for CXCR3 chemokines and IFN-γ. Reactions were run on MyiQ single color real-time PCR detection system (Bio-Rad). Expression of each target gene was normalized to GAPDH, and fold expression over vehicle control was calculated using the 2^−ΔΔCt method (16).

Statistical analysis

All statistical analyses and graphics used Prism software (GraphPad). Two-way ANOVA with Benferroni post hoc tests (time/treatment) was performed to analyze growth curves.

Results

AMO inhibits the growth of bladder cancer cells

MB49 bladder tumor cells were inoculated s.c. into syngeneic C57BL/6 mice. AMO was administered intermittently (three times weekly) at the site of tumor inoculation or into the tumor cell mass beginning either on the day of tumor cell inoculation (Fig. 1A–C) or 9 d later (Fig. 1D). Intratumor AMO markedly slowed tumor growth in WT mice but not in A2A_AR−/− mice (Fig. 1B). This finding indicates that AMO targets A2A and/or A2B receptors in mice and does not have any direct effect on the growth of MB49 cells. Although AMO blocks A1, A2A, and A2B receptors with similar affinities, the elimination of the effects of AMO to slow tumor growth in A2A_AR−/− mice suggests that A1AR blockade does not contribute to the effects of AMO on tumor growth. These findings are consistent with low expression of A1ARs on cells of the immune system. These findings also rule out the possibility that the effects of AMO are mediated by some action not involving adenosine receptor blockade. It is notable that acute AMO treatment slows tumor growth more effectively than A2A_AR−/− deletion (Fig. 1C). This may reflect an adaptive response by the mouse immune system to persistent adenosine receptor deletion and suggests that receptor deletion may not be a good surrogate for acute pharmacological receptor blockade.

In the clinical setting, most tumors are detected and treated only when tumor cells have proliferated enough to produce a detectable mass. Hence, to evaluate the clinical relevance of adenosine receptor blockade, AMO was injected into MB49 tumors 9 d after implantation of tumor cells, at which time the average size of tumors was between 150 and 250 mm^3. As shown in Fig. 1D, intratumor injection of AMO effectively slowed the growth of established bladder tumors.

Suppression of tumor growth by AMO is associated with T cell activation

Antitumor immune responses have been associated with enhanced intratumor expression of IFN-γ and IFN-γ–inducible CXCR3 chemokines that are chemotactic to lymphocytes and elicit...
angiostatic effects (13). Activation of the $A_{2A}$AR is thought to facilitate tumor survival by disinhibiting effector functions of activated T cells that express inhibitory $A_{2A}$ARs (3, 6). $A_{2B}$AR signaling is not thought to influence T cells directly. Activation of the $A_{2B}$AR may promote tumor survival by stimulating angiogenesis (8) or by inhibiting DC maturation and Ag presentation (17, 18). Hence, we reasoned that AMO treatment might directly and indirectly enhance T cell responses by virtue of blocking $A_{2A}$ and/or $A_{2B}$ receptors on T cells and DCs, respectively. We performed flow cytometric analysis of tumor-associated lymphocytes. AMO significantly increased CD69 (Fig. 2A) and IFN-γ (Fig. 2B) expression in tumor-associated CD4+ and CD8+ T cells. T cells isolated from MB49 tumors grown in $A_{2A}$&$A_{2B}$AR−/− mice also expressed high CD69 and IFN-γ, similar to WT mice treated with AMO. Moreover, AMO had no effect on the already activated lymphocytes derived from tumors grown in $A_{2A}$&$A_{2B}$AR−/− mice. Nor did AMO have any effect on the rate of MB49 tumor grown in RAG1−/− mice that lack mature T and B cells (Fig. 2C). The results in RAG1−/− mice rule out the possibility that AMO prevents tumor growth by direct antiangiogenic effects on the vasculature; rather, the effects of AMO to slow the growth of bladder tumors requires the participation of T cells. It is possible that T cell activation depends on other cells that participate in their activation, such as Ag-presenting DCs. To test this possibility, we examined the effects of ATL801 on the activation of NK cells and DCs in MB49 tumors of RAG1−/− mice. As shown in detail in Supplemental Fig. 1, ATL801 had no effect on the activation of NK cells or CD11b+ DCs, but significantly increased the activation of CD11b− DCs, which include subtypes that capture dying cells and cross present Ags to both CD4+ and CD8+ T cells (19). Hence, both CD4 and CD8 T cell activation in response to $A_{2B}$AR blockade may be secondary to CD11b− DC activation.

