

Antimetastatic Effects of Blocking PD-1 and the Adenosine A_{2A} Receptor

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Abstract

Adenosine targeting is an attractive new approach to cancer treatment, but no clinical study has yet examined adenosine inhibition in oncology despite the safe clinical profile of adenosine A_{2A} receptor inhibitors (A_{2A}Ri) in Parkinson disease. Metastasis is the main cause of cancer-related deaths worldwide, and therefore we have studied experimental and spontaneous mouse models of melanoma and breast cancer metastasis to demonstrate the efficacy and mechanism of a combination of A_{2A}Ri in combination with anti-PD-1 monoclonal antibody (mAb). This combination significantly reduces metastatic burden and prolongs the life of mice compared with either monotherapy alone. Importantly, the combination was only effective when the tumor expressed high levels of CD73, suggesting a tumor biomarker that at a minimum could be used to stratify patients that might receive this combination. The mechanism of the combination therapy was critically dependent on NK cells and IFN γ , and to a lesser extent, CD8⁺ T cells and the effector molecule, perforin. Overall, these results provide a strong rationale to use A_{2A}Ri with anti-PD-1 mAb for the treatment of minimal residual and metastatic disease. *Cancer Res*; 74(14); 3652–8. ©2014 AACR.

Introduction

Tumor-induced immunosuppression is a major hurdle to the efficacy of current cancer therapies. Perhaps because of their remarkable clinical efficacy against a broader range of cancers, recent successes with immune checkpoint blockade inhibitors such as anti-CTLA-4 (ipilimumab; refs. 1 and 2) and anti-PD-1/PDL1 (nivolumab, MK-3475/MPDL3280A, MDX-1105; refs. 3–5) are revolutionizing cancer treatment. Immune checkpoints refer to a plethora of inhibitory pathways hard-wired into the immune system that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiologic immune responses in peripheral tissues to minimize collateral damage. Ipilimumab and nivolumab generated very effective responses in advanced melanoma when used in combination (6), but the former triggered a significant number of immune-related adverse events, even when used as single therapy (7), which may limit its potentially broader use in combinations. Because of the number of immunosuppressive

mechanisms utilized by tumors, it is becoming increasingly apparent that targeting multiple immunosuppressive pathways has the potential to enhance therapeutic efficacy without an increase in adverse events associated with excessive inflammation/autoimmunity (8).

One promising molecule that is a new target in preclinical studies is immunosuppressive adenosine. This metabolite is produced by the CD73 ectoenzyme expressed on host suppressor cells and tumor cells (9). Inhibition of CD73 using a monoclonal antibody (mAb) has been shown to reduce tumor growth and metastasis by enhancing antitumor immunity (10). Both host and tumor-expressed CD73 are important suppressive mechanisms, because knockdown or overexpression of CD73 on tumor cells can modulate tumor growth and metastasis (9–11). In addition, CD73^{-/-} mice are protected from transplanted and spontaneous tumors (10, 12, 13). In humans, high CD73 expression in triple negative breast cancer has been shown to be a negative prognostic marker and correlates with a high risk of metastasis (14, 15). The protumor effects of CD73 are believed to be largely because of adenosine-mediated immunosuppression. Adenosine binds to four known receptors A₁, A_{2A}, A_{2B}, and A₃, with the activation of A_{2A} and A_{2B} receptors known to suppress the effector functions of many immune cells (10). In the microenvironment of the tumor, both A_{2A} and A_{2B} receptor activation has been demonstrated to suppress antitumor immunity (11, 16, 17) and increase the spread of CD73⁺ tumors (9). In addition, either A_{2A} or A_{2B} blockade with small molecule antagonists can reduce tumor metastasis. Because A_{2A} receptor expression is increased in lymphocytes following activation (18), we hypothesized therapies that liberate lymphocyte effector responses, such as anti-CTLA-4, anti-PD-1, and anti-Tim3 (19–21), may also increase

