# Adenosine A2a receptor inhibition increases the anti-tumor efficacy of anti-PD1 treatment in murine hepatobiliary cancers


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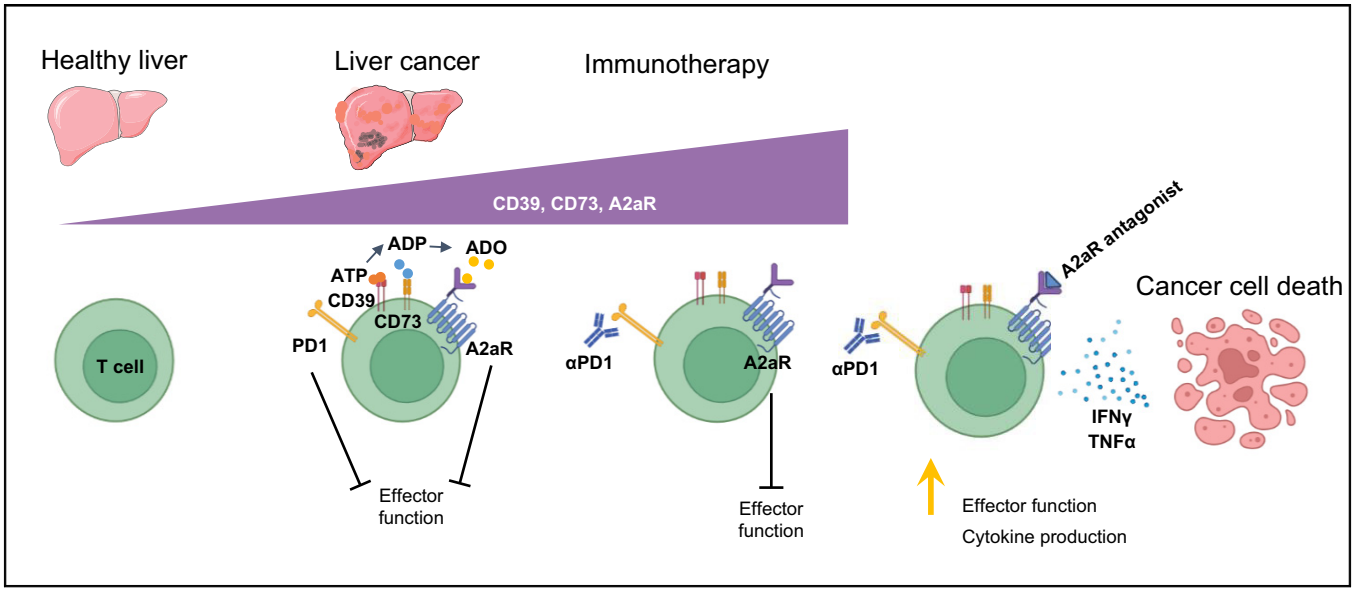
## Graphical abstract




## Highlights

- Adenosine-related genes are highly expressed in patients with liver cancer.
- A2aR expression on T cells increases in tumor-bearing and ICI-treated mice.
- A2aR blockade enhances the anti-tumor effect of ICIs.

## Impact and implications

Adenosine A2a receptor (A2aR)-expressing T cells in the liver increased in tumor-bearing mice and after anti-PD1 treatment. The combination of an A2aR inhibitor and anti-PD1 treatment had potent anti-tumor effects in two murine models of orthotopic liver cancer. Adenosine A2a receptor blockade promotes immunotherapy efficacy in murine models, highlighting putative clinical benefits for advanced stage liver cancer patients.

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Research article

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# Adenosine A2a receptor inhibition increases the anti-tumor efficacy of anti-PD1 treatment in murine hepatobiliary cancers

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**Backgrounds & Aims:** The efficacy of immune checkpoint inhibitor (ICI) therapy for liver cancer remains limited. As the hypoxic liver environment regulates adenosine signaling, we tested the efficacy of adenosine A2a receptor (A2aR) inhibition in combination with ICI treatment in murine models of liver cancer.

**Methods:** RNA expression related to the adenosine pathway was analyzed from public databases. Peripheral blood mononuclear cells of 13 patients with hepatocellular carcinoma (HCC) were examined by flow cytometry. The following murine cell lines were used: SB-1, RIL175, and Hep55.1c (liver cancer), CT26 (colon cancer), and B16–F10 (melanoma). C57BL/6 and BALB/c mice were used for orthotopic tumor models and were treated with SCH58261, an A2aR inhibitor, in combination with anti-PD1 therapy.

