Adenosine 2B receptor expression on cancer cells promotes metastasis

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Running title: A2BR promotes cancer metastasis

Word Count: 5686

Key words: A2B adenosine receptor, CD73, metastasis, cancer

Conflict of interest: MJS has research agreements with Medimmune and Bristol Myers Squibb. Other authors declare that they have no conflict of interest.

Classification: Microenvironment and Immunology
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Funding

Mark J. Smyth and John Stagg were supported by a Susan Komen for the Cure Grant. Mark J. Smyth was supported by a National Health and Medical Research Council of Australia (NH&MRC) Senior Principal Research Fellowship, and Cancer Research Institute Clinical Team Strategy Grant. Deepak Mittal was supported by a QIMR Berghofer - “Weekend to End Women’s Cancers” Grant. Debottam Sinha is supported by IPRS (GUIPRS and GUPRS) from the Griffith University, Australia.
Abstract

Adenosine plays an important role in inflammation and tumor development, progression and responses to therapy. We show that an adenosine 2B receptor inhibitor (A2BRi) decreases both experimental and spontaneous metastasis and combines with chemotherapy or immune checkpoint inhibitors in mouse models of melanoma and triple-negative breast cancer (TNBC) metastasis. Decreased metastasis upon A2BR inhibition is independent of host A2BR and lymphocytes and myeloid cells. Knockdown of A2BR on mouse and human cancer cells reduces their metastasis in vivo and decreases their viability and colony forming ability, while transiently delaying cell cycle arrest in-vitro. The pro-metastatic activity of adenosine is partly tumor A2BR-dependent and independent of host A2BR expression. In humans, TNBC cell lines express higher A2BR than luminal and Her2+ breast cancer cell lines and high expression of A2BR is associated with worse prognosis in TNBC. Collectively, high A2BR on mouse and human tumors promotes cancer metastasis and is an ideal candidate for therapeutic intervention.
Introduction

Adenosine generated by ectoenzyme CD73 under hypoxic conditions has been described as an important regulator of immune responses in cancers and inflammatory disorders (1, 2). Adenosine binds to four different G-protein-coupled adenosine receptor subtypes A1, A2A, A2B and A3 with different affinities allowing for diversity and cellular specificity in signaling pathway activity (3). Antagonists of A2A and A2B adenosine receptors (A2AR and A2BR) have been shown to reduce tumor metastasis in mouse models of melanoma, ovarian, bladder and breast carcinoma (4, 5). A2BR is a low affinity receptor but its expression is highly transcriptionally regulated by factors such as hypoxia inducible factor (HIF)-1α associated with inflammatory hypoxia (6) suggesting its role in tumor promotion.

In-vitro studies using cancer cell lines have shown an opposing role of A2BR on cancer cell proliferation and apoptosis, an effect that could depend on the concentration of adenosine receptor agonist, level of A2BR and the type of cancer cell lines. For example, NECA, 5′-(N-Ethylcarboxamido)adenosine, a non-specific adenosine receptor agonist was shown to activate caspase-3 and induce apoptosis in ovarian cancer cell lines with high A2BR expression (7). Similarly, an adenosine analogue NECA was shown to enhance caspase-dependent apoptosis in response to chemotherapy in a human osteosarcoma cell line (8) and these effects were reversed by the A2BR antagonist PSB603 and siRNA knockdown to A2BR. Interestingly, a recent study also proposed tumor-promoting role of adenosine in an adenosine receptor-independent fashion (9). In contrast, activation of A2BR was shown to induce proliferation of prostate cancer cells
In-vivo studies have been less convincing regarding the mechanism of A2BR antagonists in controlling tumors (12). A non-selective adenosine receptor antagonist (aminophylline) and a selective A2BR antagonist ATL801 was shown to reduce the growth of bladder and breast cancers when injected intratumorally (5). Similarly, activation of A2BR enhanced the growth of primary melanoma. This effect was reversed by the A2BR antagonist PSB1115, and was associated with the reduced accumulation of myeloid derived suppressor cells (MDSCs) and increased NK cell and CD8+ T cell infiltration in the tumor tissue (13). Antagonists for A2AR and A2BR were effective in reducing the metastasis of CD73 expressing tumors, but not CD73-negative tumors, suggesting CD73 as a putative biomarker for selecting patients for both these therapies (4). Interestingly, unlike A2AR, A2BR blockade had no effect on NK cell function in mouse model of metastasis, suggesting a NK cell-independent mechanism contributing to increased metastasis of CD73+ tumors (4). A2BR-induced VEGF production by MDSCs enhanced angiogenesis and promoted tumor growth in melanoma and Lewis lung carcinoma models (14, 15). Further studies from Novitskiy et.al. suggested that dendritic cells (DC) activation via A2BR differentiated DC so that they expressed high levels of angiogenic and immunosuppressive factors such as VEGF, IL-10, TGF-beta, and IDO. When injected into the tumor bearing mice, these DCs increased the number of blood vessels and tumor weight (16). Another study proposed A2BR receptor as a Fra-1 target gene contributing to metastatic activity of breast cancer cells (17).