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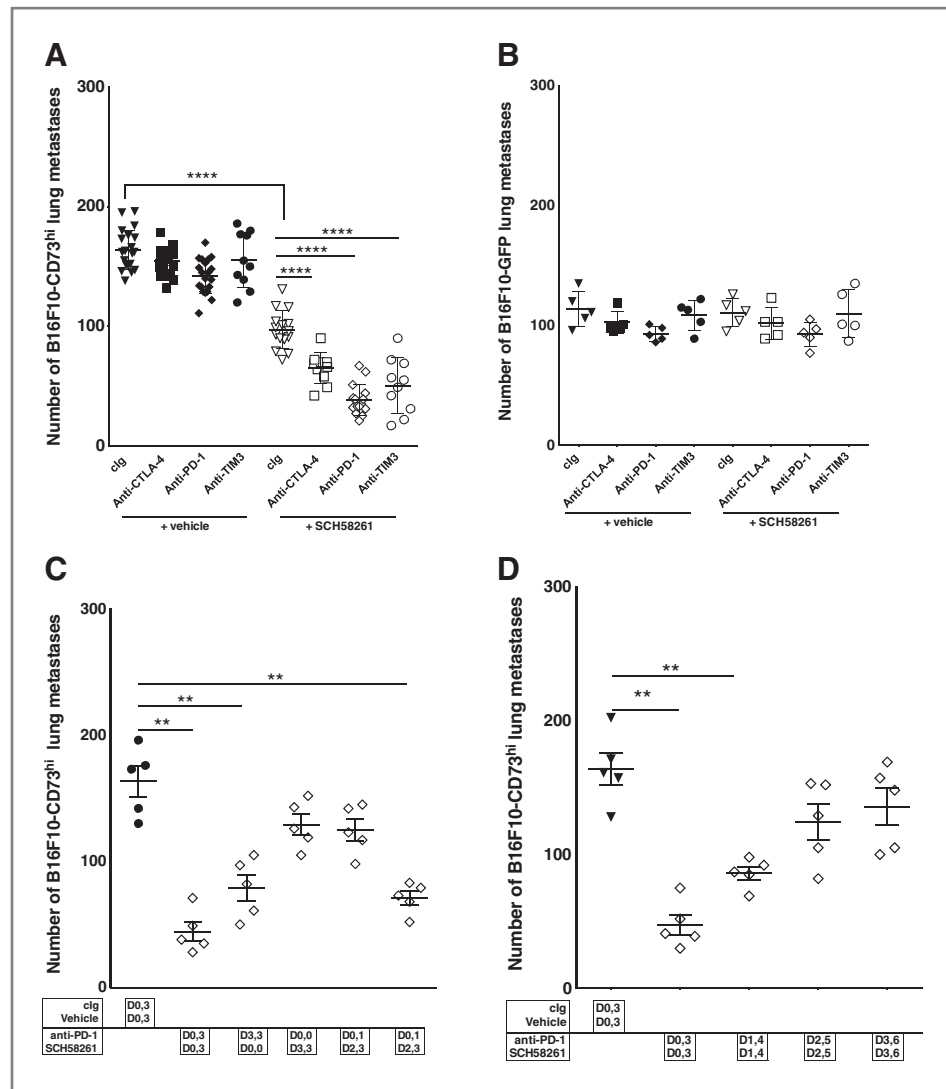
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Figure 1. Anti-PD-1 and A_{2A}Ri suppress experimental B16F10-CD73^{hi} lung metastasis. C57BL/6 WT mice were injected intravenously with B16F10-CD73^{hi} (A, C, and D) melanoma cells (1×10^5 cells) or B16F10-GFP (2×10^5 cells; B) on day 0. A and B, on day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of vehicle, A_{2A}Ri (SCH58261, 1 mg/kg), clg (2A3, 250 μ g), anti-CTLA-4 (UC10-4H10, 250 μ g), anti-PD-1 (RMP1-14, 250 μ g), anti-Tim3 (RMT3-23, 250 μ g), or the combination as indicated. C and D, anti-PD-1 and A_{2A}Ri combination is optimal following early coincident therapy. Mice were treated with intraperitoneal injections of vehicle, A_{2A}Ri (SCH58261, 1 mg/kg), clg (2A3, 250 μ g), anti-PD-1 (RMP1-14, 250 μ g), or the combination once or twice on days 0 to 6 after tumor inoculation as indicated. Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Results are pooled from one to four experiments and means \pm SEM of 5 to 20 mice per group are shown. Improved metastatic control of the combination was statistically significant compared with SCH58261 alone as indicated (****, $P < 0.0001$; **, $P < 0.01$; Mann-Whitney test).



the effects of A_{2A}-mediated immunosuppression. In this study, we investigated whether dual immune checkpoint blockade and A_{2A}R inhibitor could increase the magnitude of immune responses to metastasis.

