

Adenosine Receptor 2A Blockade Increases the Efficacy of Anti-PD-1 through Enhanced Antitumor T-cell Responses

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Abstract

Immunotherapy is rapidly emerging as a cancer treatment with high potential. Recent clinical trials with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies (mAbs) suggest that targeting multiple immunosuppressive pathways may significantly improve patient survival. The generation of adenosine by CD73 also suppresses antitumor immune responses through the activation of A_{2A} receptors on T cells and natural killer (NK) cells. We sought to determine whether blockade of A_{2A} receptors could enhance the efficacy of anti-PD-1 mAb. The expression of CD73 by tumor cells limited the efficacy of anti-PD-1 mAb in two tumor models, and this was alleviated with concomitant treatment with an A_{2A} adenosine receptor antagonist. The blockade of PD-1 enhanced A_{2A} receptor expression on tumor-infiltrating CD8⁺ T cells, making them more suscep-

tible to A_{2A}-mediated suppression. Thus, dual blockade of PD-1 and A_{2A} significantly enhanced the expression of IFN γ and Granzyme B by tumor-infiltrating CD8⁺ T cells and, accordingly, increased growth inhibition of CD73⁺ tumors and survival of mice. The results of our study indicate that CD73 expression may constitute a potential biomarker for the efficacy of anti-PD-1 mAb in patients with cancer and that the efficacy of anti-PD-1 mAb can be significantly enhanced by A_{2A} antagonists. We have therefore revealed a potentially novel biomarker for the efficacy of anti-PD-1 that warrants further investigation in patients. Because our studies used SYN-115, a drug that has already undergone phase IIb testing in Parkinson disease, our findings have immediate translational relevance for patients with cancer. *Cancer Immunol Res*; 3(5); 506–17. ©2015 AACR.

Introduction

The importance of the immune system in the control of cancers is increasingly being recognized. Tumors use multiple inhibitory pathways to suppress the immune response and thus lead to "immune escape" (1). The clinical success of therapies that block these inhibitory pathways, such as anti-PD-1 (2) and anti-CTLA-4 (3) mAbs, underlines the potential of immunotherapy to treat cancer in a more specific fashion with fewer adverse events than with conventional cancer therapies.

One mechanism of tumor-induced immune suppression is the generation of adenosine by the ectoenzyme CD73 (4). In terms of the generation of adenosine, CD73 sits downstream of the

ectoenzyme CD39 that catalyzes the degradation of ATP into AMP via the intermediate ADP. AMP then acts as a substrate for CD73, resulting in the production of immunosuppressive adenosine (4). A major enzyme for the degradation of adenosine is adenosine deaminase (ADA). ADA can be expressed on the cell surface when bound to CD26; however, CD26 expression is reduced on tumor cells (5). Thus, the tumor microenvironment contains concentrations of adenosine sufficient to inhibit antitumor T-cell responses (6).

CD73 is endogenously expressed on epithelial cells, endothelial cells, and some immune subsets, and its expression has also been observed in several cancer types, including colon cancer, melanoma, breast cancer, ovarian cancer, and leukemia (4). Notably, the expression of CD73 by tumor cells in patients with triple-negative breast cancer correlates with an increased risk of metastasis and consequently poor patient outcomes (7, 8). The drivers of CD73 expression within the tumor microenvironment include hypoxia, type I IFNs, TNF α , IL1 β , prostaglandin (PG)_{E2}, and TGF β (4). Moreover, it has been shown that certain chemotherapies, including anthracyclines, can drive CD73 expression, contributing to chemoresistance of tumor cells (8). The protumoral effect of CD73 has been validated in preclinical models showing that targeting CD73 with a mAb can reduce primary tumor growth and metastasis (9). Moreover, anti-CD73 has been shown to enhance the efficacy of chemotherapy and other immunotherapies (8, 10).

Although targeting CD73 directly has shown great therapeutic promise, there is as yet no clinical development of anti-CD73 mAbs. However, because the immunosuppressive effects of CD73 are mediated by adenosine, targeting the downstream adenosine

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receptors has high translational potential. Activation of A_{2A} and A_{2B} receptors is known to suppress multiple immune cells, including the proliferation, cytokine production, and cytotoxicity of T cells (4, 11). Notably, in the context of cancer, activation of either A_{2A} or A_{2B} receptors has been shown to reduce antitumor immunity (6, 12, 13) and enhance the metastasis of CD73⁺ tumors (14). Moreover, antagonists targeting the A_{2A} and A_{2B} receptors have undergone clinical trials for other indications and have shown a good safety profile (15, 16).

In this study, we investigated whether blockade of A_{2A} receptors could enhance the efficacy of anti-PD-1. Because PD-1 blockade is known to enhance T-lymphocyte function, and A_{2A} expression is increased on T lymphocytes following activation (17), we hypothesized that A_{2A} blockade would enhance the efficacy of anti-PD-1 by augmenting antitumor T-lymphocyte responses.

Materials and Methods

Cell lines and mice

The C57BL/6 mouse breast carcinoma cell line AT-3 was obtained from Dr. Trina Stewart (Griffith University, Nathan, QLD, Australia; ref. 18), MC38 cells were obtained from Dr. Nicole Haynes (Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia), and 4T1.2 cells were obtained from Prof. Robin Anderson (Peter MacCallum Cancer Centre). Tumor cell lines were transduced to express chicken ovalbumin peptide as previously described (19, 20). These cell lines were originally obtained from the ATCC, actively passaged for less than 6 months, and were authenticated using short tandem repeat profiling. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the use of cell lines in Cancer research were followed. Tumor lines were also verified to be *Mycoplasma* negative by the Victorian Infectious Diseases References Lab (Melbourne, VIC, Australia) by PCR analysis. Tumor cells were grown in DMEM supplemented with 10% FCS, GlutaMAX, and penicillin/streptomycin. For *in vivo* experiments, the indicated numbers of cells were resuspended in PBS and injected s.c. in a 100- μ L volume. C57BL/6 and BALB/C wild-type (WT) mice were obtained from the Animal Resources Centre (Perth) and the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia), OT-I mice, perforin^{-/-}, and IFN γ ^{-/-} mice were bred in-house at the Peter MacCallum Cancer Centre, and A_{2A}^{-/-} mice were bred at St. Vincent's Hospital (Melbourne, VIC, Australia).

Vectors and tumor cell transduction

Murine CD73 cDNA (Open Biosystems) was cloned into the pMSCV-Cherry retroviral vector. Retroviruses were made by transfection of either pMSCV-Cherry or pMSCV-Cherry-CD73 in combination with the pMD2.G envelope vector into HEK293gagpol cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. AT-3ova^{dim} and MC38ova^{dim} were transduced with pMSCV Cherry-CD73 as previously described (14).

Antibodies, agonists, and antagonists

5'-(N-Ethylcarboxamido)adenosine (NECA) and SCH58261 were purchased from Sigma. mAbs RMP1-14 (anti-PD-1 mAb), 2A3 (Isotype control), and RMT3-23 (anti-TIM-3) were purchased from BioXcell. 145-2C11 (anti-CD3 mAb) used for splenocyte

stimulation was purchased from BD Pharmingen and SIINFEKL peptide was purchased from GenScript.

OT-I cocultures

OT-I splenocytes were isolated and labeled with CFSE (1 μ mol/L) as per the manufacturer's instructions (eBioscience). CFSE-labeled OT-I splenocytes were cocultured with Mitomycin-treated (5 μ mol/L) MC38 ova tumor cells in the presence of SIINFEKL peptide (10 ng/mL). To indicated wells, NECA (1 μ mol/L), SCH58261 (1 μ mol/L), anti-PD-1 (50 μ g/mL), or 2A3 isotype control were added. After 4 days, supernatants were taken for analysis of cytokine content and cells were analyzed by flow cytometry for CFSE dilution, expression of Granzyme B, and T-cell activation markers.