Thus far, research has supported the role of A2BR in promoting tumor growth, however
its comparative role and importance on cancer cells, immune cells, endothelial cells and other host cells has not been addressed. In this study, using several mouse and human models of cancer metastasis, we comprehensively show that A2BR expression on cancer cells, but not on host cells including endothelial or immune cells, promotes tumor metastasis. Further, A2BR overexpression is associated with worse prognosis in human triple-negative breast cancers. Importantly, an A2BR antagonist enhances the therapeutic efficacy of immune checkpoint inhibitors and chemotherapy, implying the combined use of A2BR antagonists for preventing and treating cancer metastasis.
Materials and Methods

Mice, Antibodies and Chemicals

C57BL/6 and BALB/c wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute for Medical Research or ARC Animal Resource Centre. C57BL/6, A2BR−/− mice were obtained from Holger Eltzschig (University of Colorado, Denver). Balb/c.Rag2−/−γc−/− mice were obtained from the Jackson Laboratory (ID: 014593) and SCID mice were originally obtained from the Animal Resources Centre (ARC, Perth, Australia). All mice were bred and maintained at the QIMR Berghofer Medical Research Institute and used between the ages of 8-14 weeks. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Chemotherapeutic drug doxorubicin was obtained from the pharmacy of the Peter MacCallum Cancer Centre, Melbourne. PSB1115 (Sigma), anti-mouse PD-1 (RMP1-14), and anti-mouse CTLA-4 (UC10-4F10) were obtained and used as described previously (4) (18). Antibodies reactive with Erk (137F5), p-Erk (T202/Y204), AKT (C67E7) and p-AKT (Ser473, D9E) were obtained from Cell Signaling Technology, USA and FOXM1 (C-20) and c-Myc were obtained from Santa Cruz Biotechnology, USA and ADORA2B and β-actin (C4/actin) were obtained from ProSci and BD Biosciences, USA respectively.

Cell culture and tumor metastasis assays

Mouse B16F10-CD73hi melanoma cells were generated from B16F10 cells (ATCC) at the Peter MacCallum Cancer Centre in 2008. LWT1 melanoma cells were generated from SM1WT1 cells at the QIMR Berghofer in 2013. 4T1.2 and E0771 mammary carcinomas
were obtained from Dr. Robin Anderson at the Peter MacCallum Cancer Centre (2004 and 2014, respectively). RM-1 prostate carcinoma cell lines, were maintained, injected, and monitored as previously described (4, 19-21). Human breast cancer cell lines MDA-MB-231, SUM159PT and MDA-MB-468 were all purchased from ATCC (2014-2015) and MDA-MB-231 derived spontaneous metastatic cell line, MDA-MB-231-HM-LNm5 was obtained from Robin Anderson (Peter MacCallum Cancer Centre, Melbourne)(2014). All cell lines were tested negative for mycoplasma and only the human cell lines were STR profiled. Cell lines were cultured in complete DMEM with 10% FBS in a 5% CO₂ incubator. Experimental and spontaneous metastasis assays were performed with mouse tumors in C57BL/6 or Balb/c WT mice or human tumors in Balb/c.Rag2⁻⁻γc⁻⁻ mice as previously described (and indicated in the figure legends) (4, 19-21).

**Lentiviral Knockdown of A2BR**

Human and mouse A2BR was knocked down using ready to use GIPZ shRNA lentiviral particles from Dharmacon GE Life Sciences (Colorado, USA) as per manufacturer’s instructions. Cells were then sorted for turbo-GFP expression and knockdown of A2BR expression was determined by RT-PCR and immunoblotting.

**Colony formation assays and cell cycle analysis**

500-1000 cells were seeded on 12 well plates and incubated for additional 7-14 days to determine colony viability. The colonies were fixed with 0.05% crystal violet for 30 minutes, washed and counted under a dissection microscope. Cell cycle perturbations
were determined using flow cytometry after staining with propidium iodide as described previously (22).

**Western blotting and real-time PCR**

Western blotting was performed as described earlier (23). The Super Signal chemiluminescent ECL-plus (Amersham) was used for protein detection. RNA was isolated from tumor cell lines by RNAzol (Sigma-Aldrich) as per manufacturer's instructions. cDNA was synthesized using 500 μM dNTPs, 500 nM Oligo-dT, 200 U of Tetro Reverse Transcriptase and 20 U of Ribosafe RNase Inhibitor (Bioline). Mouse Adenosine receptor expression was analyzed using SensiFAST SYBR Lo-ROX (Bioline) and gene specific primer for mouse adenosine receptors and normalized to mouse RPL32 expression. Housekeeping gene HPRT was used to determine human adenosine receptor expression in human breast cancer cell lines. List of RT-PCR primer sequence is in Supplementary Table 2.

**Gene expression analyses**

In order to evaluate the expression of A2BR as a prognostic indicator, gene expression was extracted and analysed from publicly available datasets as previously described (24) where the PAM50 molecular subtype classifier (24) was applied. Relapse free survival (RFS) was taken as the survival endpoint for each dataset, if unavailable, disease-free or distant metastases were used. Kaplan-Meier survival curves are constructed using tertiles
of gene expression values and differences evaluated using Log-rank p values pooled across the tertiles. Cox regression analyses were also used to evaluate prognostic associations of the gene as a continuous variable. If more than one probe set was present per gene, the probe set with the highest variance was used. To achieve a comprehensive comparison between datasets, a robust linear scaling was applied to each gene (24). All analyses have been performed using R version 3.2.3 (http://www.r-project.org/).