Materials and Methods

Mice

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 perforin-deficient (pfp^{-/-}) and C57BL/6 DEREK (FoxP3-DTR-GFP) mice were bred in-house at the QIMR Berghofer Medical Research Institute. All mice were maintained at the QIMR Berghofer Medical Research Institute and used between the ages of 6 to 14 weeks. Groups of 5 to 10 mice per experiment were used for experimental and spontaneous tumor metastasis assays. These group sizes were used to ensure adequate power to detect biologic differences. All experiments were approved by the

QIMR Berghofer Medical Research Institute Animal Ethics Committee.

Cells

The C57BL/6 B16F10 (ATCC) and B16F10-CD73^{hi} melanomas and 4T1.2 mammary carcinomas were derived and maintained as described (9, 10). Tumor cells were grown in DMEM supplemented with 10% FCS, glutamax, and penicillin/streptomycin. For *in vivo* experiments, the indicated number of cells were resuspended in PBS and injected in a 200 μ L volume.

Antibodies and antagonists

Purified anti-mouse PD-1 mAb (RMP1-14), anti-mouse CTLA-4 mAb (UC10-4F10), anti-mouse Tim3 (RMT3-23) and control Ig (2A3) were purchased from BioXCell (West Lebanon) and used in the schedule and dose as indicated. SCH58261 was purchased from Sigma and used at 1 mg/kg i.p. per dose. Anti-IFN γ (H22), Rabbit anti-asialoGM1 antibody (Wako

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Chemicals), anti-CD4 (GK1.5), and anti-CD8 β (53-6.7) were resuspended in PBS and injected at the indicated time points.

In vivo treatments

For spontaneous metastasis and postsurgery survival experiments, 5×10^4 4T1.2 tumor cells were inoculated into the fourth mammary fat-pad of BALB/c mice. On day 20 to 22, mice were anesthetized, the primary tumor was surgically removed, the wound was closed with surgical clips, and treatments commenced immediately as indicated. For experimental metastasis, 1 or 2×10^5 B16F10 or B16F10-CD73^{hi} cells were injected intravenously in 200 μ L PBS and the treatment commenced immediately after (day 0) and day 3. B16F10 and B16F10-CD73^{hi} macrometastases on the surface of the lungs were counted using a dissecting microscope (Nikon SMZ 745T).

Flow cytometry

Eight- to 12-week-old C57BL/6 mice were injected with 2.5×10^5 B16F10-CD73^{hi} cells intravenously into the tail vein (10 mice per group). Mice were treated with SCH58261 (1 mg/kg) or anti-PD-1 (250 μ g/mouse) or the combination of both or with isotype control mAb (clone 2A3, 250 μ g/mouse) on day 0 and day 3. Mice were sacrificed on day 5 and lungs were collected after perfusion with PBS and mechanically dissociated using the Medimachine (Dako) to obtain cell suspensions. Hematopoietic cells were then purified using a discontinuous Percoll gradient and stained for flow cytometry.

The following antibodies were used: CD3 PerCPeFluor710 (500A2), CD8 APCeFluor780 (53-6.7), Foxp3 eFluor605NC (FJK-16s), NK1.1 CD106a PE (1D4B), CD45.2 PECy7 (104), CD4 Alexa Fluor 700 (RM4-5) from BD Biosciences and NK1.1 Alexa Fluor 488 (PK13) from Biolegend. Stained cells were acquired and analyzed by multicolor flow cytometry using an LSR Fortessa (BD Biosciences).

Analysis of adenosine receptor expression by RT-PCR

Total RNA was isolated from cancer cell lines and flow cytometry-sorted splenic lymphocytes by using RNeasy (Sigma-Aldrich) as per the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA was reverse transcribed in 20 μ L reaction containing 500 μ mol/L dNTP mix, 500 nmol/L Oligo-dT, 200U of Tetro Reverse Transcriptase and 20 U of Ribosafe RNase Inhibitor (Bioline) at 42°C for 50 min. The reactions were heated at 85°C for 5 minutes, cDNA product was diluted to 10 ng/ μ L and 2 μ L of cDNA was subjected to 10 μ L real-time PCR reaction using SensiFAST SYBR Lo-ROX Kit (Bioline) and primers (Sigma-Aldrich) on Applied Biosystems ViiA 7 Real-Time PCR system. Ct values for A_{2A} receptor were compared with the housekeeping gene RPL32 using the following mouse primers: L32 forward-TTCCTGGTCCACAATGTCAAG, L32 Reverse- TGTGAGCGATCTCAGCAC, A_{2A} receptor Forward- CAGAGTTCATCTTCAGCCTC, A_{2A} receptor Reverse-CACCCAGCAAATCGCAATG.