**Tumor growth inhibition by AMO is CXCR3 dependent**

IFN-γ induces IFN-inducible CXCR3 chemokines, which attract effector T cells to the tumor microenvironment and inhibit angiogenesis (20). To determine if AMO treatment increases expression of IFN-γ and IFN-γ-inducible chemokines, we used quantitative real-time PCR to quantify mRNAs for IFN-γ and CXCR3 chemokines in MB49 tumors that had received three injections of vehicle or AMO beginning 9 d after tumor cell inoculation. Fig. 3A shows that AMO significantly increased transcription within MB49 tumors of IFN-γ and the IFN-γ-inducible chemokine CXCL10, but not CXCL9 or CXCL11. We examined tumor lymphocytes by flow cytometry in the same tumors. AMO significantly increased the percentage of tumor CD8 and CD4 T cells that are CXCR3+, but did not increase the percentage of CXCR3+ NKT or NK cells (Fig. 3B). These data are consistent

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**FIGURE 2.** Adaptive immune responses are required for tumor growth inhibition by AMO. CD69 (A) and intracellular IFN-γ (B) expression were evaluated by flow cytometry to detect the activation status of T cells from PBS- or AMO-treated MB49 tumors grown for 3 wk in WT animals. Data are pooled from three independent experiments, each with three to four mice per treatment group. C, MB49 bladder carcinoma cells (10⁷) were inoculated s.c. in the right flank of RAG1−/− mice. Intratumor injections of 200 μM AMO in 100 μl PBS were performed three times weekly starting from day 2 or 3 after tumor cell injection. n = 13. *p < 0.05, **p < 0.01.
with the idea that the CXCL10 that accumulates in tumors injected with AMO is chemotactic to CXCR3+ T lymphocytes. As can be seen in Fig. 3C, chemotaxis of CXCR3+ T cells appears to be required for antitumor activity because AMO has no effect on tumor growth in CXCR3−/− mice. Overall, these observations suggest that AMO enhances the production of IFN-γ and CXCL10 and enhances tumor killing by a CXCR3-dependent processes.

Tumor growth inhibition by AMO is due to A2BAR blockade

We next sought to determine the relative contributions of A2A and A2B receptor blockade by AMO on the rate of MB49 tumor growth. Fig. 4 shows that, surprisingly, the effect of AMO on MB49 tumor growth is completely abrogated in A2BAR−/− mice. The deletion of the A2A receptor does not decrease the rate of tumor growth, and the effect of AMO to slow tumor growth was preserved in A2AR−/− mice. These data indicate that AMO slows MB49 tumor growth predominantly by blocking A2B receptors.

AMO inhibits tumor growth by effects on BM-derived cells

The A2BAR is expressed on vascular smooth muscle and endothelial cells, as well as on hematopoietic cells (4). As noted above, CD11b+ DCs are activated by A2BAR blockade, and activation of DCs may be responsible for increasing the production of IFN-γ by CD4+ and CD8+ T cells and downstream production of the CXCR3 chemokine CXCL10. On this basis, we hypothesized that blockade of A2BARs on hematopoietic cells is required for tumor growth inhibition by AMO. CXCR3 is expressed by both endothelial and hematopoietic cells (20) and can directly inhibit the growth of endothelial cells by a signaling pathway that is partly CXCR3 dependent (21, 22). To determine if AMO acts by signaling through CXCR3 on immune or nonimmune cells, we transferred BM from WT or CXCR3−/− donors to lethally irradiated WT recipients. Seven to 8 wk after BM transplantation, mice were inoculated with MB49 cancer cells and treated with either AMO or PBS. As shown in Fig. 5A, AMO significantly inhibited tumor growth in recipient mice that received WT marrow but failed completely to inhibit tumor growth in mice lacking CXCR3 only in BM cells. In a similar set of experiments, we found that targeted deletion of the A2BAR only in BM cells also completely prevented the effect of AMO to reduce tumor growth (Fig. 5A). These results indicate that A2BARs and CXCR3 found on BM cells are required for AMO-mediated suppression of tumor growth.