**Statistical analysis**

Statistical analysis was achieved using Graphpad Prism Software. Data was considered to be statistically significant where the p value was equal to or less than 0.05. Data were compared using a one-way ANOVA, Mann-Whitney U test or unpaired t test. Differences in survival were evaluated using a Log-rank (Mantel-Cox) test. Unless specified, all experiments were repeated at least twice with similar results.
Results

High ADORA2B expression is associated with worse patient outcome

CD73-adenosine signaling pathway has been implicated in promoting tumor growth and metastases (1, 2, 25-27). We have previously shown that high CD73 expression is associated with a poor prognosis in the primary triple-negative breast cancer (TNBC) subtype and its overexpression in these tumors predicted chemoresistance to doxorubicin therapy (26). Here we analyzed publicly available gene expression data for associations between A2BR expression and prognosis. High A2BR was significantly associated with poorer survival in breast cancer patients, and this effect seemed to be strongest in the TNBC patients (Fig. 1), suggesting that A2BR is a tumor target of therapeutic interest for some breast cancers. Interestingly, high A2BR was not statistically associated with poor survival in the luminal or HER2+ subtypes (Fig.1),

A2BRi suppresses experimental and spontaneous metastasis alone and in combination with other therapies

We analyzed the effect of A2BR inhibition in mouse models of experimental and spontaneous metastasis with cells expressing high levels of CD73. Consistent with our previous study (4), a selective antagonist of A2BR (A2BRi, PSB1115) significantly decreased experimental lung metastasis in the B16F10-CD73\textsuperscript{hi} melanoma (Fig. 2A, B), CD73\textsuperscript{+} TNBC EO771 mammary adenocarcinoma (Fig. 2C), and metastatic BRAF\textsuperscript{V600E} melanoma, LWT1 (Fig. 2D). A2BRi post surgical resection (day 20 after tumor inoculation) also significantly improved the survival of mice bearing highly metastatic 4T1.2 breast tumors (Fig. 2E).
We next assessed the combinatorial effect of A2BRi with anti-PD-1 or anti-CTLA-4 monoclonal antibodies (mAb) in both melanoma and mammary cancer models of metastasis. We found that the combination of anti-PD-1 or anti-CTLA-4 with A2BRi was superior to either monotherapy alone in decreasing lung metastasis (Fig. 3A) and improving the survival of 4T1.2 tumor bearing mice (Fig. 3B). Interestingly, the triple combination of A2BRi, anti-PD-1 and anti-CTLA-4 was more effective in the spontaneous model of 4T1.2 breast cancer metastasis, but not in the experimental metastasis of B16F10 melanoma, when compared with dual combination therapy (Fig. 3A, B). Chemotherapy remains a standard of care treatment for TNBC patients. Therefore, we investigated whether A2BRi improved the therapeutic effect of standard chemotherapeutic drug doxorubicin. A2BRi in combination with doxorubicin was more effective in improving the survival of 4T1.2 tumor bearing mice when compared to either monotherapy alone (Fig. 3C). Collectively, this data suggests that A2BRi is an attractive anti-metastatic therapy and can improve survival when used in combination with standard chemotherapy and immune checkpoint inhibitor mAbs.

**A2BRi suppression of metastasis is independent of T/NK cells and myeloid cells**

A2B receptor is induced by hypoxia and has a broad cellular distribution in tumor microenvironment, including myeloid cells (28). Therefore, we hypothesized that the anti-metastatic effect of A2BRi might be dependent on immune cells. Unlike anti-asGM1 antibody treated mice alone, depletion of NK cells by using anti-asGM1 antibody did not greatly increase the metastatic burden in lungs of mice treated with A2BRi (Supplementary Fig. S1A). Similarly, depletion of CD8$^{+}$ T cells alone or in combination with CD4$^{+}$ T cells and/or NK cells had no effect on the number of B16F10-CD73$^{hi}$

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metastases or survival against 4T1.2 in groups of mice treated with A2BRi, indicating that T/NK cells are not required for anti-metastatic activity of A2BRi (Supplementary Fig. S1A, B). Furthermore, A2BR blockade with PSB1115 was still effective in the absence of T and NK cells in E0771, another metastatic model of mammary carcinoma (Supplementary Fig. S2). Furthermore, A2BRi did not lose its therapeutic activity in groups of mice depleted of CD11c⁺ dendritic cells (using CD11c-DOG transgenic mice, Supplementary Fig. S1C) and macrophages (using clodronate liposomes, Supplementary Fig. S1D), suggesting that these immune cells are dispensable for A2BRi suppression of cancer metastasis.