Statistical analysis

Statistical differences were analyzed by Mann-Whitney test with $P < 0.05$ considered significant. A log-rank test was

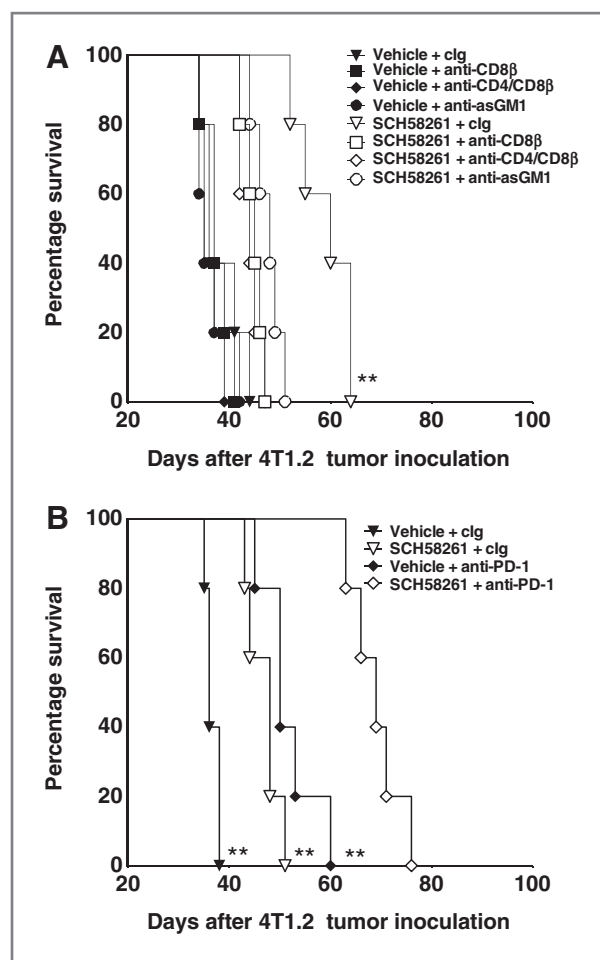


Figure 2. Anti-PD-1 and A_{2A} Ri suppress spontaneous 4T1.2 lung metastasis. Groups of 5 female BALB/c mice were injected in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (5×10^4). A, on day 20, the primary tumor was resected and mice were treated intraperitoneally with vehicle or SCH58261 (1 mg/kg) as indicated on day 20, 23, 26, and 29. Some groups of mice were depleted of NK cells or T cells by treatment with anti-asGM1, anti-CD8 β , or anti-CD4 and anti-CD8 β (100 μ g i.p.) on days 20, 21, 28, 35, and 42 as indicated. A_{2A} Ri requires both NK cells and T cells to suppress 4T1.2 lung metastasis. B, on day 18, the primary tumor was resected and mice were treated intraperitoneally with vehicle, SCH58261 (1 mg/kg), clg (2A3, 250 μ g), anti-PD-1 (RMP1-14, 250 μ g), or combinations as indicated on day 18, 21, 24, and 27. Survival of the mice was monitored. Asterisk indicates the combination group treated with clg is significantly different to all other groups (log-rank test, **, $P < 0.01$; A) and the statistically significant difference in groups compared with the combination treated group (by log-rank test, **, $P < 0.01$; B).

performed to assess the statistical significance of differences between survival curves. Prism (Graph pad Software) was used for graphs and statistical analysis.

Results and Discussion

A_{2A} R and PD-1 blockade combine to suppress experimental lung metastases

We have recently demonstrated the therapeutic activity of the A_{2A} Ri, SCH58261, in protecting mice from B16F10-CD73^{hi}