Analysis of adenosine receptor expression by RT-PCR

RNA was isolated from T lymphocytes using the Qiagen RNeasy Mini Kit as per the manufacturer's instructions. To generate cDNA, mRNA was added to 1 μ L of oligo dT and incubated at 65°C for 5 minutes. RNA was then added to a mixture of 4 μ L 5 \times cDNA synthesis buffer (Promega), 2 μ L 10 nmol/L dNTP mix (Promega), 1 μ L 0.1 mol/L DTT (Promega), 1 μ L RNase inhibitor (Promega), 1 μ L Reverse Transcriptase (Promega), and 1 μ L RNAase free H₂O. cDNA was generated at 50°C for 50 minutes and 85°C for 5 minutes before storage at -20°C until analysis of mRNA expression by qRT-PCR. Adenosine receptor expression was determined using the TaqMan Gene Expression Master Mix and primers for A_{2A} and A_{2B} as previously described (9). qRT-PCR for L32 and IFN γ was conducted with SYBR Green (Agilent) as per the manufacturer's instructions using the following primers: L32 forward: TTCCTGGTCCACAATGTCAAG, L32 reverse: TGTGAGCGATCTCAGCAC. IFN γ forward: GCAACAGCAAGCGCAAAAAG and IFN γ reverse: AGCTCATTGAATGCTTGGCG.

Analysis of tumor-infiltrating immune subsets

After euthanizing mice, tumors were excised and digested using a mix of 1 mg/mL collagenase type IV (Sigma-Aldrich) and 0.02 mg/mL DNAase (Sigma-Aldrich). After 30 minutes digestion at 37°C, cells were twice passed through a 70- μ m filter. Single-cell suspensions were then prepared and analyzed by flow cytometry as previously described (14). IFN γ and Granzyme B expression was determined directly *ex vivo* using antibody clones XMG1.2 and GB11, respectively.

Determination of cAMP content of human PBMCs

PBMCs from a normal donor were isolated using Ficoll and cultured for 10 days in IMDM medium (Life Technologies) containing 30 ng/mL of OKT3 (eBioscience) and 500 U of recombinant human IL2 (Prometheus Laboratories). Activated PBMCs were resuspended in serum-free IMDM containing 500 μ mol/L IBMX (Sigma) and 100 μ mol/L Ro 20-1724 (Sigma) to block phosphodiesterases. SYN115 at 10 μ mol/L was added to the cells 15 minutes before the addition of the A_{2A} selective agonist CGS21680 (1 μ mol/L). Cells were incubated with the compounds for 1 hour at 37°C and cAMP production was assayed using the cAMP Glo kit (Promega), according to the manufacturer's instructions.

Statistical analysis

Statistical differences were analyzed by the Mann-Whitney test with $P < 0.05$ considered statistically significant.

Results

Combination treatment with anti-PD-1 and A_{2A} blockade enhances the IFN γ production of CD8 $^+$ T cells *in vitro*

To explore the link between the A_{2A} and PD-1 suppressive pathways, we first assessed the *in vitro* effector functions of ova-specific CD8 $^+$ OT-I cells when in coculture with MC38ova dim tumor cells and SIINFEKL peptide (10 nmol/L). SIINFEKL peptide was added to the cultures because robust activation of naïve OT-I cells was not observed in the absence of SIINFEKL peptide. Cocultures were performed in the presence or absence of the adenosine analogue NECA (1 μ mol/L), the A_{2A} receptor antagonist SCH58261 (1 μ mol/L), anti-PD-1 mAb (50 μ g/mL), and/or isotype control (2A3). In the presence of NECA, the combination of anti-PD-1 and SCH58261 significantly enhanced IFN γ production ($P < 0.05$) when compared with single treatment with either agent (Fig. 1A). In the absence of exogenous NECA, neither single nor dual blockade of A_{2A} and PD-1 significantly enhanced IFN γ levels, indicating that in this assay exogenous adenosine (NECA) was required (Supplementary Fig. S1A). In contrast to the effects on IFN γ production, neither anti-PD-1 nor SCH58261 had a significant effect on proliferation as measured by CFSE dilution in this setting (Fig. 1B). The effect of A_{2A} and PD-1 blockade on the cytotoxic activity of OT-I T cells was also investigated. Although it was observed that NECA showed a trend to decrease the expression of Granzyme B by CD8 $^+$ OT-I cells, and that Granzyme B expression could be significantly enhanced by the addition of either SCH58261 or anti-PD-1, there was no further increase in Granzyme B expression following dual PD-1 and A_{2A} blockade (Supplementary Fig. S1B). Furthermore, the cytotoxic activity of OT-I cells against MC38ova dim tumor cells following either A_{2A} or PD-1 blockade as measured by ^{51}Cr release or CD107a staining was not modulated (Supplementary Fig. S1C and S1D). Taken together, these data indicate that combined blockade of A_{2A} and PD-1 has the capacity to enhance IFN γ production by CD8 $^+$ T lymphocytes in the presence of the adenosine analogue NECA and led us to evaluate this combination therapy *in vivo*.

A_{2A} blockade enhances the efficacy of anti-PD-1 mAb against CD73 $^+$ tumors

Because we hypothesized that adenosine may limit the efficacy of PD-1 blockade, we investigated whether the expression of CD73 by tumor cells rendered them more resistant to anti-PD-1 mAb. CD73 was ectopically expressed in the breast carcinoma cell line AT-3ova dim and the colon carcinoma MC38ova dim (Supplementary Fig. S2A). Although both tumor lines expressed A_{2B} , and to a lesser extent, A_1 receptors, the expression of A_{2A} was very low on AT-3 ova dim tumor cells and undetectable on MC38 ova dim tumor cells (Supplementary Fig. S2B). Furthermore, exposure of these tumor lines to NECA *in vitro* did not modulate their proliferation (Supplementary Fig. S3A) or phenotype, including expression of PD-L1 (Supplementary Fig. S3B). Taken together, this suggests that any effect of A_{2A} blockade would be mediated by modulation of the tumor microenvironment rather than tumor cells themselves.

Tumors were injected s.c. into mice and treated at day 14 once they were established (20–50 mm 2). Treatment of AT-3ova dim tumors with anti-PD-1 mAb resulted in a profound and significant reduction in primary tumor growth (Fig. 2A) with a mean reduction in tumor size at day 14 after therapy of 81% (Fig. 2B). However, the efficacy of anti-PD-1 mAb against AT-3ova dim CD73 $^+$ tumors was significantly less than against AT-3ova dim control tumors, with a mean reduction of tumor size of only 31% (Fig. 2B). These data thus show that the expression of CD73 reduced the efficacy of anti-PD-1 mAb single-agent therapy.

We hypothesized that the increased resistance of AT-3ova dim CD73 $^+$ tumors to anti-PD-1 monotherapy was due to A_{2A} -mediated suppression of antitumor immune responses induced by anti-PD-1 mAb. To investigate this possibility, we determined whether treatment of mice with the A_{2A} antagonist SCH58261 could enhance the therapeutic efficacy of anti-PD-1. Although A_{2A} blockade alone had no effect on the growth of AT-3ova dim CD73 $^+$ tumors as a single therapy (Fig. 2A), it significantly enhanced the efficacy of anti-PD-1 mAb in reducing the growth of AT-3ova dim CD73 $^+$ tumors (Fig. 2A). The combination of anti-PD-1 and SCH58261 (median survival, 40 days) also significantly increased the survival of mice compared with both isotype control/vehicle-