**A2BR on mouse cancer cells promote metastasis**

We were encouraged to examine the function of A2BR on various mouse cancer cell lines. Suitable mAbs for detecting mouse and human A2BR by flow cytometry were not available. At the mRNA level, we found variable expression of A2BR on different cancer cell lines (Supplementary Fig. S3A). Additionally, A2BR appeared to be more highly expressed on cancer cells than other adenosine receptors (Supplementary Fig. S3 B, C). To assess the function of A2BR on cancer cells, we used lentiviral vectors to knockdown (KD) mouse A2BR expression from the mammary carcinomas EO771 and 4T1.2 (Fig. 4A-C), the LWT1 melanoma (Supplementary Fig. S4C, E) and the RM1 prostate carcinoma (Supplementary Fig. S4D, E). Based on the A2BR expression at mRNA and protein levels in A2BR KD cells, we selected sh3 A2BR KD cells for EO771 cells and sh1 A2BR KD cells for other cell types in subsequent studies (Fig. 4 and Supplementary Fig. S4).
To evaluate the impact of A2BR loss on cancer cells, both shRNA (sh) control and A2BR KD cells were examined for their metastatic potential in-vivo. A2BR KD cells produced significantly fewer experimental metastases in mouse mammary carcinoma, melanoma and prostate carcinoma models (Fig. 4D, E, F), consistent with what we observed after treatment with A2BR antagonist, PSB1115. Mice orthotopically inoculated with 4T1.2 A2BR KD mammary carcinoma survived longer than those inoculated with sh control 4T1.2 mammary carcinoma, post surgical resection (Fig. 4G). The consistent effect of A2BR KD in reducing metastatic potential across various mouse cancer cell lines, and all three shRNA hairpins showing variable but significantly decreased metastatic burden compared to the sh control in the EO771 mammary carcinoma (Fig. 4D), suggested that the observed phenotype was not an off-target effect of the lentiviral hairpins. Interestingly, the knockdown of A2BR did not affect the growth of primary tumors in EO771 and 4T1.2 mammary carcinoma models (Supplementary Fig. S4A, B).

**Host A2BR does not contribute significantly to metastasis**

To further evaluate the role of host A2BR expression in controlling metastasis, we injected sh control and A2BR KD EO771 cells in WT mice and treated them with vehicle or A2BRi (PSB1115) or injected the same lines into A2BR\(^{-/-}\) mice. Addition of A2BRi reduced the number of EO771 lung metastases in sh control cells, but not in the sh3 A2BR KD cells (Fig. 5A). Additionally, sh control and sh3 A2BR KD EO771 cells produced similar number of lung metastases in WT and A2BR\(^{-/-}\) mice (Fig. 5A), suggesting that host A2BR expression does not contribute to the anti-metastatic activity of A2BRi. Similar results were obtained in the 4T1.2 spontaneous metastasis model in which the addition of A2BRi improved the survival of mice injected with sh control.
4T1.2 cells, but not sh1 A2BR KD cells (Fig. 5B). Furthermore, pro-metastatic activity of adenosine receptor agonist NECA was dependent partly on tumor A2BR as NECA increased the number of lung metastases of control sh E0771 tumor cells, but only partly increased the metastasis of sh3 A2BR KD E0771 tumor cells (P<0.007). The numbers of lung metastases were similar in WT and A2BR KO mice after NECA treatment suggesting that adenosine does not have its pro-metastatic effect through host A2BR (Fig. 5C).

**A2BR on human cancer cells promote metastasis**

Given that high A2BR expression correlated with poor outcome in human breast cancer (Fig. 1), we next studied the function of A2BR expression on human breast cancer cells. We first performed A2BR mRNA expression analysis in breast cancer cell lines using the publically available database GOBO (29). Using the GOBO dataset, we found that A2BR mRNA expression was associated with human TNBC cell lines (30), with basal like subtype exhibiting the highest expression, while hormone receptor positive cell lines were negative for A2BR mRNA expression (Supplementary Fig. S5). Consistent with A2BR mRNA expression, CD73 was highly expressed in Basal B subtypes of breast cancer, including (data not shown). Next, we examined whether A2BRi reduced experimental metastasis in human TNBC tumor xenograft settings. A2BRi at a higher dose was slightly more effective in decreasing lung metastatic burden than at the lower dose (Fig. 6A, B). A2BRi reduced experimental metastasis of both the parental and its metastatic variant MDA-MB-231-HM5 (CD73\textsuperscript{hi}A2BR\textsuperscript{hi}) cells (Fig 6A-C). Moreover, we also found a similar result when CD73\textsuperscript{+} SUM159PT, an invasive metastatic cell line derived from anaplastic breast tumor was treated with A2BRi (Supplementary Fig. S6A),
suggesting the effect of A2BRi-mediated metastatic inhibition is not cell line specific. To study the effect of A2BR expression on MDA-MB-231 cells in metastasis, A2BR KD cells were generated (Fig. 6D-E) and sh control and sh3 A2BR KD MDA-MB-231 cells were examined for their metastatic potential in-vivo. Knockdown of A2BR slowed down the growth of primary MDA-MB-231 tumors marginally but it did not reach statistical significance (Fig. 6F). Notably, consistent with the data from the mouse cancer cell lines, A2BR KD MDA-MB-231 cells produced fewer experimental lung metastases compared to sh control cells (Fig. 6G). Collectively, our data strongly suggest that inhibition of A2BR either using small molecule inhibitor or gene knockdown impaired lung metastases in human breast cancer cell lines.

**A2BR regulates p-ERK activation, cell viability and cell cycle progression**

Next we assessed the mechanism of the effect of A2BR expression on tumor metastasis. A2BR knockdown reduced the long-term colony formation ability (Fig. 7A-C), which may be associated with the decreased viability in-vitro (Fig. 7D-F) of mouse and human A2BR KD cancer cell lines. One of the common mechanisms for cancer cells to metastasize is via epithelial-mesenchymal transition (EMT), however we did not observe any consistent difference in the expression of EMT markers between control and A2BR KD cells (data not shown). Reduced A2BR expression decreased the migration and invasion capability of EO771 cells (Supplementary Fig. S6B, C).