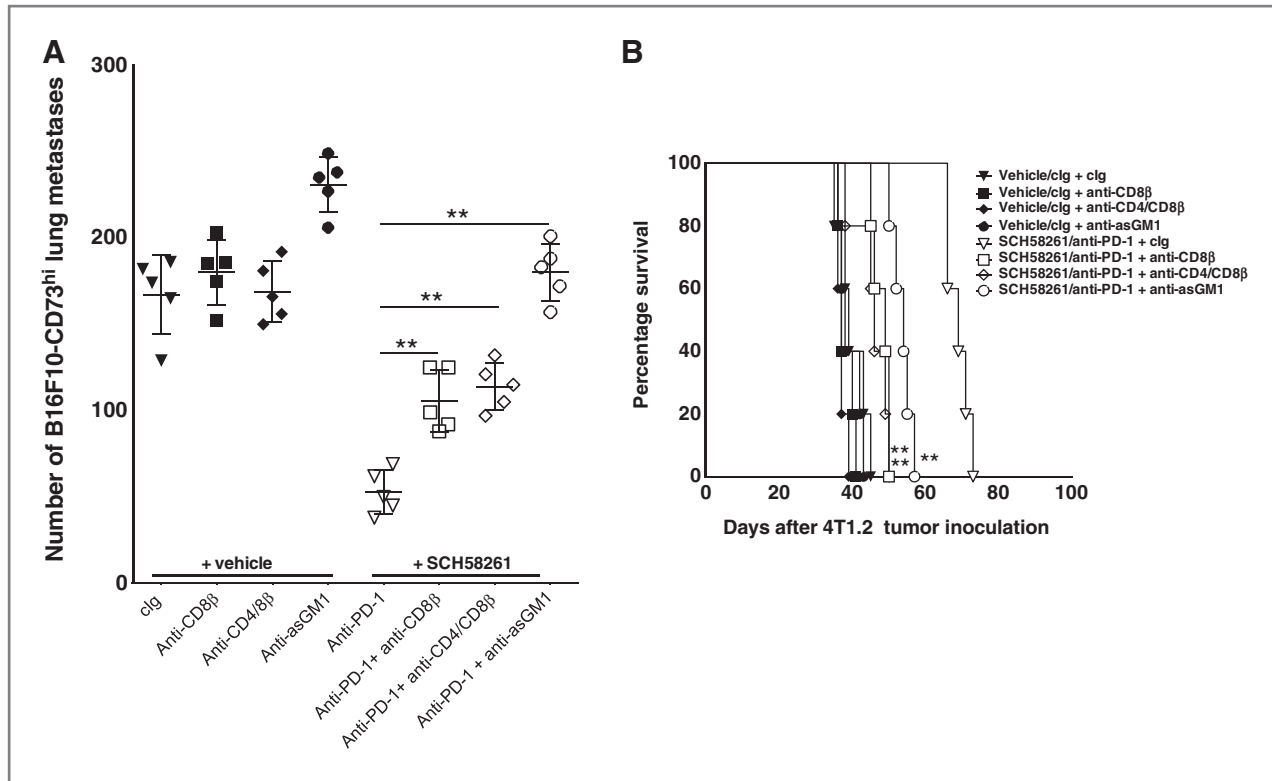


Figure 3. Anti-PD-1 and A_{2A}Ri suppression of experimental and spontaneous metastasis requires T cells and NK cells. **A**, C57BL/6 WT mice were injected intravenously with B16F10-CD73^{hi} melanoma cells (1×10^5 cells) on day 0. On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of vehicle, A_{2A}Ri (SCH58261, 1 mg/kg), clg (2A3, 250 μg), anti-PD-1 (RMP1-14, 250 μg), or the combination as indicated. Some groups of mice were depleted of NK cells or T cells by treatment with anti-asGM1, anti-CD8β or anti-CD4, and anti-CD8β (100 μg i.p.) on days -1, 0, and 7 as indicated. Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means \pm SEM of 5 mice per group are shown. Loss of metastatic control of the combination was statistically significant as indicated (**, $P < 0.01$, Mann-Whitney test). **B**, groups of 5 female BALB/c mice were injected in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (5×10^4). On day 22, the primary tumor was resected and mice were treated with intraperitoneal injections of vehicle, SCH58261 (1 mg/kg), clg (2A3, 250 μg), anti-PD-1 (RMP1-14, 250 μg), or combinations as indicated on day 22, 25, 28, and 31. Some groups of mice were depleted of NK cells or T cells by treatment with anti-asGM1, anti-CD8β or anti-CD4, and anti-CD8β (100 μg i.p.) on days 21, 22, 29, 36, and 43 as indicated. Survival of the mice was monitored. Asterisk indicates the combination group treated with clg is significantly different to all other groups as determined by log-rank test; **, $P < 0.01$.