AMO inhibits tumor growth by effects on cells

A2BAR receptor activation is known to stimulate the release of angiogenic factors from myeloid cells (20). In addition, chemokines that activate CXCR3 receptors are known to produce angiostatic responses, suggesting that CXCR3 activation can reduce the infiltration of vascular endothelial precursors in tumors. Therefore, we hypothesized that A2BAR blockade on immune cells would decrease tumor angiogenesis. As can be seen in Fig 5B, flow cytometric analysis revealed that AMO significantly reduces CD34+CD45+ vascular endothelial precursor cells in tumors grown in mice reconstituted with WT but not A2BAR−/−

**FIGURE 3.** AMO activates the IFN-γ-CXCR3 signaling pathway to inhibit tumor growth. A and B, Nine days after inoculation of 10^5 MB49 bladder cells, tumors were injected three times with PBS or 200 μM aminophylline in 100 μl PBS on days 9, 11, and 14. On day 15, tumors were harvested and assayed for mRNA expression or FACS analysis. A, Relative transcript levels of IFN-γ and IFN-inducible chemokines CXCL9, CXCL10, and CXCL11 were detected by qPCR. B, Frequencies of CXCR3+ lymphocyte populations were detected by flow cytometry. C, MB49 bladder carcinoma cells (10^5) were inoculated s.c. in the right flank of CXCR3−/− mice and treated with intratumor injections of 200 μM aminophylline in 100 μl PBS three times weekly. n = 7. *p < 0.05, **p < 0.001.
or CXCR3−/− BM-derived cells. These findings suggest that AMO inhibits tumor growth in part increasing the release of angiostatic factors from immune cells.

Selective A2BAR blockade with ATL801 inhibits bladder cancer growth in a CXCR3-dependent manner

AMO is a nonselective antagonist of A1, A2A, and A2B receptors. The results of experiments in which A2A or A2B receptors were selectively deleted suggest that the A2BR is the primary target of AMO. To test this further, we evaluated a novel A2BR-selective antagonist ATL-801. As shown in Fig. 6, the selective A2BAR blocker ATL801 significantly slows by 50% MB49 growth in WT or A2AAR−/− mice but not in A2BAR−/− mice. We also tested the effects of selective A2BAR blockade on established tumors. As can be seen in Fig. 7A, ATL801 significantly inhibited the growth of established tumors, and this was associated with a significant increase in IFN-γ, CXCL10 transcript levels, and numbers of CXCR3+ T cells. ATL801 also slightly increased the fraction of CXCR3+ NK cells, but this effect was not statistically significant. The antitumor effects of ATL801 were absent in RAG1−/− or CXCR3−/− mice, although there was a slight insignificant inhibition of tumor growth in RAG1−/− mice that could be related to enhanced infiltration of CXCR3+ NK cells. Overall, these results suggest that selective A2BR blockade by AMO and ATL801 produces similar tumoricidal immunological response in bladder tumors.

AMO and ATL801 inhibit the growth and spontaneous metastases of 4T1 breast cancer cells from mammary fat to lung

As a second cancer model, we examined 4T1-12B breast cancer cells expressing luciferase because: 1) unlike MB49, they can easily be inoculated into their natural niche (mammary fat pads); 2) they can spontaneously metastasize to the lungs as in aggressive human breast cancers; 3) their metastasis can be quantitatively detected by luminescence and; and 4) their genetic background, BALB/c, differs from the genetic background of MB49 cells, C57BL/6. We injected 4T1-12B cells into syngeneic BALB/c recipients. AMO produced a 40% decrease in the rate of breast tumor growth (Fig. 8A, top panel). Because A2BAR−/− mice on a BALB/c background are not available, we tested the effects of selective A2BAR blockade on tumor growth using ATL801. Intratumor injection ATL801 produced a reduction in the rate of tumor growth similar to what is observed after AMO administration (Fig. 8A, bottom panel). These findings suggest that A2BAR blockade is primarily responsible for inhibition of the growth of not only MB49 bladder cells but also 4T1 breast tumor cells. In addition, AMO reduced by >85% the number of luciferase+ 4T1-12B tumor cells that spontaneously metastasized to the lung as detected by bioluminescence (Fig. 8B).

Discussion

The current study demonstrates for the first time, to our knowledge, that selective intratumoral A2BR blockade can reduce the growth rate of two syngeneic cancers, MB49 bladder and 4T1 breast.
A2B receptor blockade within primary mammary fat tumors also inhibits metastasis of breast cancer cells to the lung. The efficacy of intratumoral injection is important because this route of administration greatly attenuates side effects that can occur as a result of systemic administration of adenosine receptor blockers.

Previous studies have established that adenosine accumulates in solid tumors to high concentrations, stimulates angiogenesis, and inhibits Th1 cytokine production, adhesion of immune cells to endothelial cells, and the activation of T cells, macrophages, and NK cells (23). Activation of A2B receptors on myeloid cells and DCs in vitro was shown to enhance secretion of vascular endothelial growth factor and suppresses DC maturation, resulting in increased tumor angiogenesis, suboptimal tumor Ag presentation, and incomplete maturation of effector T cells (18). The transfer of A2BAR-stimulated DCs into tumors was found to increase tumor growth (17).