Next, in order to further understand the function of A2BR, we performed a metastasis specific gene expression array on control and A2BR KD EO771 cells and observed downregulation of metalloproteinases and other markers in A2BR KD cells
(Supplementary Table 1). Validation of the PCR array data, confirmed downregulation of FXYD5, a cell membrane glycoprotein known to reduce cell adhesion and drive metastasis (31). This was observed in both mouse and human A2BR KD cancer cells compared to their sh control cells, suggesting the role of FXYD5 in promoting A2BR-dependent metastasis (32) (Supplementary Fig. S6D, E). Moreover, consistent with reduced colony forming ability and delay in cell proliferation following A2BR depletion in multiple cancer lines, we found reduced phospho-ERK1/2 (Thr202/Tyr204) signaling (Fig. 7G), but not phospho-STAT3 (Tyr705) or phospho-AKT (S473) (data not shown). This was associated with reduced c-MYC and FOXM1 activity (Fig. 7G); both are downstream effectors of MAPK signaling. Reduced FOXM1 expression in A2BR KD cells encouraged us to examine cell cycle profile of sh control and A2BR KD cells. Interestingly, both for mouse and human breast carcinoma cells, we observed A2BR knockdown was associated with transient delay in cell cycle progression with cell arresting in S/G2 delay (Fig. 8H, I). Overall, these results suggest that A2BR may drive cancer metastasis by reducing cell adhesion and MAPK-dependent signaling activation. The reduced viability and colony formation ability of A2BR KD cancer cells in-vitro may be associated with the observed transient delay in cell cycle progression.
Discussion

The CD73-adenosine pathway has gained considerable therapeutic interest in recent years with mAbs and small molecule inhibitors targeting CD73 and adenosine receptor inhibitors demonstrating anti-tumor activity in mouse models of cancer metastasis (4, 19, 33-36). Since other studies (4, 10, 13, 15, 17) had indicated A2BRi had anti-tumor activity, we were interested to know whether that activity was directed at A2BR on the tumor or the host. We first demonstrated that A2BRi suppressed experimental and spontaneous metastasis of CD73⁺ mouse models of TNBC, melanoma, and prostate cancer, and that this anti-metastatic activity of A2BRi was independent of lymphocytes (NK cells and T cells) and myeloid cells (CD11c+ and clodrolip sensitive macrophages). Furthermore, the A2BRi (PSB1115) was effective in reducing the metastasis of human TNBC xenografts in immunodeficient mice and was effective even in A2BR-deficient mice. Knockdown of A2BR on mouse and human cancer cell lines confirmed the critical role for tumor A2BR, rather than host A2BR, in metastasis and response to adenosine. Previously, CD73 overexpression was shown to be associated with poor clinical outcome for patients with ovarian (27), prostate (25) and TNBC (26). Using datasets from the same TNBC patients that had poor prognosis with increased CD73 expression, we observed that overexpression of A2BR was associated with poorer prognosis in TNBC. In fact, both CD73 and A2BR were highly expressed by Basal B type TNBC, suggesting that A2BR, like CD73, might be a therapeutic target in CD73⁺ cancers.
Previous data suggested that the anti-tumor activity of A2BRi relies on its ability to lower accumulation of tumor-infiltrating MDSCs, enhanced DC activation and restore an efficient anti-tumor T cell response that may be dependent on IFN-inducible chemokine CXCL10 and its ligand CXCR3 (5, 13). We did not test the functional importance of CXCL10, but in several mouse models by functionally depleting individual immune cell populations or using mice deficient in host A2BR, the effect of A2BRi on host cells did not appear as critical as the tumor A2BR expression. The discrepancy in results obtained here could be attributed to different cancer cell lines and different A2BRi used in previous studies, but previous studies did not attempt to directly and functionally dissect the role of host and tumor A2BR. More importantly, previous studies were focussed on primary tumor models and used intratumor and peritumor injections of A2BRi that are not practical clinically in metastatic settings. In contrast to A2BRi, anti-CD73 anti-tumor activity requires both host and tumor CD73 expression (37), and A2ARi anti-metastatic activity is entirely dependent on hematopoietic cells, particularly myeloid cells and NK cells which express a high level of A2AR (4, 19) (38).

Knockdown of A2BR on various mouse and human tumor cell lines confirmed the critical role of tumor, as opposed to host A2BR. Reduced metastasis of the A2BR knockdown cancer cells was in part explained by their induced cell cycle arrest and reduced viability and colony-forming ability demonstrated *in-vitro*. The reduced lung metastatic burden with mouse A2BR KD cells was similar to that obtained with A2BRi PSB1115 treated parental tumor cells suggesting that all the effect of A2BRi was dependent on tumor A2BR expression. Overexpression of A2BR in endothelial cells under hypoxic conditions has been associated with increased vascular permeability and
enhanced angiogenesis (39). Furthermore, in a Lewis lung carcinoma model, A2BR-deficient mice reportedly have reduced primary tumor growth and intratumor VEGF compared to the WT mice, suggesting a role of endothelial A2BR in promoting primary tumor growth under hypoxic conditions (14). But we found that injection of control and A2BR KD tumor cells did not significantly affect primary tumor growth in WT mice and produced similar number of lung metastasis in WT and A2BR KO mice. These data demonstrated that the mechanism of A2BRi in reducing metastasis was independent of host A2BR.