**Results:** RNA expression of *ADORA2A* in tumor tissues derived from patients with HCC was higher than in tissues from other cancer types. A2aR$^+$ T cells in peripheral blood from patients with HCC were highly proliferative after immunotherapy. Likewise, in an orthotopic murine model, A2aR expression on T cells increased following anti-PD1 treatment, and the expression of A2aR on T cells increased more in tumor-bearing mice compared with tumor-free mice. The combination of SCH58261 and anti-PD1 led to activation of T cells and reductions in tumor size in orthotopic liver cancer models. In contrast, SCH58261 monotherapy was ineffective in orthotopic liver cancer models and the combination was ineffective in the subcutaneous tumor models tested. CD4$^+$ T-cell depletion attenuated the efficacy of the combination therapy.

**Conclusion:** A2aR inhibition and anti-PD1 therapy had a synergistic anti-tumor effect in murine liver cancer models.

**Impact and implications:** Adenosine A2a receptor (A2aR)-expressing T cells in the liver increased in tumor-bearing mice and after anti-PD1 treatment. The combination of an A2aR inhibitor and anti-PD1 treatment had potent anti-tumor effects in two murine models of orthotopic liver cancer. Adenosine A2a receptor blockade promotes immunotherapy efficacy in murine models, highlighting putative clinical benefits for advanced stage liver cancer patients.



## Introduction

Liver cancer is the sixth most common cancer and the third leading cause of cancer-related death worldwide.[1] There are two main types of primary liver cancer: cholangiocarcinoma and hepatocellular carcinoma (HCC). The most common type of liver tumor occurs due to metastasis from primary tumors outside the liver[2] and these metastatic cancers often do not respond well to immune checkpoint inhibitor (ICI) therapy.[3,4] While ICI therapy has become the standard of care for the treatment of primary liver cancer, the efficacy of mono-therapeutic agents remains limited.[5–7] Hypoxia, which is a well-known characteristic of liver cancer, likely contributes to poor ICI therapy outcomes.[8] There are several factors contributing to hypoxia in liver tumors including: anatomy with the portal vein bringing 70% of the blood supply to the liver,[9,10] fibrosis which impairs blood perfusion, and treatment-induced hypoxia with therapies such as trans-arterial chemoembolization (TACE).

A2a adenosine receptor (A2aR) antagonists represent an interesting tool to enhance anti-tumor immunity, especially in the context of ICI therapy and hypoxia.[11] The A2aR has been shown to prevent excessive collateral tissue damage by overactive T cells and myeloid cells in non-infected but inflamed normal tissues of vital organs.[12] In addition, A2aR antagonists have long been proposed as a therapeutic tool to unleash tumor-reactive T and natural killer (NK) cells.[12,13] More recently Fong and colleagues described encouraging observations of tumor regression, disease control, and survival of patients with otherwise refractory renal cell cancer with progressive disease after treatment with an A2aR antagonist.[14]

Adenosine, the natural ligand for A2aR, is generated from extracellular ATP through the activity of CD39 and CD73.[15] Modulating CD39[16,17] and CD73[18,19] have also been investigated to improve T cell function in cancer.[20]

Keywords: Liver cancer; immunotherapy; adenosine receptor.
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Targeting the adenosine-A2aR-cAMP axis in combination with immunotherapy has been reported to enhance the anti-tumor effect in preclinical models. Indeed, the blockade of CD73 enhances the efficacy of anti-programmed death 1 (PD1) and anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) treatment in subcutaneous tumor and chemically induced tumor models.[21] The co-blockade of CD73 and A2aR inhibition has shown synergistic anti-tumor effects with A2aR considered a putative immunomodulatory molecule.[22]

In this study, we examined adenosine-related gene expression in liver cancer and investigated the efficacy of an A2aR inhibitor in preclinical murine liver cancer models.