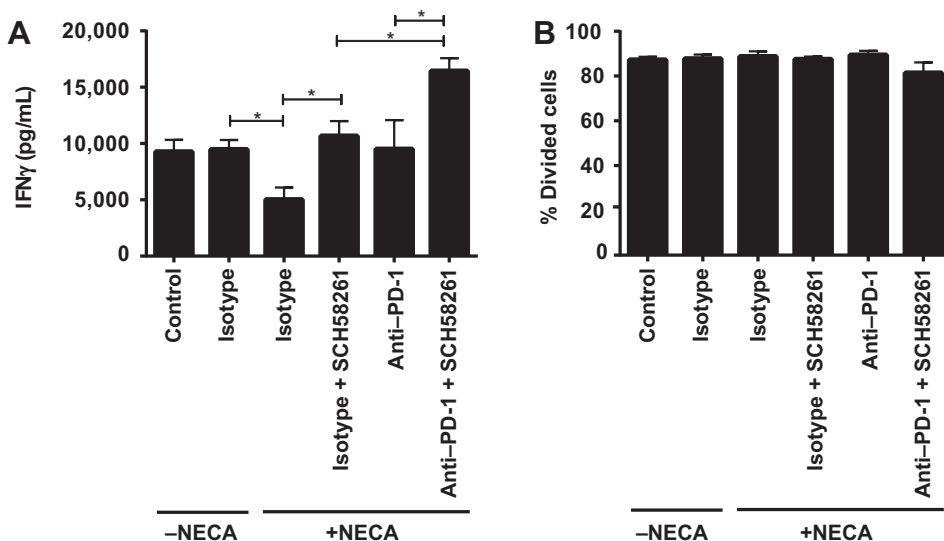
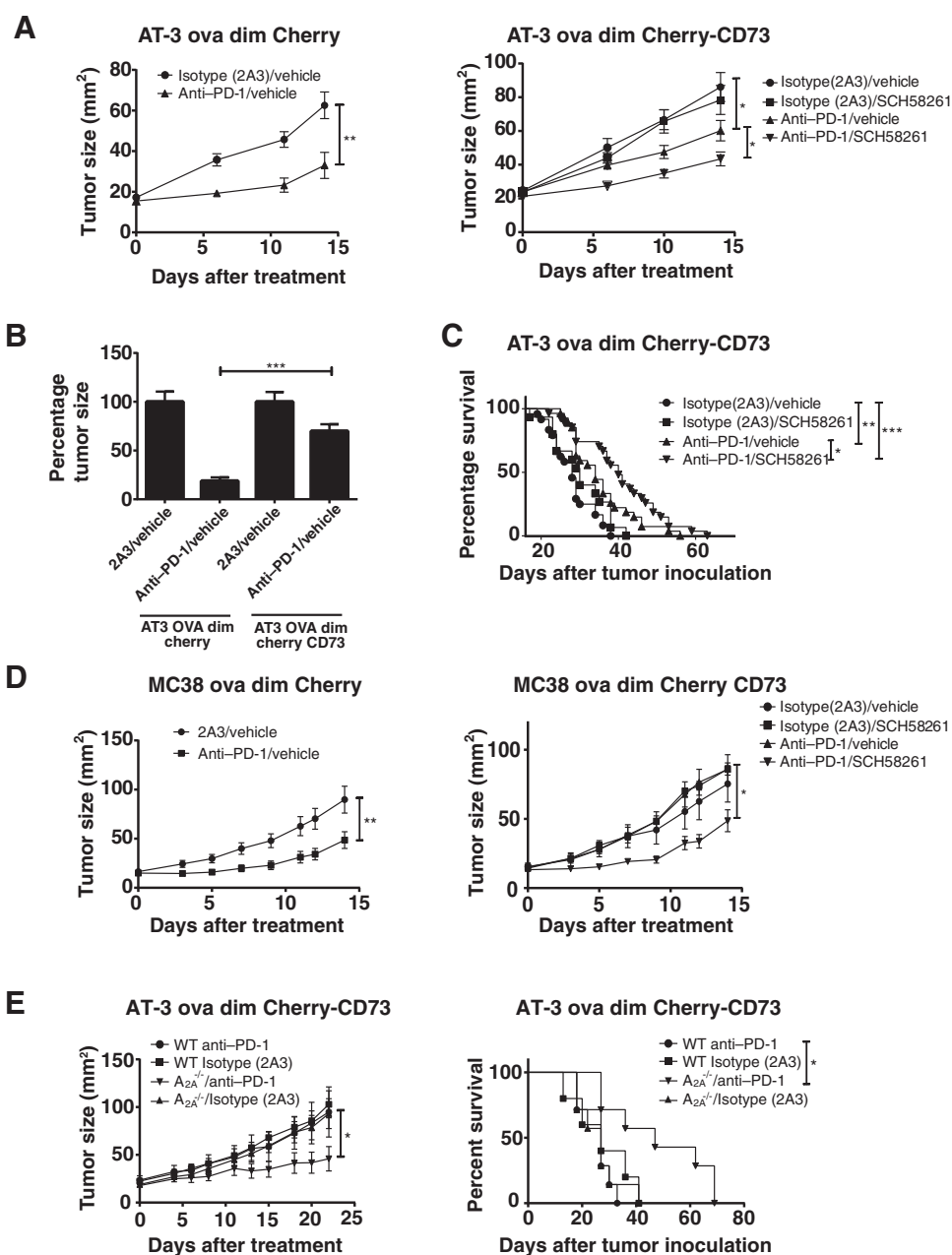


Figure 1.

Combination of A_{2A} receptor and PD-1 blockade enhances IFN γ production of OT-I splenocytes cocultured with MC38ova dim tumor cells. CFSE (1 μ mol/L)-labeled OT-I splenocytes were cultured with Mitomycin C (5 μ mol/L)-treated MC38ova dim tumor cells and SIINFEKL peptide (10 ng/mL) in the presence or absence of anti-PD-1 (50 μ g/mL), SCH58261 (1 μ mol/L), NECA (1 μ mol/L), and/or 2A3 isotype control (50 μ g/mL) for 4 days. Supernatants were collected and IFN γ concentration was determined by ELISA (A), and the percentage of TCR β^+ CD8 $^+$ cells that had divided was determined by flow cytometry (B). Data are pooled from four independent experiments and presented as mean \pm SEM. *, $P < 0.05$.

**Figure 2.**

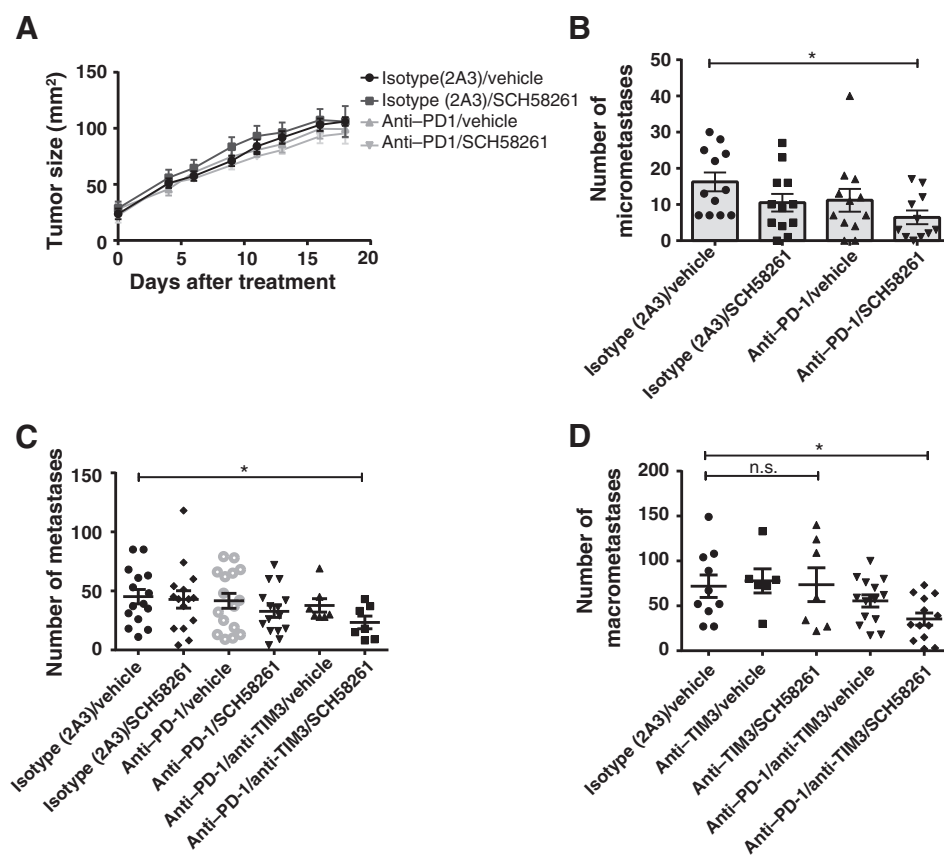
A_{2A} receptor blockade enhances the efficacy of anti-PD-1 against established tumors. A–E, C57BL/6 WT or (E) A_{2A}^{-/-} mice were injected s.c. with 5×10^5 AT-3ova^{dim} Cherry (A and B), 5×10^5 AT-3ova^{dim} Cherry-CD73⁺ (A, B, and E), 1×10^6 MC38ova^{dim} Cherry (D), or 1×10^6 MC38ova^{dim} Cherry-CD73⁺ (D) tumor cells. A–E, mice were treated i.p. at days 14, 18, 22, and 26 with either isotype control (2A3; 200 μ g/mouse) or anti-PD-1 mAb (RMP1-14, 200 μ g/mouse) and where indicated SCH58261 (1 mg/kg) or vehicle control at days 14 to 29. Tumor growth and survival were monitored. A, tumor growth presented as means of 11 to 17 mice per group \pm SEM. B, the mean percentage tumor size \pm SEM at day 14 after therapy for 11 to 17 mice per group. C, survival of mice is shown from four pooled independent experiments ($n = 15$ –27 per group). Survival was determined as when tumors grew to a size greater than 100 mm². D and E, data represented as the mean \pm SEM of 5 to 12 mice per group for tumor growth curves and 11 to 13 mice per group for survival graph. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