A question outstanding is how tumor A2BR might functionally control tumor metastasis. The decrease in p-ERK1/2, FOXM1 and c-Myc expression after A2BR knockdown suggested that tumor A2BR KD decreased metastasis by affecting the Raf/MEK/ERK pathway. There was also a reduction in the expression of FXYD5, a regulator of cell-cell adhesion and a marker of poor prognosis in ovarian carcinomas (31) and invasiveness in breast carcinoma (40). In fact, A2BR has been suggested to negatively regulate cell adhesion by modulating the localization of Rap1 protein (41). Others have suggested that A2BR controls carcinoma proliferation via HIF-1α activation, indicating that A2BR may have several mechanisms to regulate tumor progression (42, 43). Both RNAi silencing and pharmacologic blockade of A2BR inhibited filopodia formation and invasive activity of breast cancer cells and correspondingly reduced tumor outgrowth in the lungs (17). Although we did observe an impact of A2BR KD on the invasive activity of mouse TNBC E0771 cells, this was not reproduced in the other A2BR KD cell lines. Further functional studies using additional knockdown or CRISPR of candidate
molecules will be required to dissect out the most important molecular changes occurring in A2BR-deficient cells that limits their metastatic potential.

Immunotherapy with immune checkpoint inhibitors such as PD-1 and CTLA-4 is changing the landscape of cancer therapy as best demonstrated in advanced melanoma, non-small cell lung cancer, and renal cell carcinoma patients (44, 45). A limitation with immune checkpoint blockade, particularly anti-CTLA-4, is immune-related adverse events due to “tumor unrelated” non-specific immunological activation (46, 47). One advantage of targeting the adenosine pathway is the safety profile of adenosine receptor inhibitors, established in phase I-III clinical trials in Parkinson’s disease (48). A recent study suggested CD73 as a biomarker for anti-PD-1 therapy (49) and CD73 and A2AR blockade can further enhance the therapeutic activity of immune checkpoint inhibitors (18, 19, 49, 50). Despite the lack of a critical role for lymphocytes in A2BRi anti-metastatic activity, the A2BRi PSB1115 improved the anti-metastatic activity of anti-PD-1 and anti-CTLA-4 mAbs in two different models of metastasis (one experimental and one spontaneous) and this may be associated with an additive direct effect of A2BRi PSB1115 on cancer cell metastasis and alleviating immune suppression by immune checkpoint inhibitors. Furthermore, the A2BRi improved the survival of mice when combined with doxorubicin. In addition, knockdown of A2BR enhanced the sensitivity of 4T1.2 mouse and MDA-MB-231 human breast carcinoma cells to doxorubicin in-vitro (Supplementary Fig. S7), suggesting that A2BR expression on cancer cells provides resistance to doxorubicin and A2BR blockade might add value in chemotherapeutic combinations.
Overall, our data from mouse and human studies demonstrates the key role of tumor A2BR, but not host A2BR, in promoting cancer metastasis and the promise of targeting A2BR with other strategies that prevent tumor metastasis.
Acknowledgements

We thank Kate Elder and Liam Town for their technical support and Camille Guillerey, Dipti Vijayan, and Eva Putz for critical reading of this manuscript.
References


Figure Legends

Figure 1. High ADORA2B gene expression is significantly associated with poorer prognosis in triple negative breast cancer (TNBC). ADORA2B in (A) all breast cancers and (B) in triple-negative breast cancers (C) estrogen receptor positive Luminal A and (D) Luminal B and (E) HER2-overexpressing subtypes. Kaplan Meier curves demonstrate prognostic effect of tertiles of gene expression with a pooled Log-rank p value (P = number of patients; E = number of events; Blue – high; Red – low; Green – medium expression). (F) The Cox regression model evaluates the gene as continuous variable, confirming the prognostic effect–hazard ratio (HR) and 95% confidence intervals (CI) for ADORA2B in all breast cancers, triple-negative breast cancers, estrogen receptor positive Luminal A and Luminal B breast cancers and HER2-overexpressing breast cancer subtypes.

Figure 2. A2BRi suppresses experimental and spontaneous lung metastasis. C57BL/6 wild type (WT) mice were injected intravenously with (A, B) B16F10-CD73\textsuperscript{hi} melanoma cells (10\textsuperscript{5} cells) (C) E0771 mammary carcinoma (2 x 10\textsuperscript{5}), or (D) LWT1 melanoma cells (5 x 10\textsuperscript{5} cells) on day 0. On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of vehicle or A2BRi PSB1115 as indicated in (A) or 1 mg/kg (B-D). Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM; (A) n = 5 mice/group (B) n = 10 mice/group are shown, pooled from two independent experiments. Improved metastatic control of PSB1115 was statistically significant by ANOVA as indicated (*** P <0.001; **** P <0.0001). (E) Groups of 5 female BALB/c WT mice were injected in the
mammary fat pad with the mammary carcinoma cell line 4T1.2 (5 x 10^4). On day 20, the primary tumor was resected and mice were treated i.p with vehicle or PSB1115 (1 mg/kg i.p.) on day 21, 24, 27, and 30. Survival of the mice was monitored. Represents two independent experiments pooled. PSB1115 is significantly different to vehicle as determined by Log-rank test, ****P < 0.0001.