## Materials and methods

### Patients' PBMCs and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) from 13 patients were used for analyses. All patients were enrolled in a clinical trial at the NIH evaluating anti-CTLA4 therapy (tremelimumab) (NCT01853618). Clinical information is provided in Table 1. PBMCs were isolated from the blood as previously described.[23] Briefly, blood was gradient-centrifuged using a lymphocyte separation medium (Ficoll Lonza), and the intermediate PBMC layer was frozen and stored in liquid nitrogen. All patients provided written informed consent, and the study design was consistent with the principles of the Helsinki Declaration. Multicolor flow cytometry was performed to study different immune cell subsets using the following antibodies (clone, manufacture): anti-CD19-BV421 (HIB19, BioLegend), anti-CD11c-BV510 (B-ly6, BD), anti-HLA-DR-BV785 (L243, BioLegend), anti-CD16-AF488 (3G8, BioLegend), anti-CD11b-PerCPCy5.5 (ICRF44, BioLegend), anti-CD3-AF700 (UCHT1, BD), anti-CD3-PECy7 (UCHT1, BD), anti-CD14-APC (M5E2, BD), anti-CD33-AF700 (WM53, BD), anti-CD56-APCFire750 (QA17A16, BioLegend), anti-FoxP3-PacificBlue (259D, BioLegend), anti-CD8-BV510 (SK1, BD), anti-CD45RA-BV650 (HI100, BioLegend), anti-CD4-BV785 (RPA- T4, BioLegend), anti-CCR7-FITC (150503, BD), anti-PD1-PE (EH12.1, BD), anti-CTLA4-PECy7 (L3D10, BioLegend), anti-Ki67-APC (Ki67, BioLegend),anti-CD39-BV605(A1, BioLegend), anti-CD73-PEDazzle594(AD2, BioLegend), and anti-A2A R-PE (7F6-G5-A2 Novus). Data for all samples were collected on a CytoFLEX LX flow cytometer (Beckman Coulter CytoFLEX Flow Cytometer) and analyzed using FlowJo software version 10.4.2 (BD Biosciences).

**Table 1. Patient characteristics (n = 13).**

| | Number |
|---|---|
| Age, median (range) | 59 (42-76) |
| Sex | |
| Male | 11 |
| Female | 2 |
| Cirrhosis | |
| Yes | 10 |
| No | 3 |
| Etiology | |
| HBV | 4 |
| HCV | 5 |
| Non-B, non-C | 4 |
| Baseline Child-Pugh score | |
| 5 | 8 |
| 6 | 1 |
| 7 | 1 |
| BCLC stage | |
| B | 7 |
| C | 6 |
| Extrahepatic disease | |
| Yes | 3 |
| No | 10 |
| Prior sorafenib | |
| Yes | 6 |
| No | 7 |

### Mice and cell lines

Six-to 10-week-old C57BL/6NCrl or BALB/C female mice were purchased from Charles River. The following murine tumor cell lines were used: Hep55.1c and RIL175 (HCC),[24,25] SB1 (cholangiocarcinoma),[26] CT26 (colon carcinoma)[24] as well as B16–F10 (melanoma).[25] Cells were cultured in RPMI 1640 GlutaMax with 10% fetal calf serum, ampicillin, and streptomycin. MTT assay (ab211091, Abcam) was performed according to the manufacturer's protocol.

### Mouse procedures and antibody treatment

Intrahepatic tumor injection was performed as described previously.[27] In brief, $2.0x10^5$ cells were resuspended in 20 ul of PBS mixed with Matrigel (Corning) and injected into the liver. A subcutaneous tumor was induced by injecting $1.0x10^6$ cells into the left flank of each mouse as previously described.[23] Treatment was initiated 5 days post tumor cell injection. The following antibodies were injected intraperitoneally into mice at a dose of 200 μg/mouse at the days indicated in the figures; anti-PD1 (clone 29F.1A12, BioXCell), anti-CD4 (clone GK1.5; BioXCell), and anti-CD8 (clone 2.43; BioXCell). The A2aR inhibitor (SCH58261, Sigma) was administered intraperitoneally at a concentration of 1 mg/kg body weight as indicated. All experiments were conducted according to local institutional guidelines and approved by the NIH Animal Care and Use Committee in Bethesda, MD.