We found that intermittent intratumoral injection of the nonselective adenosine receptor antagonist AMO or the A2BAR selective antagonist ATL801 reduces the growth of bladder and breast cancers in syngeneic immunocompetent mice. The use of mice lacking A2AARs, A2BARs, or both indicates that the effects of intermittent intratumoral injection of AMO or ATL801 on the growth of MB49 and 4T1 tumors are primarily mediated by host A2BARs on BM-derived immune cells. The results suggest that A2BAR blockade increases tumor Ag cross presentation by DCs, probably to enhance killing of tumors by cytotoxic agents.

Effects of A2A receptor blockade on tumor growth

A2AAR deletion has been previously reported to inhibit the growth of strongly immunogenic melanomas, and this effect was attributed to deletion of inhibitory A2AARs on CD8+ T cells (6). In the current study, A2AAR deletion had no significant effect on the growth of bladder tumors or on the antitumor effect of AMO. This may indicate that A2AAR blockade is most effective in highly immunogenic tumors. By contrast, the primary effect of A2BAR blockade appears to be on CD11b+ DCs, probably to enhance tumor Ag presentation and cytokine-mediated T cell activation. We found that the effects of the nonselective antagonist AMO are completely blocked in mice lacking A2BARs, but only partially blocked in mice lacking A2AARs. These findings indicate that in bladder and breast cancer models and possibly in general, A2BAR blockade may prove to be more effective than A2AAR blockade for inhibiting tumor growth. These findings suggest that either selective A2BAR blockers such as ATL801 or nonselective A2A and A2B adenosine receptor blockers such as AMO might be more useful as antitumor drugs than selective A2AAR blockers. It will be interesting to determine in future studies if A2BAR blockade enhances killing of tumors by cytotoxic agents.

Regulation of A2BAR expression

The A2BAR promoter contains a hypoxia-inducible factor-1α (HIF-1α) response element that drives expression of the receptor in hypoxic cells including endothelial and epithelial cells (24) and DCs (25). Specific deletion of HIF-1α on myeloid cells can delay spontaneous tumor growth and enhance adaptive immune responses (26). It is possible that deletion of HIF-1α acts in part by reducing the expression of immunosuppressive A2BARs. This
The idea is consistent with our findings of tumor suppression mediated by blockade of A2B ARs that may be upregulated in DCs in a hypoxic tumor environment.

A2B AR receptors on myeloid and tumor cells

By taking advantage of C57BL/6 mice lacking A2A or A2B adenosine receptors, we were able to demonstrate that host and not tumor cell adenosine receptors are the targets through which AMO inhibits bladder tumor growth in the current study. Because BALB/c mice lacking A2B ARs are not available, we were not able to genetically define the receptor targets of AMO in 4T1 tumors on a BALB/C genetic background, but experiments with ATL801 suggest that A2B AR blockade is also important for inhibition of the growth and metastasis of breast cancer cells. We cannot rule out direct effects of A2B AR blockade on breast cancer cells. In fact, A2B ARs have been reported in 4T1 breast cancer cells as well as colorectal carcinoma and T24 bladder carcinoma (1, 5, 27). Primary colorectal carcinoma cells upregulate A2B AR mRNA expression under hypoxic conditions and undergo apoptosis upon A2B AR blockade (1). A2B AR activation on 4T1 breast cancer cells has been shown to increase their metastatic potential (5), which may contribute to strong inhibition of metastasis by AMO despite its modest effect on slowing primary tumor growth (Fig. 8). A2B AR activation on human T24 bladder cancer cells increases cAMP, Ca2+ accumulation, and IL-8 production in vitro, which can be prevented by theophylline or selective A2B AR antagonist (27). Therefore, in 4T1 breast cancer cells and some other types of tumors, A2B AR blockade may directly slow tumor growth and metastatic potential in addition to enhancing host antitumor immune responses.