**Figure 3. Immune checkpoint blockade and chemotherapy enhances A2BRi suppression of experimental and spontaneous metastasis.** (A) Groups of 5-20 C57BL/6 wild type (WT) mice were injected intravenously with B16F10-CD73^hi melanoma cells (10^5 cells) on day 0. Mice were treated with intraperitoneal injections of vehicle, A2BRi (PSB1115, 1 mg/kg i.p), cIg (2A3, 250 μg i.p.), α-CTLA-4 (UC10-4F10, 250 μg i.p.), α-PD-1 (RMP1-14, 250 μg i.p.), or their combination on days 0 and 3 after tumor inoculation as indicated. Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5-20 mice per group are shown. Represents four independent experiments pooled. Improved metastatic control over PSB1115 alone was statistically significant by ANOVA as indicated (**P<0.01; ***P<0.001; **** P <0.0001). (B, C) Groups of 5 female BALB/c WT mice were injected in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (5 x 10^4). On day 18 the primary tumor was resected and mice were treated i.p with vehicle or PSB1115 (1 mg/kg i.p.), cIg (2A3, 250 μg i.p.), α-CTLA-4 (UC10-4F10, 250 μg i.p.), α-PD-1 (RMP1-14, 250 μg i.p.), or their combination as indicated on day 18, 21, 24, and 27. Doxorubicin was administered i.v. at 2 mg/kg on days 18 and 25. Survival of the mice
was monitored. Improved survival over PSB1115 alone was statistically significant by Log-rank test as indicated (*P<0.05, **P<0.01).

**Figure 4. A2BR expression on mouse cancer cell promotes metastasis.**

RT-PCR expression of mouse A2BR shown as relative A2BR KD after lentiviral KD of A2BR in (A) EO771 and (B) 4T1.2 cells with three different small hairpins. RPL32 was used as a reference gene. Means ± SEM, n = 4-9; **P<0.01, ***P<0.001, ****P<0.0001. (C) Immunoblot analysis of A2BR expression in EO771 and 4T1.2 cells knocked down for A2BR. β-actin served as a loading control. (D) 2 x 10^5 EO771 sh control (ctrl sh) and A2BR KD (sh1, sh2, sh3) cells, (E) LWT1 melanoma cells or (F) RM1 prostate carcinoma cells (sh control and sh1 A2BR KD cells) were injected intravenously and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface (means ± SEM, n = 10-11, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ANOVA or Mann-Whitney tests). (G) 5 x 10^4 4T1.2 sh control (ctrl sh) and sh1 A2BR KD cells were injected in the mammary fat pad of Balb/c WT mice and the primary tumor was resected on day 18 and the survival of mice was monitored and analyzed by Log-rank test (n = 15 mice/group; *P<0.05).

**Figure 5. Addition of A2BRi does not provide additional therapeutic benefit against A2BR KD cells.**

(A) C57BL/6 wild type WT mice were injected intravenously with 2 x 10^5 E0771 control (ctrl sh) and A2BR KD (Sh3) cells on day 0. In some groups of mice, on day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of vehicle or A2BRi (PSB1115, 1 mg/kg i.p) as indicated. Metastatic burden was quantified in the
lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5-10 mice per group are shown. NS = not significant (B) 5 x 10^4 4T1.2 sh control (ctrl sh) and sh1 A2BR KD cells were injected in the mammary fat pad of Balb/c WT mice. On day 18, the primary tumor was resected and mice were treated i.p with vehicle or PSB1115 (1 mg/kg i.p.) as indicated on day 19, 22, 24, and 27. Survival of mice was monitored and analyzed by Log-rank test (n = 10-15 mice/group; **P < 0.01). (C) C57BL/6 wild type WT or A2BR^-/- mice were injected intravenously with 2 x 10^5 E0771 Ctrl sh or sh3 A2BR KD cells on day 0. On day 0 and 3 after tumor inoculation, mice were treated with injections of vehicle or NECA (0.05 mg/kg i.p) as indicated. Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5 mice per group is shown (****P<0.0001, ANOVA). NS = not significant.

Figure 6. A2BR expression on human tumor cells promotes metastasis.

(A, B) 1 x 10^6 MDA-MB-231 and (C) 5 x 10^5 MDA-MB-231 HM5 (lymph node metastatic variant of MDA-MB-231) cells were injected intravenously in Balb/c.Rag2^-/-.γc^-/- immunodeficient mice and mice were treated intraperitoneally with vehicle or A2BRi (A) (1 mg/kg) or (B, C) (10 mg/kg) on day 0 and day 3. Metastatic burden was quantified in the lungs after 6 weeks (A,B) or 4 weeks (C) by counting colonies on the lung surface. (D) RT-PCR expression of human A2BR shown as relative A2BR KD after lentiviral KD of A2BR in MDA-MB-231 cells with three small hairpins. Human HPRT was used as a reference gene. (E) Immunoblot analysis of human A2BR expression in sh control (ctrl) and A2BR KD MDA-MB-231 cells. β-actin served as a loading control. (F) 1 x 10^6 MDA-MB-231 sh control and sh3 A2BR KD cells were injected in the mammary fat pad.
of immunodeficient SCID mice and primary tumor growth was monitored. (G) 8 x 10⁵ MDA-MB-231 sh control and sh3 A2BR KD cells were injected intravenously in Balb/c.Rag2⁻/⁻γc⁻/⁻ immunodeficient mice and metastatic burden was quantified in the lungs after 8 weeks by counting colonies on the lung surface. Means ± SEM, n = 5-9 mice/group, *P<0.05, **P<0.01; Mann-Whitney test).