### Isolation of murine lymphocytes and flow cytometry

Lymphocytes from the liver and spleen of mice were isolated as previously described[23] and analyzed by multicolor flow cytometry. Flow cytometry was performed on a CytoFLEX LX platform and results were analyzed using FlowJo software. The following antibodies were used in this study (clone, manufacture): anti-F4/80-Alexa Fluor 700 (BM8, BioLegend), anti-B220-Alexa Fluor 700 (RA3-6B2, BioLegend), anti-Cd11b-Alexa Fluor 700 (M1/70, BioLegend), anti-Cd3-Alexa Fluor 594 (17A2, BioLegend) anti-Cd4-BV605 (GK1.5, BioLegend), anti-Cd8-BV786 (53-6.7, BD), anti-Foxp3-BV421 (MF-14, BioLegend), anti-Cd62l-PerCP/Cy5.5 (MEL-14, BioLegend), anti-Cd44-BV510 (IM7, BioLegend), anti-NK1.1-BV510 (PK136, BioLegend), anti-A2Ar-FITC (7F6-G5-A2, NOVUS), anti-Cd73-APC (TY11.8, BioLegend), anti-Cd39-PECy7 (Duha59, BioLegend), anti-Cd19-PerCP Cy5.5(6D5, BioLegend), anti-PD1-APC/Cy7 (29F.1A12, BioLegend), anti-Cd107a- PE (1D4B, BD), anti-CD69-BV650 (H1.2F3, BioLegend), anti-Ifnγ-BV421 (XMG1.2, BioLegend), anti-Tnfα-PerCPCy5.5 (MP6-XT22, BioLegend), anti-GZMB-FITC (GB11, BioLegend), and anti-perforin-APC (S16009A, BioLegend).

### RNA sequencing analysis using a public database

*ADORA1, ADORA2A, ADORA2B, ADORA3, ENTPD1,* and *NT5E* expression were analyzed from different tumor tissues using TCGA (The Cancer Genome Atlas) datasets.[28–30]