treated mice and anti-PD-1/vehicle-treated mice (median survival, 28 and 34 days, respectively; Fig. 2C). To extend these observations to another tumor model, we also compared the efficacy of anti-PD-1 mAb against established MC-38ova^{dim} and MC38ova^{dim} CD73⁺ tumors. Similarly as with the AT-3ova^{dim} tumors, CD73 expression on MC38ova^{dim} tumor cells reduced the efficacy of anti-PD-1 mAb treatment (Fig. 2D). Furthermore, as with AT-3ova^{dim} CD73⁺ tumors, the resistance of MC38ova^{dim} CD73⁺ tumors to PD-1 blockade was overcome when anti-PD-1 was given in combination with SCH58261 (Fig. 2D). This result indicated that A_{2A} blockade could enhance the efficacy of anti-PD-1 mAb in two distinct tumor models. To confirm that the A_{2A} antagonist SCH58261 was acting through host A_{2A} receptors, we determined the efficacy of anti-PD-1 mAb in WT and A_{2A}^{-/-} mice. Indeed, anti-PD-1 mAb therapy had

significantly increased efficacy in A_{2A}^{-/-} mice in terms of both primary tumor growth and survival (Fig. 2E), confirming the importance of host A_{2A} receptors in limiting the antitumor immune response induced by PD-1 blockade.

Having clearly demonstrated that the combination of PD-1 and A_{2A} blockade could treat established primary tumors, we next investigated the ability of this combination to treat the less immunogenic 4T1.2 tumor cell line. To examine this, we injected the 4T1.2 breast cancer line, which expresses CD73 endogenously, into the fourth mammary fat pad of WT mice. Mice were treated with either isotype control (2A3) or anti-PD-1 and SCH58261 or vehicle control. The combination of anti-PD-1 and SCH58261 was ineffective in reducing the primary tumor growth of 4T1.2 tumors (Fig. 3A), perhaps attributable to the low level of PD-1

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**Figure 3.**

A_{2A} blockade enhances the antimetastatic activity of PD-1/TIM-3 blockade. A and B, 5×10^4 4T1.2 tumor cells were injected into the fourth mammary fat pad. Seven days after injection mice were treated with either anti-PD-1, 2A3 isotype control (200 μ g/ mouse), and either SCH58261 (1 mg/kg) or vehicle control. Antibody therapies were repeated 4 and 8 days after the onset of therapy and SCH58261/vehicle was given daily from day 0 to 22 after onset of therapy. A, tumor growth shown as mean \pm SEM of 5 to 7 mice per group of a representative experiment. B, lung metastases were evaluated following sectioning of the lungs at day 23 after therapy (day 30 after injection) and shown as the mean \pm SEM of 11 to 12 mice per group. C and D, 1×10^5 4T1.2 cells were injected i.v. into WT BALB/c mice. At days 3 and 7 after tumor injection mice were treated with 2A3 isotype control (200 μ g/mouse), anti-PD-1 (200 μ g/mouse), and/or anti-TIM-3 (200 μ g/mouse) and at days 3 to 13 with SCH58261 (1 mg/kg) or vehicle control. Lungs were harvested at day 14 and the number of metastases enumerated. Data shown as the mean \pm SEM of 7–15 mice per group. *, $P < 0.05$.

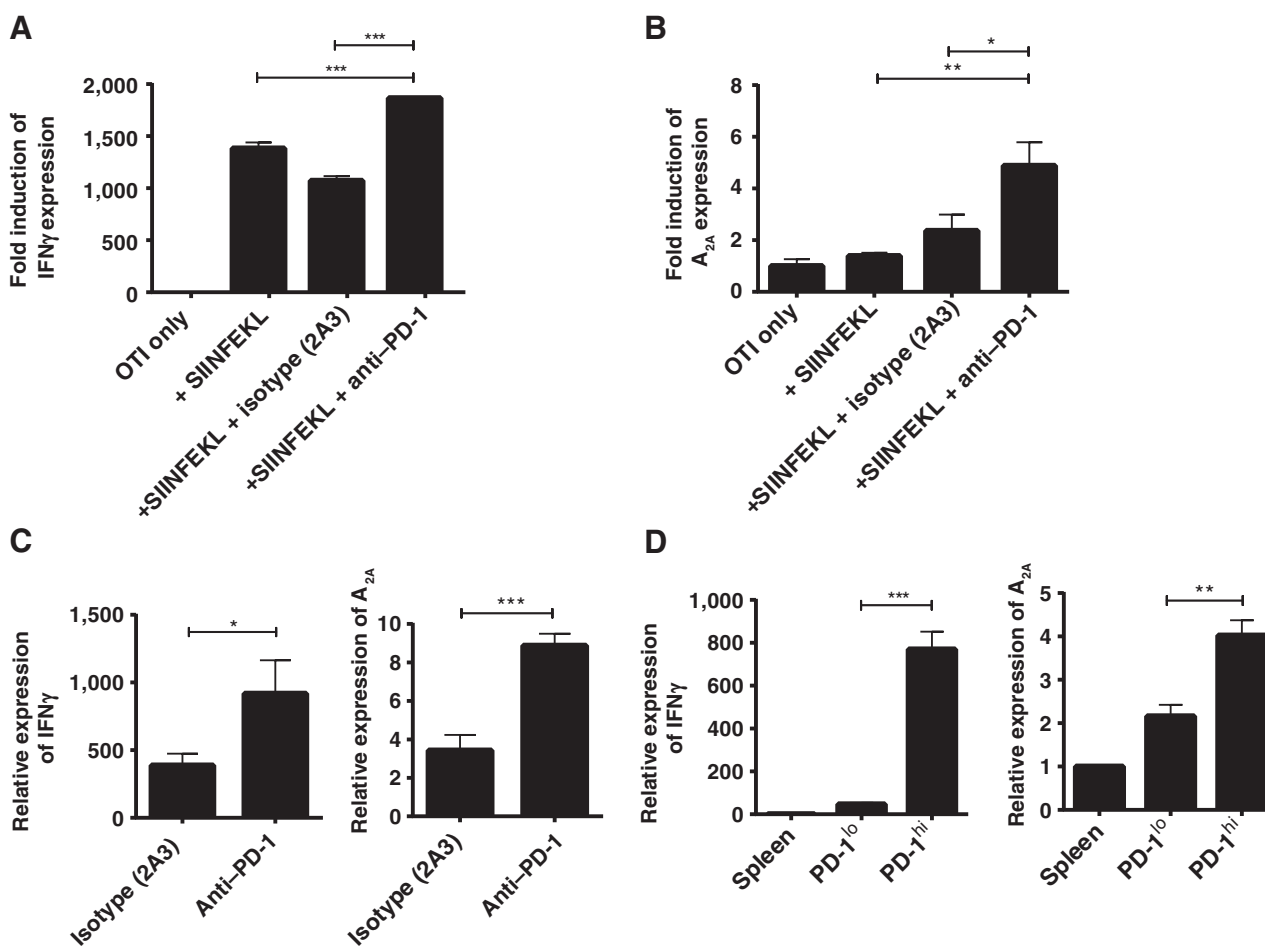
expression on T cells in this tumor model (Supplementary Fig. S4), and therefore reflecting the lower immunogenicity of this tumor line. However, the combination of anti-PD-1 and SCH58261 significantly reduced the number of spontaneous metastases in this setting (Fig. 3B). We next investigated the ability of anti-PD-1 and SCH58261 to treat established 4T1.2 lung metastases. 4T1.2 tumor cells were injected i.v., and after 3 days, mice were treated with a combination of anti-PD-1 or 2A3 isotype control mAb and SCH58261 or vehicle control. Neither anti-PD-1 nor SCH58261 was effective in reducing the number of metastases when given as a single agent (Fig. 3C). When anti-PD-1 mAb and SCH58261 were given in combination, there was a trend toward a reduction in metastases although this did not reach statistical significance.