**Figure 7. A2BR knockdown on cancer cells decreases colony formation, viability and phospho-ERK activation**

The effect of A2BR depletion on colony formation was determined using crystal violet staining in (A) E0771, (B) 4T1.2 and (C) MDA-MB-231 control and A2BR KD cells. The number of colonies was counted using a dissection microscope. The viability of (D) E0771, (E) 4T1.2 and (F) MDA-MB-231 control and A2BR KD cells after seven days of culture was determined by MTS assay. Absorbance was recorded at 495 nM using a HT Microplate spectrophotometer. Data pooled from two independent experiments (A-F) (means ± SEM, ***P<0.001, ****P<0.0001; Mann-Whitney test). (G) Representative immunoblots showing the expression of total ERK1/2, p-ERK1/2, FOXM1 and c-MYC in control and A2BR KD cells in EO771, 4T1.2 and MDA-MB-231 cells. β-actin was used as a loading control. (H) Representative flow cytometry plot showing cell cycle phases in EO771 control (Ctrl sh) and A2BR KD (sh3) cells in culture after staining with propidium iodide. (I) Bar graph shown as percentage of E0771 and MDA-MB-231 control and A2BR KD cells in G1, S and G2 phase at 12 h, 24 h and 48 h in cell culture (means ± SEM, *P<0.05, **P<0.01; ***P<0.001, ****P<0.0001; unpaired t-test between control and A2BR KD cells at indicated time points and cell cycle).
Figure 1

A. ADORA2B All

Logrank $p = 0.00029$

Cumulative Survival

P = 4189
E = 2429

Years

B. ADORA2B TNBC

Logrank $p = 0.0063$

P = 951
E = 519

Years

C. ADORA2B LumA

Logrank $p = 0.069$

P = 1350
E = 834

Years

D. ADORA2B LumB

Logrank $p = 0.56$

P = 1238
E = 737

Years

E. ADORA2B Her2+

Logrank $p = 0.85$

P = 650
E = 339

Years

F. ADORA2B

- ALL HR: 1.2 pval: 0.0048 95% CI: 1.1 - 1.4
- LumA HR: 1 pval: 0.82 95% CI: 0.75 - 1.4
- LumB HR: 1.2 pval: 0.19 95% CI: 0.92 - 1.5
- Her+ HR: 0.91 pval: 0.54 95% CI: 0.67 - 1.2
- TNBC HR: 1.4 pval: 0.013 95% CI: 1.1 - 1.7

Log2 Hazard Ratio

-4
-2
0
2
4
Figure 2

A

Number of B16F10-CD73hi lung metastases

B

Number of B16F10-CD73hi lung metastases

C

Number of EO771 lung metastases

D

Number of LWT1 lung metastases

E

Percent survival

Days after 4T1.2 tumor inoculation
Figure 3

A

Number of B16F10-CD73

lung metastases

vehicle + clg
vehicle + α-CTLA-4
vehicle + α-PD-1
α-CTLA-4 + α-PD-1
α-CTLA-4 + PSB1115
α-PD-1 + PSB1115
α-CTLA-4 + α-PD-1 + PSB1115

B

Percent survival

Days after 4T1.2 tumor inoculation

vehicle
vehicle + clg
vehicle + α-CTLA-4
vehicle + α-PD-1
α-CTLA-4 + α-PD-1
α-CTLA-4 + PSB1115
α-PD-1 + PSB1115
α-CTLA-4 + α-PD-1 + PSB1115

C

Percent survival

Days after 4T1.2 tumor inoculation

vehicle
vehicle + clg
DOX
PSB1115 + clg
PSB1115 + DOX

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Figure 4

A. E0771

B. 4T1.2

C. E0771 and 4T1.2

D. Number of E0771 lung metastases

E. Number of LWT1 lung metastases

F. Number of RM1 lung metastases

G. Percent survival

Legend:

- Ctrl sh
- sh1
- sh2
- sh3

** p < 0.01
*** p < 0.001
**** p < 0.0001

Beta-actin Western Blotting

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Figure 5

A

Number of E0771 lung metastases

WT

A2BR−

B

Percent survival

Days after 4T1.2 inoculation

C

Number of E0771 lung metastases

WT

A2BR−
Figure 6

A

B

C

D

E

F

G

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 7

A. E0771

Number of E0771 colonies

B. 4T1.2

Number of 4T1.2 colonies

C. MDA-MB-231

Number of MDA-MB-231 colonies

D. E0771

Relative cell viability

E. 4T1.2

Relative cell viability

F. MDA-MB-231

Relative cell viability

G. E0771, 4T1.2, MDA-MB-231

Protein expression levels (A2BR, pERK1/2, ERK1/2, c-MYC, FOXM1, β-Actin)

H. Ctrl sh, sh3

Flow cytometry analysis (G0/G1, S, G2)

I. E0771

Cell cycle distribution

J. MDA-MB-231

Cell cycle distribution
Adenosine 2B receptor expression on cancer cells promotes metastasis

Deepak Mittal, Debottam Sinha, Deborah Barkauskas, et al.

Cancer Res  Published OnlineFirst May 24, 2016.

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Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-16-0544

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