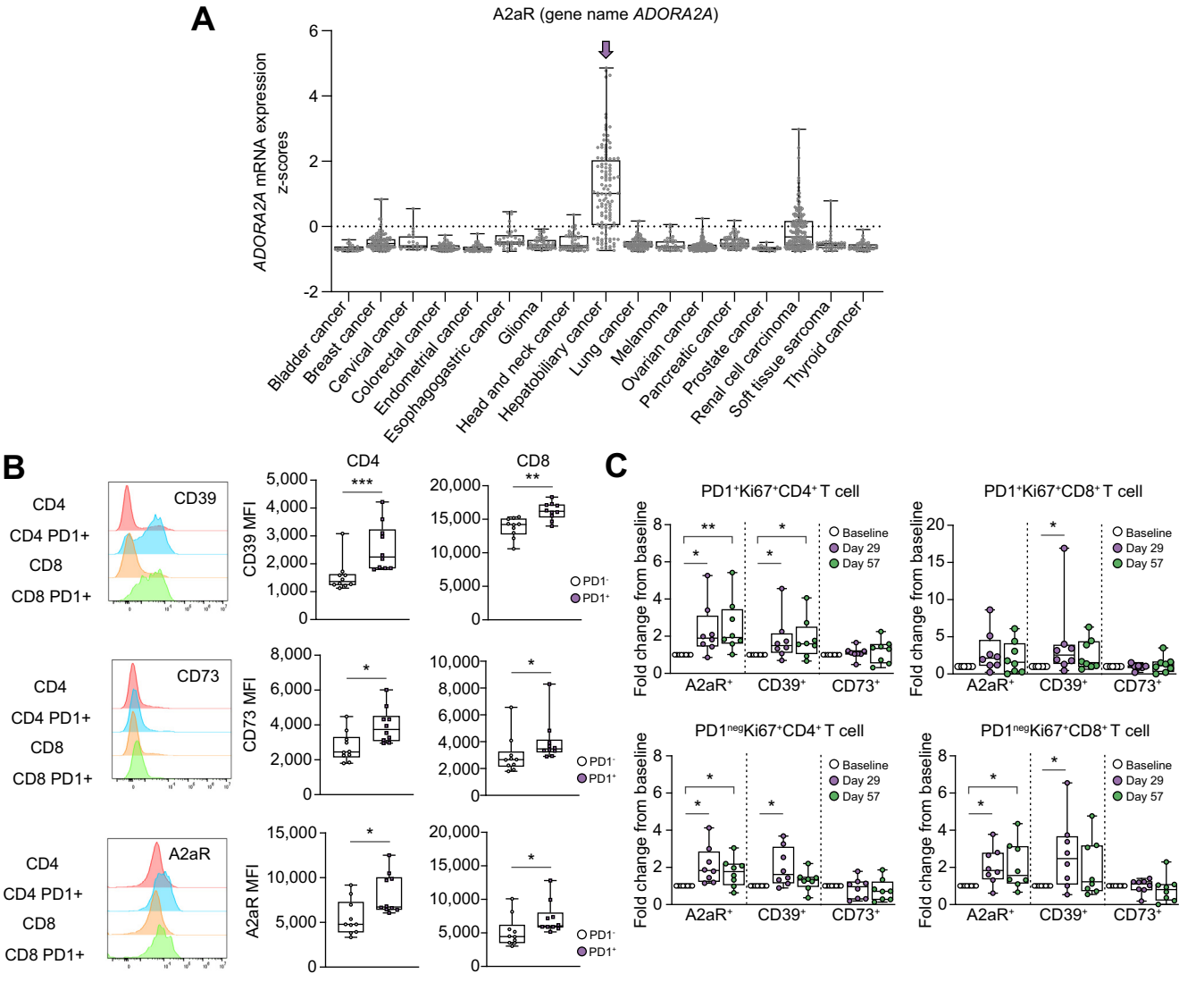


**Fig. 1. A2aR expression is increased in hepatobiliary cancer and ICI therapy increases adenosine-related protein expression in T cells.** (A) The expression level of ADORA2A in tumor tissue of each cancer type was analyzed using TCGA datasets. (B–C) PBMCs of 13 patients with HCC who underwent immunotherapy were analyzed with flow cytometry at baseline, the first day of the second course (C2D1 day 29), and the first day of the third course (C3D1 day 57). The MFI of CD39, CD73, and A2aR of CD4 and CD8 cells with or without PD1 expression at baseline (B). Fold changes in the frequency of each T-cell subset from baseline to day 29 and day 57 were calculated for eight patients (C). *$p$ <0.05, **$p$ <0.01, ***$p$ <0.001 (B) Mann-Whitney $U$ test (C) Kruskal-Wallis and Dunn's *post hoc* test. HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TCGA, The Cancer Genome Atlas.

### Statistical analysis

For statistical analyses, PRISM (GraphPad, Ver8.4.3.) software was used. For the comparison of two groups, a Student's $t$-test (for the parametric test) and the Mann-Whitney $U$ test (for the non-parametric test) were used. For multiple comparisons, one-way ANOVA and Tukey's *post hoc* test (for the parametric test) and Kruskal-Wallis and Dunn's *post hoc* test (for the non-parametric test) were utilized. For multiple comparisons in two categorical variables, two-way ANOVA with a *post hoc* Sidak test was used. All statistical information is described in the Fig. legends.

## Results

### A2aR is highly expressed in tumor tissue from patients with HCC and on T cells following ICI treatment

To examine the adenosine pathway in liver cancer tissue, we analyzed the mRNA expression of adenosine receptors and enzymes that metabolize and bind adenosine.[15] CD39 and CD73 are enzymes that degrade ATP to ADP, and ADP to adenosine, respectively.[16,18] The expression of *ADORA1* (encoding the adenosine A1 receptor), *ADORA2A* (encoding the adenosine A2a receptor), *ADORA2B* (encoding the adenosine A2b receptor), *ADORA3* (encoding the adenosine A3 receptor), *ENTPD1*