It has previously been reported that $CD8^+$ T cells coexpress PD-1 and TIM-3 in the 4T1 breast cancer model and that anti-TIM-3 can significantly enhance the efficacy of PD-1 blockade (21). We therefore hypothesized that the inclusion of anti-TIM-3 in the treatment regimen may liberate antitumor T-cell responses sufficiently for A_{2A} blockade to become effective. Indeed, while anti-PD-1 and anti-TIM-3 showed a trend to reduce the number of tumor metastases, only the inclusion of A_{2A} blockade along with anti-PD-1 and anti-TIM-3 resulted in a significant reduction of tumor metastases (Fig. 3C). The combination of anti-TIM3 and SCH58261 had no significant effect on tumor metastases in the absence of anti-PD-1 (Fig. 3D), indicating that the blockade of multiple immunosuppressive pathways was required for the eradication of established 4T1.2 tumor metastases. Taken togeth-

er, these data highlight the ability of A_{2A} blockade to enhance the efficacy of checkpoint blockade.

PD-1 blockade enhances A_{2A} expression on $CD8^+$ tumor-infiltrating lymphocytes

Because A_{2A} expression is known to be increased following T-cell receptor (TCR) stimulation and PD-1 ligation is known to dampen TCR signaling (17, 22), we hypothesized that PD-1 blockade may increase A_{2A} expression on T lymphocytes and thus make them more susceptible to A_{2A} -mediated immunosuppression. To investigate this possibility, the expression of A_{2A} on OT-I splenocytes cocultured with (PD-L1 expressing) MC38ova^{dim} tumor cells *in vitro* was examined in the presence or absence of PD-1 blockade. A_{2A} expression was determined by qRT-PCR due to the lack of an antibody specific for the A_{2A} receptor suitable for flow cytometry. The expression of IFN γ was used as a positive control because it has previously been shown that PD-1 blockade enhances IFN γ production (23). As expected, PD-1 blockade significantly increased the expression of IFN γ by $CD8^+$ OT-I cells cocultured with MC38ova^{dim} tumor cells (Fig. 4A). Notably, PD-1 blockade also significantly enhanced the expression of A_{2A} receptors on $CD8^+$ OT-I cells compared with $CD8^+$ OT-I cells treated with an isotype control antibody (Fig. 4B). To investigate whether A_{2A} upregulation following PD-1 blockade also occurred *in vivo*, $CD8^+$ and $CD4^+$ T cells were isolated from AT-3ova^{dim} CD73⁺ tumors following treatment of mice with either anti-PD-1 or isotype control mAb. Notably, $CD8^+$ T cells isolated from tumors of mice treated with

**Figure 4.**

PD-1 blockade enhances A_{2A} receptor expression on CD8⁺ TILs. A and B, SIINFEKL pulsed OT-I T cells were cocultured with Mitomycin C (5 μ mol/L)-treated MC38ova^{dim} tumor cells in the presence or absence of SIINFEKL peptide (10 ng/mL) and either anti-PD-1 (50 μ g/mL) or 2A3 isotype control (50 μ g/mL). After 16 hours of coculture, cells were spun down, lysed, and analyzed for IFN γ (A) and A_{2A} (B) mRNA. Data shown are triplicate values \pm SD from one individual experiment representative of $n = 5$. C, TCR β^+ CD8⁺ lymphocytes were FACS sorted from AT-3ova^{dim} CD73⁺ tumors of mice 2 days after the mice were treated with either isotype control or anti-PD-1 mAb. D, TCR β^+ CD8⁺PD-1^{hi} and TCR β^+ CD8⁺PD-1^{low} cells were isolated from AT-3ova^{dim} CD73⁺ tumors at day 14 after tumor inoculation. C and D, cells were isolated from 5 mice per group and then pooled and lysed. IFN γ and A_{2A} expression was analyzed by qRT-PCR. Data are expressed relative to expression of naïve splenic CD8⁺ cells and presented as mean of triplicates \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

anti-PD-1 had significantly elevated expression of both IFN γ and the A_{2A} receptor compared with those isolated from isotype control-treated mice (Fig. 4C). Furthermore, isolation of the CD8⁺PD-1^{hi} and CD8⁺PD-1^{low} subsets revealed that the PD-1^{hi} population had significantly elevated expression of A_{2A} compared with PD-1^{low} counterparts (Fig. 4D). The increase in A_{2A} expression following PD-1 blockade was restricted to CD8⁺ T cells from within the tumor microenvironment as CD8⁺ T cells isolated from spleens and draining lymph nodes displayed no increase in A_{2A} expression (Supplementary Fig. S5A and S5B). In contrast, while CD4⁺ T cells displayed a trend for increased expression of IFN γ following PD-1 blockade, there was no increase in A_{2A} expression on these cells (Supplementary Fig. S5C). However, because the CD4⁺ tumor-infiltrating lymphocyte (TIL) population contains a significant proportion of foxp3⁺ cells (~50%), the possibility that PD-1 blockade modulates A_{2A} expression on either the foxp3⁻ effector cells or foxp3⁺ regulatory T cells cannot be discounted.

Taken together, these data indicate that PD-1 blockade enhances the expression of A_{2A} receptors on tumor-infiltrating CD8⁺ T cells and therefore increases their susceptibility to adenosine-mediated immunosuppression. These data may explain why A_{2A} blockade enhances the antitumor efficacy of anti-PD-1 but has little impact on the primary growth of AT-3ova^{dim} CD73⁺ tumor cells when administered as a single therapy (Fig. 2).

Combination of PD-1 and A_{2A} blockade significantly enhances the IFN γ production of tumor-infiltrating CD8⁺ T lymphocytes

To characterize the mechanism by which A_{2A} blockade enhanced the efficacy of PD-1 mAb *in vivo*, the number and phenotype of TILs were investigated in mice bearing AT-3ova^{dim} CD73⁺ tumors treated with either an isotype control antibody (2A3) or anti-PD-1 mAb and either SCH58261 or vehicle control. TILs were analyzed at days 1, 2, and 7 after treatment. Although the proportion of TCR β^+ CD8⁺, TCR β^+ CD4⁺foxp3⁻, and TCR β^+ CD4⁺foxp3⁺ TILs was not modulated by either

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anti-PD-1 alone or in combination with A_{2A} blockade (Supplementary Fig. S6A–S6C), we observed that the combination therapy significantly enhanced CD8⁺ T-cell effector functions. Analysis of TILs at day 1 showed that treatment with anti-PD-1 significantly increased the expression of IFN γ and Granzyme B by CD8⁺ TILs, which was not further enhanced following combination treatment with SCH58261 (Fig. 5A). However, 2 days following treatment, a significantly higher proportion of the CD8⁺ T cells isolated from mice treated with the combination therapy were IFN γ ⁺ and Granzyme B⁺ compared with those isolated from mice treated with anti-PD-1 mAb alone (Fig. 5A and B). The trend toward increased IFN γ production by CD8⁺ TILs in the combination group extended to day 7 after therapy with only the combination therapy giving a significantly enhanced proportion of IFN γ ⁺ CD8⁺ TILs compared

with isotype control/vehicle-treated mice (Supplementary Fig. S7). Thus, although the initial response to anti-PD-1 mAb was not affected by A_{2A} blockade, the induced CD8⁺ T-cell response was maintained by the A_{2A} antagonist SCH58261. In contrast, no increased expression of IFN γ was seen on CD4⁺ TILs following either anti-PD-1 mAb single-agent therapy or anti-PD-1 mAb/SCH58261 combination therapy at either time point (Supplementary Fig. S8A). We also investigated the effect of A_{2A} blockade on the expression of PD-1 on TILs. SCH58261 had no significant effect on PD-1 expression either in combination with PD-1 blockade (Supplementary Fig. S6) or on its own (Supplementary Fig. S9A). Moreover, A_{2A} blockade did not affect the expression of PD-L1 on tumor cells (Supplementary Fig. S9B), further suggesting that the efficacy of PD-1/A_{2A} blockade was due to enhanced T-cell effector function.