Biological effects of methylxanthines

AMO is a complex of theophylline and ethylenediamine with high aqueous solubility. Theophylline is one of many similar methylxanthines, including caffeine and theobromine, that primarily exert their biological effects by blockade of adenosine receptors, but that also share weak activity as inhibitors of cAMP phosphodiesterase. Theophylline has long been used to treat lung disorders, such as asthma and COPD, as well as apnea (28). Although high doses of AMO can produce CNS toxicities (29), this is unlikely to occur as a result of local delivery of AMO into tumors as was used in this study. Systemic delivery of theophylline has been shown to reduce B16F10 melanoma growth by a mechanism that was associated with decreased neovascularization (30, 31). However, the effects of intratumor delivery of AMO or theophylline have not been previously explored. In the current study, the amount of AMO administered directly into tumors (100 μl of 200 μM; 0.36 μg) is 500 times lower than a typical systemic therapeutic dose (10 mg/kg; 200 μg/20 g mouse). Therefore, intratumor delivery of AMO could be a very safe approach to treating solid tumors. It is notable also that injection of AMO into breast tumors markedly reduced the appearance of tumor metastases. This suggests that the generation of activated T cells by AMO injection into a single solid tumor might reduce tumor metastatization at distal sites.

A2B receptor–IFN-γ–CXCR3 signaling

In the current study, we identified a novel mechanism (IFN-γ/CXCL10/CXCR3 axis) by which A2B AR antagonism by AMO or ATL801 may inhibit tumor growth. Our data are consistent with several recent studies demonstrating CXCR3-dependent T cell antitumor activity. Anderson et al. (32) demonstrated that IL-7 induces CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. Neutralization of CXCL9, CXCL10, or IFN-γ reduced CXCR3-expressing activated T cells infiltrating lung tumors (33). The finding that A2B AR signaling regulates this pathway may be particularly helpful for designing combination therapies that can be used along with AMO or other adenosine receptor blockers to evoke synergistic reduction of tumor growth and metastasis. For example, IL-7 immunotherapy appears to use the same chemokine pathway to inhibit tumor growth as AMO (32), and hence, IL-7 and AMO are unlikely to be synergistic. Our preliminary studies show that AMO or ATL801 have no effect on regulatory T cell frequency in the tumor microenvironment (unpublished observation). Therefore, AMO may be productively used in conjunction with TGF-β blockers or anti-CD25 Ab therapy, which diminish regulatory T cell accumulation in the tumors and enhance antitumor cytotoxicity. In addition, AMO can reverse immunosuppression by adenosine released from dead cells. Hence, intratumor injection of AMO may be particularly effective in combination with cytotoxic radiation or chemotherapy.

In conclusion, intratumor AMO or ATL801 can reverse immunosuppressive effects of adenosine and inhibit tumor growth through activation of adaptive immune responses in a CXCR3-dependent manner, which is possibly due to the indirect effect of increased DC activity. These findings suggest that the A2B AR may be a useful target for facilitating the recruitment and activation of T cells and inhibiting angiogenesis in solid tumors.

FIGURE 8. AMO and ATL801 inhibit the growth and metastasis of 4T1 breast cancer cells. 4T1-12B breast cancer cells (10^5) expressing luciferase were injected into the mammary fat pads of BALB/c mice and treated with AMO (A, top panel, n = 13) or ATL801 (A, bottom panel, n = 5) three times weekly starting on day 0. B, Lung metastasis was quantified in kidney and lung following luciferin injection based on luminescence detected using an IVIS 200 imaging system (Caliper Life Sciences). Representative of n = 5. ANOVA and post hoc tests were performed to test significance differences between AMO and PBS. *p < 0.05, **p < 0.01, ***p < 0.001.
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Disclosures
J.L. has a consulting relationship with Forest Labs, the supplier of ATL801.

References
To determine if there is any activation of innate immune components after A2BAR blockade in the absence of the adaptive immune system we measured cell surface expression of CD69 on NK cells and CD86 on dendritic cells from tumors grown in RAG1-/- mice. NK cells are CD45+/NK1.1+/CD11bdim and DC's are CD45+/NK1.1+/CD11c+. Although ATL801 failed to increase surface expression of CD69 on NK cells or CD86 on myeloid cells expressing CD11c, it significantly increased the expression of CD86 on CD11b+ DCs (supplemental Figure 1). CD11b- DC's are known to capture dying cells and cross present antigens to T cells.

Supplemental Figure 1: ATL801 enhances the activation of tumor-associated CD11b+ DCs but not CD11b+ DCs or NK cells in RAG1-/- mice. MB49 bladder carcinoma cells (10^5) were inoculated. s.c. in the right flank of RAG1-/- mice and treated with intratumor injections of vehicle control (VC) or 1μM ATL801 in 100 μl PBS six times over 16 days. Expression of A) CD86 and B) CD69 were detected by flow cytometry as activation markers on tumor-associated dendritic cells and NK cells, respectively. **P<0.01, N=4 per group.