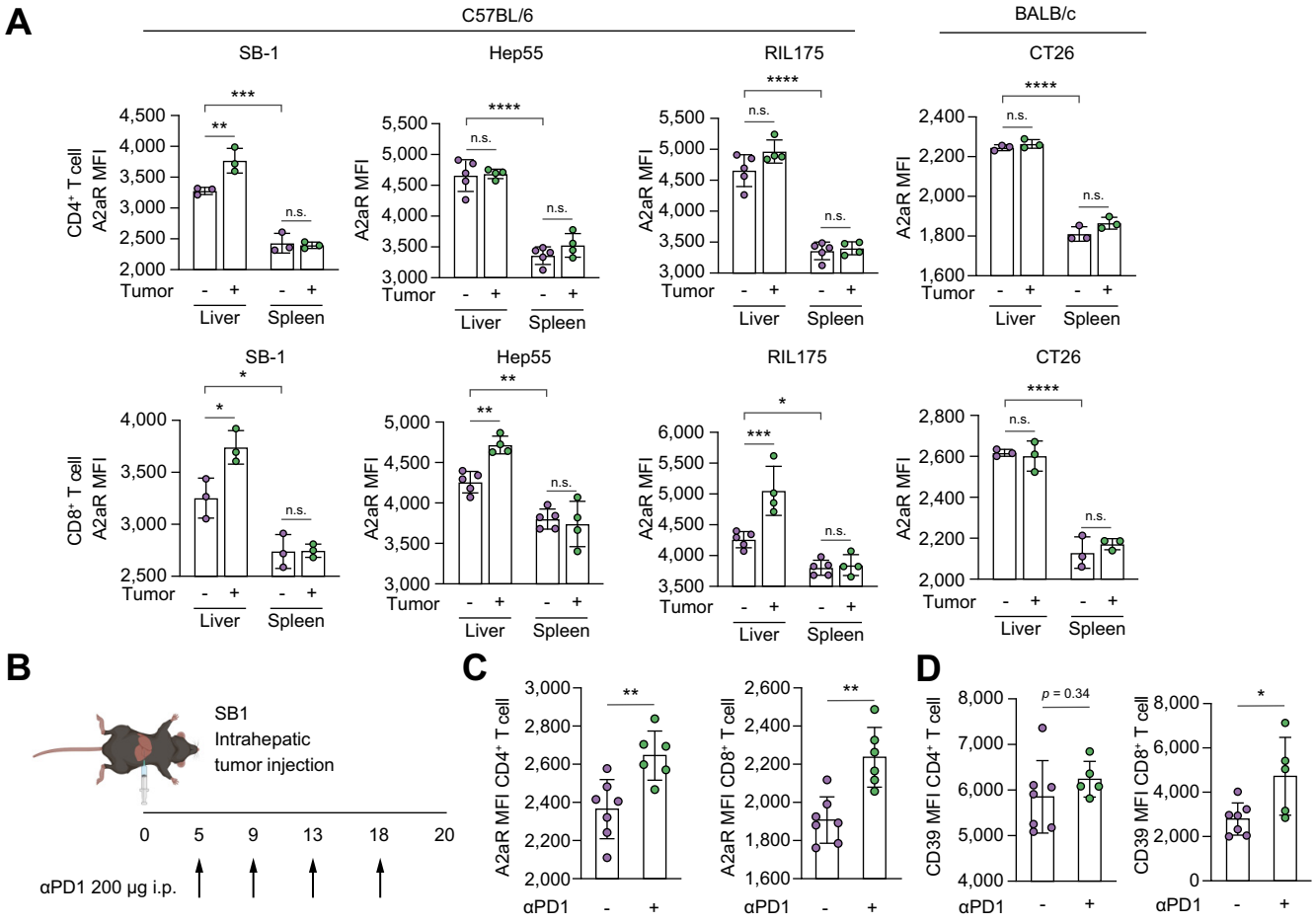


**Fig. 2. Orthotopic tumors and anti-PD1 treatment increase the expression of adenosine-related proteins in hepatic T cells in murine models.** (A) SB-1 cell, Hep55.1c cell, and RIL175 cells were injected into the livers of C57BL/6 female mice. CT26 cells were injected into the livers of BALB/C female mice. The lymphocytes of the liver and spleen were isolated and analyzed with multicolor flow cytometry. The MFI of A2aR in CD4 and CD8 cells was analyzed; n = 3-5/group. (B-D) SB-1 cells were injected into the liver of C57BL/6 female mice and treated with anti-PD1. (B) The experimental settings. (C-D) The lymphocytes of the liver and spleen were isolated and analyzed with multicolor flow cytometry. The MFI of A2aR and CD39 in CD4 and CD8 cells was analyzed. n = 6-7/group; n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ***$p < 0.001$. (A) Two-way ANOVA with *post hoc* Sidak's test. (C-D) Mann-Whitney *U* test. MFI, mean fluorescence intensity.

(encoding CD39), and *NT5e* (encoding CD73) in different types of cancers were analyzed using TCGA datasets.[29] *ADORA2A* expression was highest in hepatobiliary cancers (Fig. 1A), while expression levels of *ENTPD1* and *NT5e* were similar to those in other cancers (Fig. S1A). The expression levels of other adenosine receptors such as *ADORA1*, *ADORA2B*, and *ADORA3* in hepatobiliary tumor tissue were also similar to those in other types of cancer (Fig. S1A).