experimental lung metastasis (9). Here we have confirmed that activity and examined A_{2A}Ri in the context of checkpoint blockade by additionally treating mice with anti-CTLA-4, anti-PD-1, or anti-Tim3 (Fig. 1A). Early treatment of B16F10-CD73^{hi} lung metastases by anti-CTLA-4, anti-PD-1, or anti-Tim3 mAb alone was relatively ineffective, compared with A_{2A}Ri treatment alone over the same period. However, each of 3 checkpoint blockade mAbs, further enhanced the activity of A_{2A}Ri when given coincidentally in combination (Fig. 1A). This combination and effect of A_{2A}Ri alone was specific for CD73^{hi} expressing B16F10, because neither A_{2A}Ri alone, nor in combination, was effective in suppressing lung metastases of the parental B16F10 cells (Fig. 1B). This suggests that combination therapy involving A_{2A}Ri may be successfully used for patients with high tumor cell expression of CD73, such as those with poor prognosis triple negative breast cancer (15). Given the promise of anti-PD-1 mAbs in the clinic, we decided to pursue further general utility and mechanism of this combination. Initially, we confirmed the effect of the combination against B16F10-CD73^{hi} melanoma metastases by varying the schedule of A_{2A}Ri and checkpoint blockade (Fig. 1C). Coincident treat-

ment of A_{2A}Ri and anti-PD-1 mAb on days 0 and 3 after tumor inoculation was superior to other schedules, although A_{2A}Ri given twice on day 0 and anti-PD-1 mAb twice on day 3; or A_{2A}Ri given on day 0 and 1 and anti-PD-1 given on days 2 and 3 were also effective. Late treatment with A_{2A}Ri seemed to be less effective (Fig. 1C) and delayed combination therapy was not significant when treatment commenced at day 2 onwards post tumor inoculation (Fig. 1D). These data indicated the combinatorial promise of checkpoint blockade and A_{2A}Ri and the potential use of tumor CD73 expression as a predictive marker of response to A_{2A}Ri or A_{2A}Ri and checkpoint blockade.

A_{2A}R and PD-1 blockade combine to suppress spontaneous lung metastases

The 4T1.2 mammary carcinoma expresses high levels of CD73 constitutively (10) and may be used as a model of primary tumor resection and metastasis to test therapies in a clinically relevant setting. We have previously shown that the A_{2A}Ri, SCH58261, can inhibit spontaneous lung metastases when given early to mice (from day 3) with primary mammary tumor intact (9). When mice were administered A_{2A}Ri four times