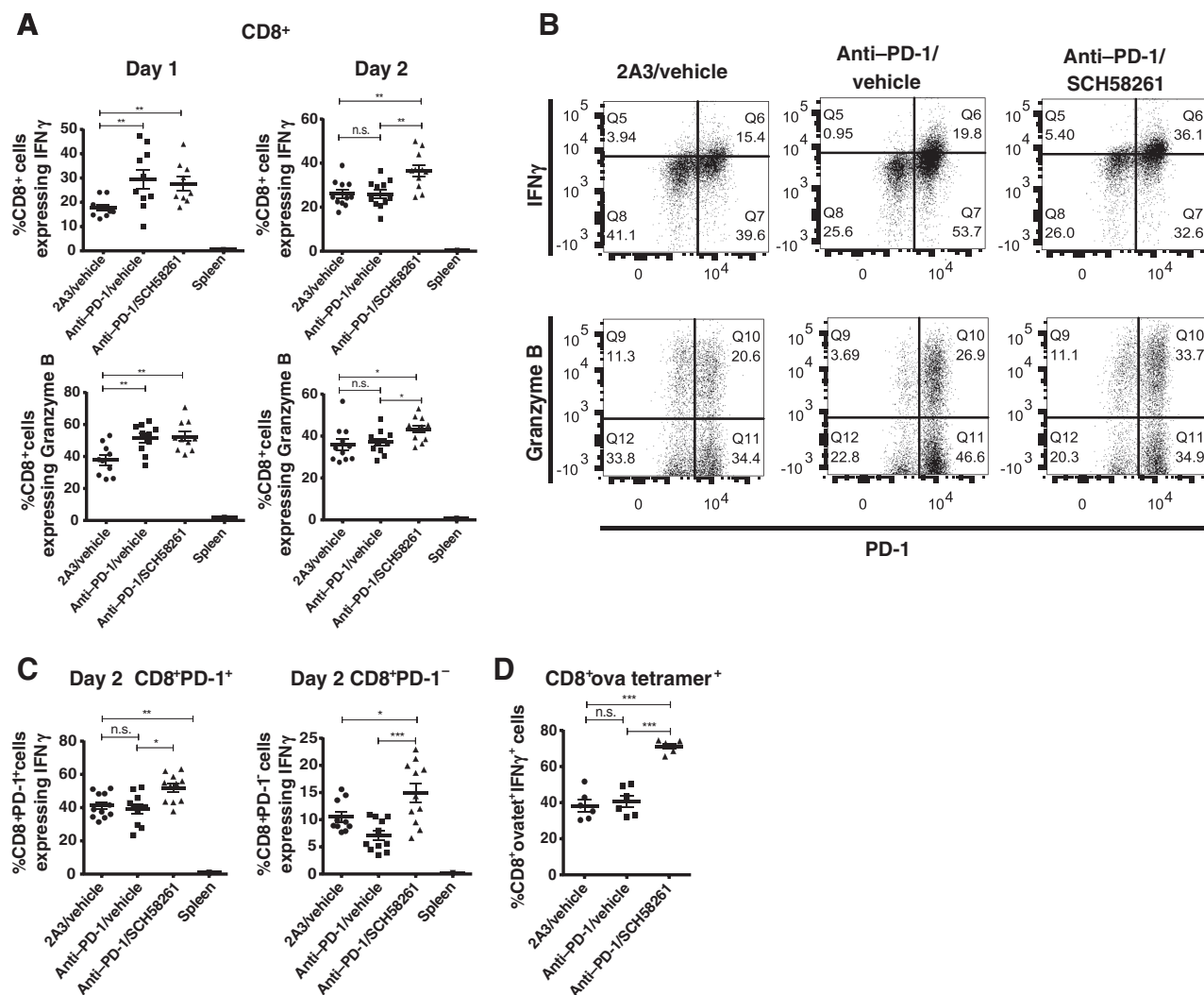


Figure 5.

Combined A_{2A} receptor and PD-1 blockade significantly enhances the IFN γ production of CD8⁺ TILs. C57BL/6 WT mice were injected s.c. with 5×10^5 AT-3ova^{dim} CD73⁺ tumor cells. After 14 days, mice were treated i.p. with either an isotype control antibody (2A3, 200 μ g/mouse) or anti-PD-1 mAb (RMP1-14, 200 μ g/mouse) and either SCH58261 (1 mg/kg) or vehicle control at days 0 and 1 after anti-PD-1 mAb therapy. TILs were isolated from AT-3ova^{dim} CD73⁺ tumors 1 to 2 days after treatment and analyzed for expression of IFN γ , Granzyme B, PD-1, and ova tetramer reactivity by flow cytometry. The expression of IFN γ and Granzyme B by splenic TCR β ⁺CD8⁺ cells is shown as a comparator. A, data shown for a minimum of 10 mice per group pooled from two separate experiments and shown as mean \pm SEM. B, representative flow cytometry analysis from one mouse per group 2 days after treatment. C and D, data represented as mean \pm SEM of $n = 5$ –11. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To further investigate the CD8⁺ T-cell populations modulated by the combination therapy, we analyzed IFN γ production and Granzyme B expression of the CD8⁺PD-1^{hi} cells and CD8⁺PD-1^{low} cells. Interestingly, dual anti-PD-1 mAb and SCH58261 treatment significantly increased the expression of IFN γ in both the CD8⁺PD-1^{hi} and CD8⁺PD-1^{low} subsets compared with anti-PD-1 alone (Fig. 5C). In contrast, Granzyme B expression was more selectively modulated by the combination therapy in the CD8⁺PD-1^{hi} population (Supplementary Fig. S8B). As with the analysis of the global CD8⁺ TIL population (Fig. 5A), the increase in IFN γ production by the CD8⁺PD-1^{hi} cells following combination therapy was seen at day 2 (Fig. 5C) but not at day 1 (Supplementary Fig. S8C), indicating that A_{2A} blockade maintained the activation induced by anti-PD-1 mAb.

We next investigated whether the antigen-specific CD8⁺ T-cell population was modulated by anti-PD-1 mAb/SCH58261 treatment. Although PD-1 blockade enhanced the proportion of CD8⁺ TILs that were ova tetramer specific, the addition of the A_{2A} antagonist did not further enhance this effect (Supplementary Fig. S8D). Notably, however, a significantly higher proportion of these ova tetramer⁺ TILs expressed IFN γ when isolated from anti-PD-1/SCH58261-treated mice compared with mice treated with anti-PD-1 mAb alone (Fig. 5D). No increase in IFN γ or Granzyme B was observed within CD8⁺ TILs in mice treated with SCH58261 in the absence of anti-PD-1, confirming that the combination of PD-1 and A_{2A} blockade was required for this effect (Supplementary Fig. S9C and S9D). Taken together, these data indicate that the enhanced efficacy of anti-PD-1 mAb/SCH58261 treatment was due to enhanced effector function of TILs as opposed to increased proliferation and/or recruitment. To determine the functional importance of increased IFN γ secretion and Granzyme B expression in terms of the antitumor immune response induced by the combination of anti-PD-1 mAb and SCH58261, the efficacy of this combination therapy was determined in IFN γ ^{-/-} and perforin^{-/-} mice. The effectiveness of anti-PD-1 mAb/SCH58261 was completely abrogated in IFN γ ^{-/-} mice but not perforin^{-/-} mice, indicating that the increased IFN γ production induced by the combination of SCH58261 and anti-PD-1 mAb was critical for its improved efficacy (Fig. 6).