Next, we analyzed A2aR, CD39, and CD73 expression on different immune cell subsets in PBMCs from 13 patients with HCC (Table 1) who were enrolled in a clinical trial testing the combination of anti-CTLA4 plus locoregional therapies (NCT01853618, Fig. S1B).[23] CD39 expression was highest on $CD8^+$ T cells. CD73 expression was highest on B cells and A2aR expression were similar on all immune cells analyzed with the highest expression on regulatory T cells (Tregs) (Fig. S1C). CD39, CD73, and A2aR expression were higher on $PD1^+CD4^+$T cells and $PD1^+CD8^+$T cells than on PD-$1^{neg}CD4^+$T cells and $PD1^{neg}CD8^+$T cells at baseline (Fig. 1B). Next, peripheral T cells from eight paired samples were examined before treatment, after one dose of anti-CTLA4 (C2D1 d29), and after two doses of anti-CTLA4 and TACE (C3D1 d57). T-cell proliferation was examined by Ki67 staining of T cells, and $PD1^+$ and $PD1^{neg}$ cells were analyzed separately. As shown in Fig. 1C, the largest increase in proliferating cells was seen in $A2aR^+$ T cells (both $PD1^+$ and $PD1^{neg}$ $CD4^+$ and $CD8^+$ T cells). Proliferating $A2aR^+$ $PD1^+CD4^+$ T cells remained elevated after two doses of anti-CTLA treatment and TACE. The proliferation of both $CD39^+$ T cells also increased upon anti-CTLA4 treatment (Fig. 1C). In contrast the frequency of $Ki67^+A2aR^{neg}$ T cells did not change upon treatment (Fig. S1D). Collectively, these results suggest that ICI therapy induces the proliferation of peripheral T cells expressing adenosine-related proteins in HCC.

### A2aR expression on hepatic T cells increases in mice with orthotopic tumors

To experimentally investigate the adenosine pathway in murine liver cancer models, we first profiled A2aR, CD39, and CD73 expressions on T cells from the liver, spleen, and blood of naïve, tumor-free mice. Expression of A2aR, CD39, and CD73 on $CD4^+$ T cells was higher in liver-derived T cells compared to blood-derived and splenic T cells (Fig. S2A). Similarly, the number of