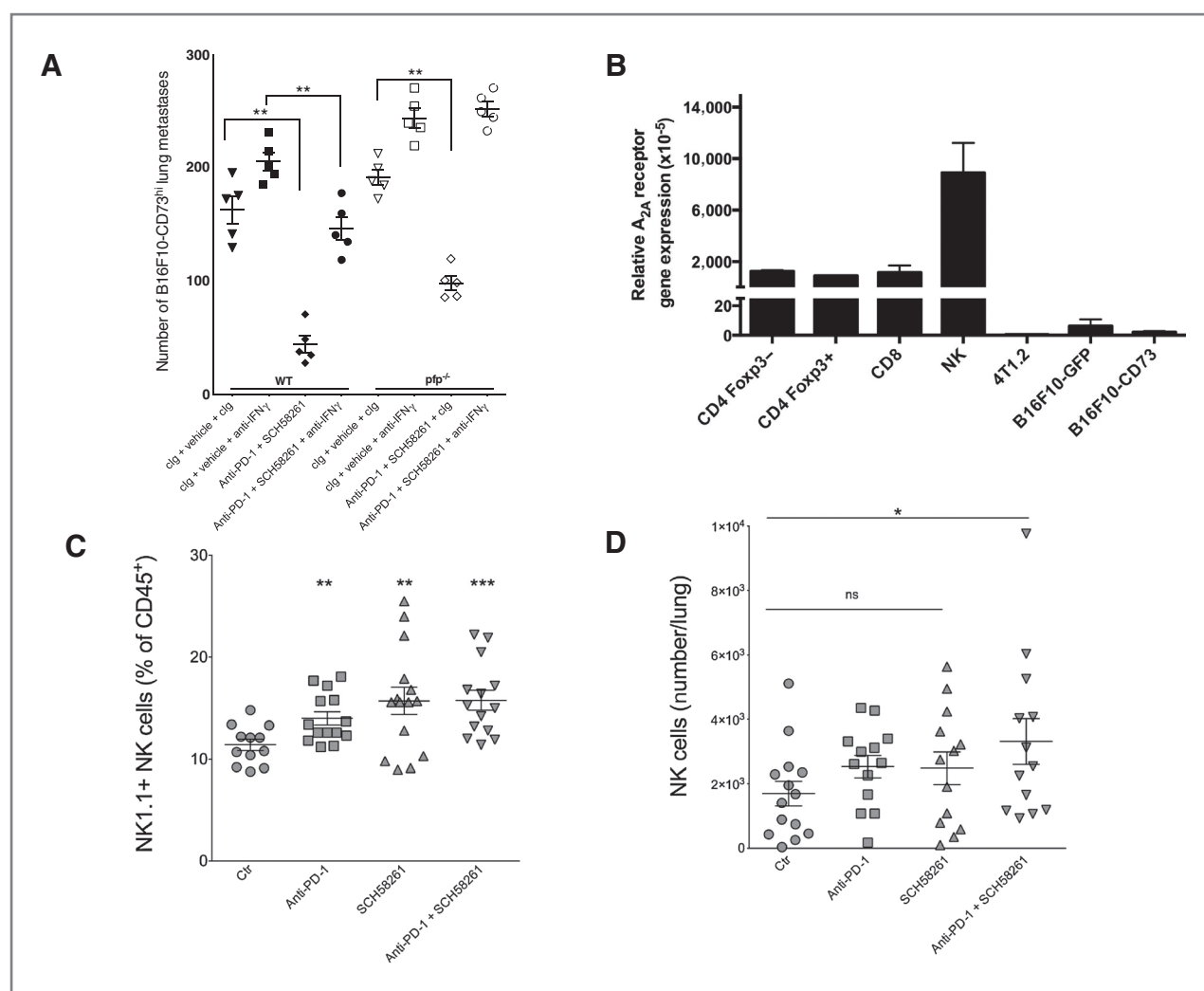


Figure 4. Anti-PD-1 and A_{2A}Ri suppression of metastasis is dependent on NK cells and requires perforin and IFN γ . **A**, C57BL/6 WT or perforin-deficient (pfp^{-/-}) mice were injected intravenously with B16F10-CD73^{hi} melanoma cells (1×10^5 cells) on day 0. On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of vehicle, A_{2A}Ri (SCH58261, 1 mg/kg), clg (2A3, 250 μ g), anti-PD-1 (RMP1-14, 250 μ g), or the combination as indicated. Some groups of mice were treated with clg or anti-IFN γ (H22, 250 μ g, i.p.) on days -1, 0, and 7 as indicated. Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means \pm SEM of 5 mice per group are shown. Improved metastatic control of the combination was statistically significant as indicated (**, $P < 0.01$; Mann-Whitney test). **B**, real-time PCR analysis of A_{2A}R mRNA expressed by splenic lymphocyte subsets as indicated and in mouse mammary (4T1.2) and melanoma cell lines (B16F10, B16F10-CD73^{hi}) transduced with control vector or CD73 retroviral vector (9). Gene expression levels were normalized to the house-keeping gene RPL32. Data shown represent the mean \pm SEM for 2 to 3 biologic replicates each with 2 technical replicates. Proportion (C) and absolute number (D) of CD3⁺ NK1.1⁺ NK cells (representative of FACS data) at day 5 in the lungs of mice injected with B16F10-CD73^{hi} melanoma cells (10^5 cells) on day 0 and treated intraperitoneally with vehicle, A_{2A}Ri (SCH58261, 1 mg/kg), clg (2A3, 250 μ g), anti-PD-1 (RMP1-14, 250 μ g), or the combination as indicated. The asterisk indicates where the combination groups were significantly different compared with their control group as determined by the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

every three days from the day of surgery, a very significant prolongation in lifespan was achieved (Fig. 2A, $P < 0.01$). By contrast, mice that were additionally depleted of NK cells or T cells (either CD8⁺ alone or CD4⁺ and CD8⁺) did not enjoy that survival benefit, indicating the nonredundant function of these lymphocyte subsets in the mechanism of antimetastatic activity of A_{2A}Ri (Fig. 2A). We next assessed the combinatorial effect of A_{2A}Ri and anti-PD-1 mAb in the same experimental model and found that anti-PD-1 and A_{2A}Ri were superior to either monotherapy alone (Fig. 2B). Thus, in both experimental and spontaneous metastases models, the combination of anti-

PD-1 mAb and A_{2A}Ri was more effective than monotherapy and significantly reduced disease burden.