Enhancement of anti-PD-1 function with the clinical trial drug SYN115

To enhance the translational relevance of our study, we also assessed the potential of SYN115, an A_{2A} antagonist that has

undergone phase IIb clinical trials (16), to enhance anti-PD-1 function. Established AT-3ova^{dim} CD73⁺ tumors were treated with an isotype control or anti-PD-1 mAb in combination with either SCH58261, SYN115, or vehicle. We observed that SYN115 had no single-agent activity, but significantly enhanced the antitumor efficacy of anti-PD-1 mAb (Fig. 7A) to a similar extent as SCH58261 (Fig. 7B). SYN115 was also able to significantly inhibit the increased cAMP concentrations mediated by the specific A_{2A} agonist CGS21680 on activated human PBMCs (Fig. 7C). Thus, an A_{2A} antagonist known to be safe and well tolerated in patients can enhance the antitumor efficacy of anti-PD-1 mAb, underlining the potential of this combination therapy to be translated into the clinic.

Discussion

It is increasingly recognized that immunotherapy is an effective strategy in several cancer types. In particular, recent results from clinical trials indicate that PD-1 blockade is a powerful emerging immunotherapeutic strategy (2). However, the success of anti-PD-1 therapy is limited to a subset of patients and thus further work is required to enhance the therapeutic benefit of anti-PD-1 and improve our understanding of where PD-1 blockade is most likely to provide patient benefit. For example, the success rate of PD-1 blockade can be improved if patients are selected on the basis of their tumors expressing the PD-1 ligand PD-L1 (2, 24).

The identification of additional biomarkers that are predictive of the response to anti-PD-1 mAb would be an important advance in our understanding, especially considering the prohibitive expense of immunotherapy. We have previously reported that CD73 is a negative prognostic marker for triple-negative breast cancer and predictive of a poor objective response to anthracycline therapy (8). Herein, we describe data indicating that the expression of CD73 by tumor cells also significantly reduces the efficacy of anti-PD-1 and that this can be overcome with blockade of A_{2A} receptors. We focused on the ability of A_{2A} blockade to enhance the efficacy of anti-PD-1 mAb because A_{2A} is upregulated on T cells following activation (17) and has an affinity for adenosine approximately 1,000-fold greater than that of A_{2B}. However, the blockade of other adenosine receptors such as A_{2B} may also enhance the therapeutic efficacy of anti-PD-1 either directly or through indirect mechanisms such as the modulation of dendritic cells (12) or myeloid-derived suppressor cells (25).

Because we have shown that the CD73:A_{2A} pathway can limit the efficacy of anti-PD-1 therapy, it would be interesting to

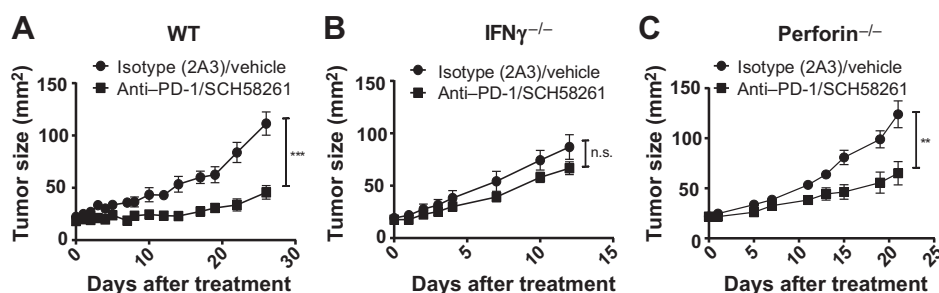
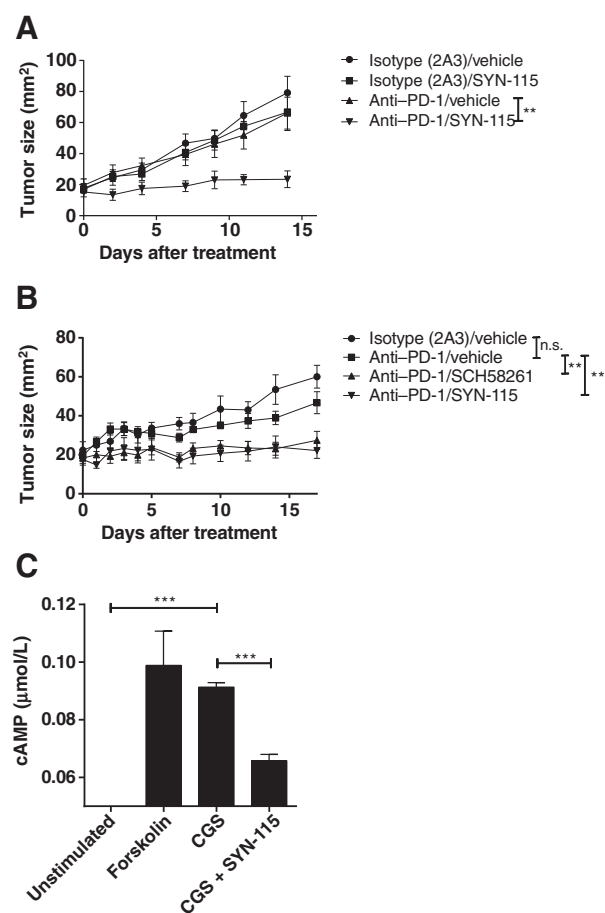


Figure 6. Combined A_{2A} receptor and PD-1 blockade is not effective in IFN γ ^{-/-} mice. AT-3ova^{dim} CD73⁺ tumor cells (5×10^5) were injected into C57BL/6 WT (A), IFN γ ^{-/-} (B), or perforin^{-/-} (C) mice. Mice were treated i.p. at days 14, 18, 22, and 26 with either isotype control (2A3; 200 μ g/mouse) or anti-PD-1 mAb (RMP1-14, 200 μ g/mouse) and where indicated SCH58261 (1 mg/kg) or vehicle control at days 14 to 29. Data represented as mean \pm SEM of $n = 6-7$. n.s., not statistically significant; **, $P < 0.01$; ***, $P < 0.001$.

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**Figure 7.**

The clinical trial A_{2A} receptor antagonist SYN115 enhances the efficacy of anti-PD-1 against established AT-3ova^{dim} CD73⁺ tumors. A and B, C57BL/6 WT mice were injected s.c. with 5×10^5 AT-3ova^{dim} CD73⁺ tumor cells. Mice were treated i.p. at days 14, 18, 22, and 26 with either an isotype control antibody (2A3, 200 µg/mouse) or anti-PD-1 mAb (RMP1-14, 200 µg/mouse) and either SCH58261 (1 mg/kg), SYN115 (1 mg/kg) or vehicle control at days 14 to 29. Data shown are mean \pm SEM of 5 mice per group for a representative experiment of $n = 3$. C, human PBMCs were activated with OKT3 and IL2 for 10 days. Cells (3×10^5) were then treated with either forskolin (250 µmol/L), CGS21680 (1 µmol/L), or SYN115 (10 µmol/L). ***, $P < 0.001$; **, $P < 0.01$; n.s., not statistically significant.

evaluate whether CD73 is a biomarker for the clinical responses to anti-PD-1 mAb therapy in patients. Recently, a phase I study reported that combining CTLA-4 and PD-1 blockade may improve the efficacy of either treatment alone (26). This is further supported by a large body of preclinical data that combining PD-1 blockade with other immunotherapies, including anti-CTLA-4 (27–30), anti-TIM-3 (21), anti-OX40 (31), anti-CD73 (10) anti-CD137 (32), anti-GITR (33), cancer vaccines (29), radiotherapy (34), chemotherapy (32), and adoptive cellular therapy (35), can significantly enhance antitumor immune responses. The results of a recent study suggested that PD-1 blockade could enhance the antimetastatic activity of A_{2A} blockade through enhanced natural killer (NK) cell responses (36). Our data indicate that A_{2A} blockade also enhances the efficacy of anti-PD-1 mAb in a primary tumor setting and warrant further investigation as a combination therapy. In contrast with these authors, how-

ever, we found that the enhanced efficacy of PD-1/ A_{2A} dual blockade was due to enhanced CD8⁺ T-cell function and found little expression of PD-1 on NK cells in the 4T1.2 tumor model. If both mechanisms are valid, this suggests that enhanced NK cell function is an indirect consequence of PD-1 blockade. Furthermore, we also show that A_{2A} blockade was able to enhance the efficacy of anti-PD-1 against established 4T1.2 lung metastases only when given in combination with anti-TIM-3. These data highlight that treatment of more established metastatic disease may require blockade of multiple inhibitory pathways.