Combination therapy suppresses metastases via NK cells, CD8⁺ T cells, and their effector functions

We next assessed the cell-mediated immune effector function responsible for the effectiveness of the A_{2A}Ri and anti-PD-1 combination in both the experimental and spontaneous metastases tumor models. In the B16F10-CD73^{hi} lung metastases model, the therapeutic effect of combined anti-PD-1 mAb and A_{2A}Ri was more dependent on NK cells, than CD8⁺ T cells, and

CD4⁺ T cells did not seem to have a critical additional role (Fig. 3A). In the surgical resection and spontaneous metastasis of 4T1.2 tumor model, both CD8⁺ T cells and NK cells were key in the optimal antitumor activity generated by the combination anti-PD-1 mAb and A_{2A}Ri (Fig. 3B). Thus, in both experimental and spontaneous tumor metastasis models, the combination of A_{2A}R and PD-1 blockade was able to engage CD8⁺ T and NK-cell effector function. This effector function was displayed by the critical need for host perforin and IFN γ pathways in the optimal antimetastatic activity of the anti-PD-1 mAb and A_{2A}Ri combination (Fig. 4A).

To our knowledge, there is no functional antibody available to detect mouse adenosine receptors. To assess the target of A_{2A}Ri, SCH58261, we analyzed the mRNA expression of A_{2A}R on B16F10-GFP and B16F10-CD73^{hi} mouse melanoma and 4T1.2 mammary carcinoma cell lines. The A_{2A}R expression on tumor cells was at minimum 1,000-fold lower than in lymphocytes and it was independent of the tumor cell expression of CD73 (Fig. 4B). Interestingly, the expression of A_{2A}R on splenic NK cells was 5-fold higher than other lymphocytes (Fig. 4B), suggesting that lymphocytes (CD4⁺ T cells, CD8⁺ T cells, and regulatory T cells) and in particular NK cells are the possible target of A_{2A}Ri in the tumor microenvironment. Our data are consistent with a previous finding where SCH58261 and NECA antagonized one another when added to NK cell:B16F10 cocultures, NECA significantly suppressing NK-cell-mediated killing activity *in vitro* (9). In addition, we also found significantly increased NK-cell proportions in the lungs of mice with 5-day B16F10-CD73^{hi} metastases and treated with monotherapy or combination therapy compared with control treated mice (Fig. 4C). NK-cell numbers were enhanced in the combination group (Fig. 4D), whereas the numbers of other immune cell populations were not significantly different between the treatment groups (data not shown).

Conclusions

We conclude that treatment of mice with a combination of A_{2A}Ri and anti-PD-1 mAb substantially reduces experimental and spontaneous metastases and increases the survival of mice, compared with either monotherapy. The antimetastatic activity of the A_{2A}Ri and combination immunotherapy is dependent upon CD73 expression on tumor cells whereas the mechanism of the combination is largely dependent on NK cells and IFN γ , and to a lesser extent on CD8⁺ T cells and perforin-dependent effector functions. This effector mecha-

nism is possibly also a combination of the reported perforin-dependent activity of A_{2A}Ri (9) and IFN γ activity of anti-PD-1 (22). Notably, adenosine or CD73 blockade has not yet been attempted in patients with cancer, despite the safety profile of A_{2A}Ri in human neurologic disease. The great promise of anti-PD-1 in the treatment of several human malignancies, including melanoma, non-small cell lung cancer and renal cancer, is being realized, and new combinations with anti-PD-1 are being explored and having further impact (6). Treatment of metastatic disease should be a priority and these results strongly advocate a combination of A_{2A}Ri with anti-PD-1 in the treatment of metastatic disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Mittal, J. Stagg, M.J. Smyth

Development of methodology: D. Mittal, B. Allard, M.J. Smyth

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Young, K. Stannard, M.W.L. Teng, B. Allard, M.J. Smyth

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Young, B. Allard, J. Stagg, M.J. Smyth

Writing, review, and/or revision of the manuscript: D. Mittal, A. Young, M.W.L. Teng, J. Stagg, M.J. Smyth

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Yong

Study supervision: M.W.L. Teng, J. Stagg, M.J. Smyth

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