The efficacy of A_{2A} antagonists when given in combination with anti-PD-1 is likely related to the fact that PD-1 blockade enhances the expression of A_{2A} receptors on tumor antigen-specific CD8⁺ TILs, therefore making them more susceptible to adenosine-mediated immunosuppression. The mechanism by which PD-1 blockade enhances A_{2A} expression is not known. However, A_{2A} expression is known to be increased in T lymphocytes following TCR activation (17) and PD-1 has been shown to suppress Ca²⁺ ERK responses immediately downstream of the TCR (22). Therefore, it seems likely that the mechanism by which PD-1 blockade increases A_{2A} expression is a direct consequence of enhanced TCR signaling, at least in part. Indeed, this hypothesis is supported by the fact that upregulation of A_{2A} on antitumor CD8⁺ T cells was seen both *in vitro* and *in vivo* following PD-1 blockade. Furthermore, because our data indicate that A_{2A} is more highly expressed on PD-1⁺ cells, the blockade of PD-1 may lead to the enrichment of the PD-1⁺ subset and consequently increase the proportion of CD8⁺ T cells susceptible to A_{2A} -mediated suppression. In this study, we did not observe that A_{2A} blockade significantly modulated PD-1 expression on CD8⁺ or CD4⁺ T cells. Although our observation appears to contrast with the observations of Cekic and Linden (37), the difference may be that in our study A_{2A} blockade was conducted in established tumors in which memory T cells have already differentiated or because the pharmacologic antagonist SCH58261 does not mediate full A_{2A} blockade. Therefore, the timing and magnitude of A_{2A} blockade may be crucial to the therapeutic outcome.

It has been proposed that the additional benefit of combined CTLA-4 and PD-1 blockade is attributable to the fact that PD-1 and CTLA-4 inhibit T-cell activation via distinct signaling pathways (38, 39). This is also the case for A_{2A} blockade. Although PD-1 signaling decreases the phosphorylation of proximal TCR signaling proteins, such as CD3 ξ , ZAP70, PI3K, and PKC θ , via the recruitment of SHP-1 and SHP-2 (38–41), A_{2A} activation acts to suppress T cells via enhanced cAMP concentrations. This process results in the activation of PKA, which consequently inhibits the activity of NFAT (42, 43), NFkB (44), and MAPK (45). Therefore, there is a precedent to evaluate the potential benefit of dual A_{2A} and PD-1 blockade in the clinic, especially among the cohort of patients who do not respond well to anti-PD-1 alone.

Although A_{2A} blockade did not enhance either the proportion of CD8⁺ T cells or the frequency of antigen-specific CD8⁺ cells, our findings indicate that the combination of PD-1 and A_{2A} blockade was able to significantly increase the production of IFN γ by antitumor T cells both *in vitro* and *in vivo*. Interestingly, while anti-PD-1 mAb single therapy was able to induce a similar upregulation of IFN γ on CD8⁺ cells 24 hours after treatment compared with combination therapy, the combination of A_{2A}

blockade and PD-1 blockade resulted in significantly more IFN γ ⁺ cells 48 hours after treatment. This indicates that A_{2A} blockade sustains the anti-PD-1 response and is likely due to the upregulation of A_{2A} receptors on CD8⁺ TILs following PD-1 blockade. Although the increase in IFN γ production was most marked in the antigen-specific CD8⁺PD-1^{hi} population, we also observed an increased production of IFN γ in the CD8⁺PD-1^{low} subset following combination therapy. The reason for this is not known but it is possible that the PD-1^{low} subset is indirectly affected because of proinflammatory cytokines secreted by the PD-1^{hi} cells, leading to increased activation of the PD-1^{low} cells themselves, as well as activation of other immune subsets, including APCs. We observed no increased expression of IFN γ by CD4⁺ T cells following PD-1 blockade despite expression of PD-1 by these TILs. This may be explained by the fact that CD4⁺ T cells are less sensitive to PD-1-mediated inhibition than CD8⁺ T cells (46). However, it should be noted that one caveat with these experiments is that we did not distinguish between foxp3⁻ and foxp3⁺ cells, which may have skewed our interpretation of these results. Nevertheless, the increased efficacy of PD-1 and A_{2A} blockade, compared with anti-PD-1 mAb alone, seems largely attributable to an increased IFN γ response from CD8⁺PD-1⁺ tumor antigen-specific TILs. Indeed, the combination therapy of PD-1/A_{2A} blockade was ineffective in IFN γ ^{-/-} mice. These results are consistent with observations by other groups that blockade of other checkpoint inhibitors, such as CTLA-4 or TIM-3 (21, 27, 47), can enhance IFN γ production by CD8⁺ TILs following anti-PD-1 mAb treatment.

Although our results suggest that the enhanced therapeutic activity of dual PD-1 and A_{2A} blockade is related to enhanced IFN γ responses, we also observed that A_{2A} blockade enhanced the expression of Granzyme B in CD8⁺ TILs when given in combination with anti-PD-1. Whether this translates to enhanced cytotoxic function is not clear, however, because we observed no evidence of PD-1 or A_{2A} blockade enhancing cytotoxic function of CD8⁺ T cells *in vitro*. This discrepancy may be explained by the activation status of the CD8⁺ T cells used in our *in vitro* assays because the cells used for typical *in vitro* "killing assays" are preactivated with high doses of IL2. It is known that activation of CD8⁺ T cells with IL2 can overcome the suppression mediated by PD-1 (46). Other groups have previously observed enhanced CTL function following PD-1 blockade (48, 49) using different experimental systems. Our experiments with perforin^{-/-} mice show that enhanced cytolytic function is not critical for the efficacy of the combination therapy. Notably, however, although the efficacy of the combination therapy was not abolished in perforin^{-/-} mice, the extent of tumor control appeared to be somewhat reduced in these mice when compared with WT controls. Thus, it remains possible that the efficacy of combination therapy *in vivo* may also be attributable in part to enhanced cytotoxic T-cell function.

In this study, we provide evidence that CD73 expression is a negative prognostic marker with regard to the therapeutic efficacy of PD-1 blockade and that A_{2A} antagonists may be a viable option

to improve the clinical efficacy of anti-PD-1 mAb therapy. Interestingly, it has recently been reported that A_{2A} blockade can also enhance the function of CTLA-4 mAb in a murine melanoma model (50). This suggests that A_{2A} blockade may be able to enhance the clinical efficacy of other checkpoint inhibitors. Several A_{2A} antagonists have undergone clinical trials for Parkinson disease, including istradefylline (KW-6002-phase III), preladenant (SCH-420814 phase II; ref. 15), and SYN-115 (phase IIb; ref. 16), the compound used in our studies. These drugs have thus far been found to be safe and well tolerated, indicating that they could be readily applied to patients with cancer. Therefore, our work using A_{2A} antagonists to block the CD73 pathway carries strong clinical relevance given that no anti-CD73 mAbs are in clinical development. Our studies have revealed a previously unreported function of SYN-115 in enhancing antitumor immunity, underlining the fact that the combination of PD-1 and A_{2A} blockade has immediate translational potential.

Disclosure of Potential Conflicts of Interest

J. Stagg has ownership interest (including patents) and is a consultant/advisory board member for Surface Oncology. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: P.A. Beavis, N. Milenkovski, P.K. Darcy

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.A. Beavis, N. Milenkovski, M.A. Henderson, L.B. John, B. Allard, P.K. Darcy

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.A. Beavis, N. Milenkovski, S. Loi, M.H. Kershaw, P.K. Darcy

Writing, review, and/or revision of the manuscript: P.A. Beavis, N. Milenkovski, M.A. Henderson, B. Allard, S. Loi, J. Stagg, P.K. Darcy

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.A. Beavis, N. Milenkovski, P.K. Darcy

Study supervision: P.A. Beavis, M.H. Kershaw, J. Stagg, P.K. Darcy

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