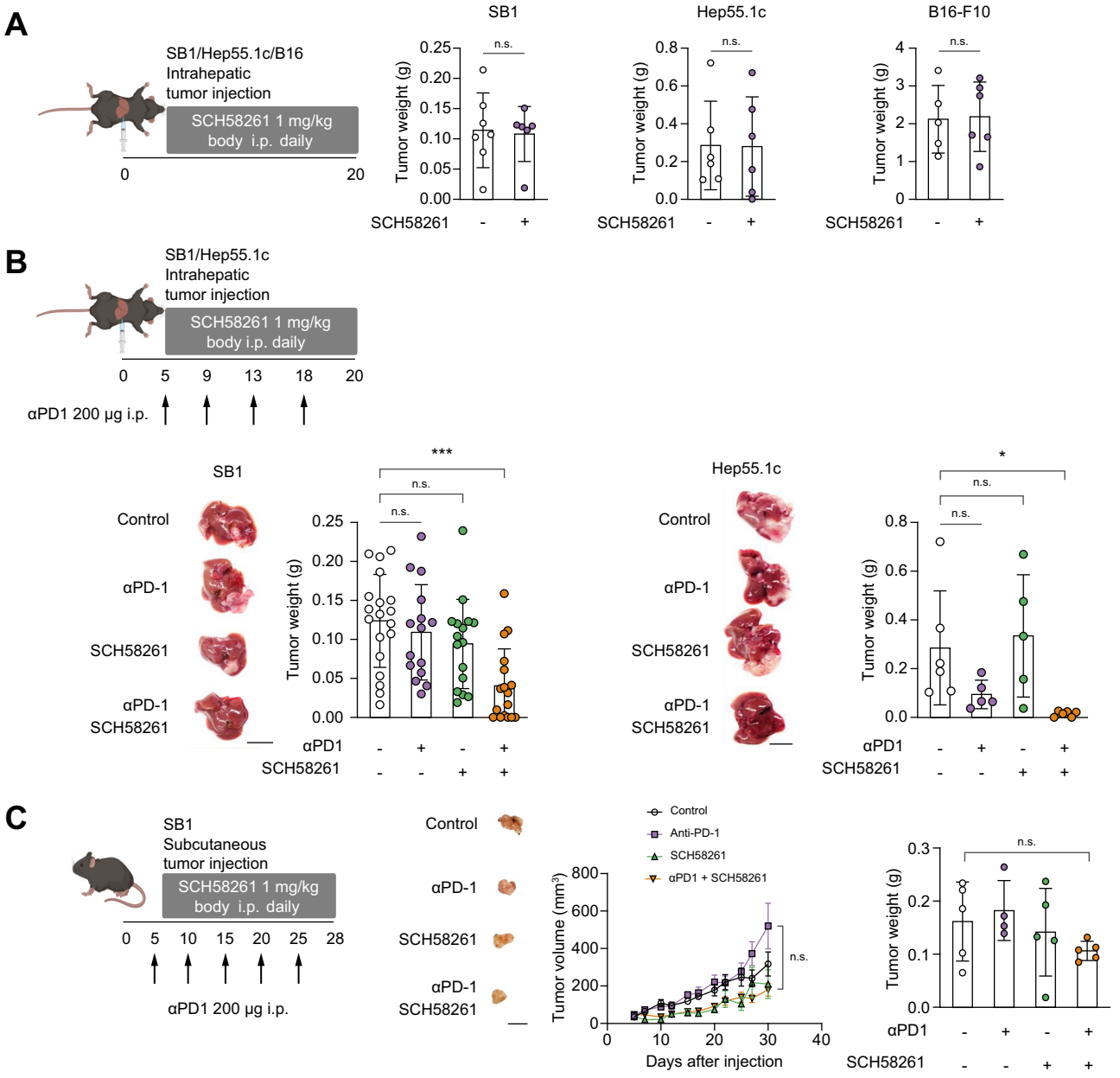


**Fig. 3. Blocking A2aR activity promotes anti-PD1 therapy against liver tumors.** (A) SB1, Hep55.1c, or B16–F10 cells were injected into the livers of C57BL/6 mice and treated with daily intraperitoneal injections of SCH58261 from 5 days after injection. Mice were analyzed 20 days after tumor injection; n = 5-7/group. (B) SB1 cells or Hep55.1c cells were injected into the liver of C57BL/6 mice and treated with SCH58261, anti-PD1, or combination therapy from 5 days after injection. The representative macro tumor image is shown. The weight of each group is shown in the right panel. For the SB-1 tumor model, n = 5-7/group/experiment and the experiment was repeated three times. For the Hep55.1c model, n = 5-6. (C) SB1 cells were injected subcutaneously into C57BL/6 mice and treated with SCH58261, anti-PD1, or combination therapy from 5 days after injection. The representative macro tumor image is shown in the middle. The tumor volume and weight of each group are shown in the right panel; n = 5/group and the experiment was repeated twice. Scale bar: 1 cm. n.s., $p$ >0.05; *$p$ <0.05; **$p$ <0.01; ***$p$ <0.001. (A) Mann-Whitney *U* test (B) (C) Kruskal-Wallis and Dunn's *post hoc* test.

$CD39^+CD8^+$ and $A2aR^+CD8^+$ T cells was higher in the liver than in the spleen, whereas most of the $CD8^+$ T cells from the liver, spleen, and blood expressed CD73 (Fig. S2A). In liver tissue, A2aR expression on T cells was higher in effector $CD44^+$ $CD62L^{neg}$ $CD4^+$ and $CD8^+$ T cells compared to their naïve $CD44^{neg}$ $CD62L^+$ T cell counterparts (Fig. S2B). Next, we investigated adenosine-related genes in primary cancer cell lines. The expression of A2aR, CD39, and CD73 was examined in three different primary murine liver tumor cell lines (SB-1, RIL 175, and Hep55.1c cells), and murine cell lines derived from tumors, which commonly metastasize to the liver: melanoma (B16–F10) and colorectal cancer (CT26). The highest CD73 expression was found on SB-1 cells (Fig. S2C). There were no differences in A2aR and CD39 expression on the cancer cell lines tested.

To investigate the change of A2aR expression on T cells from tumor-bearing mice, we analyzed A2aR expression on $CD4^+$ and

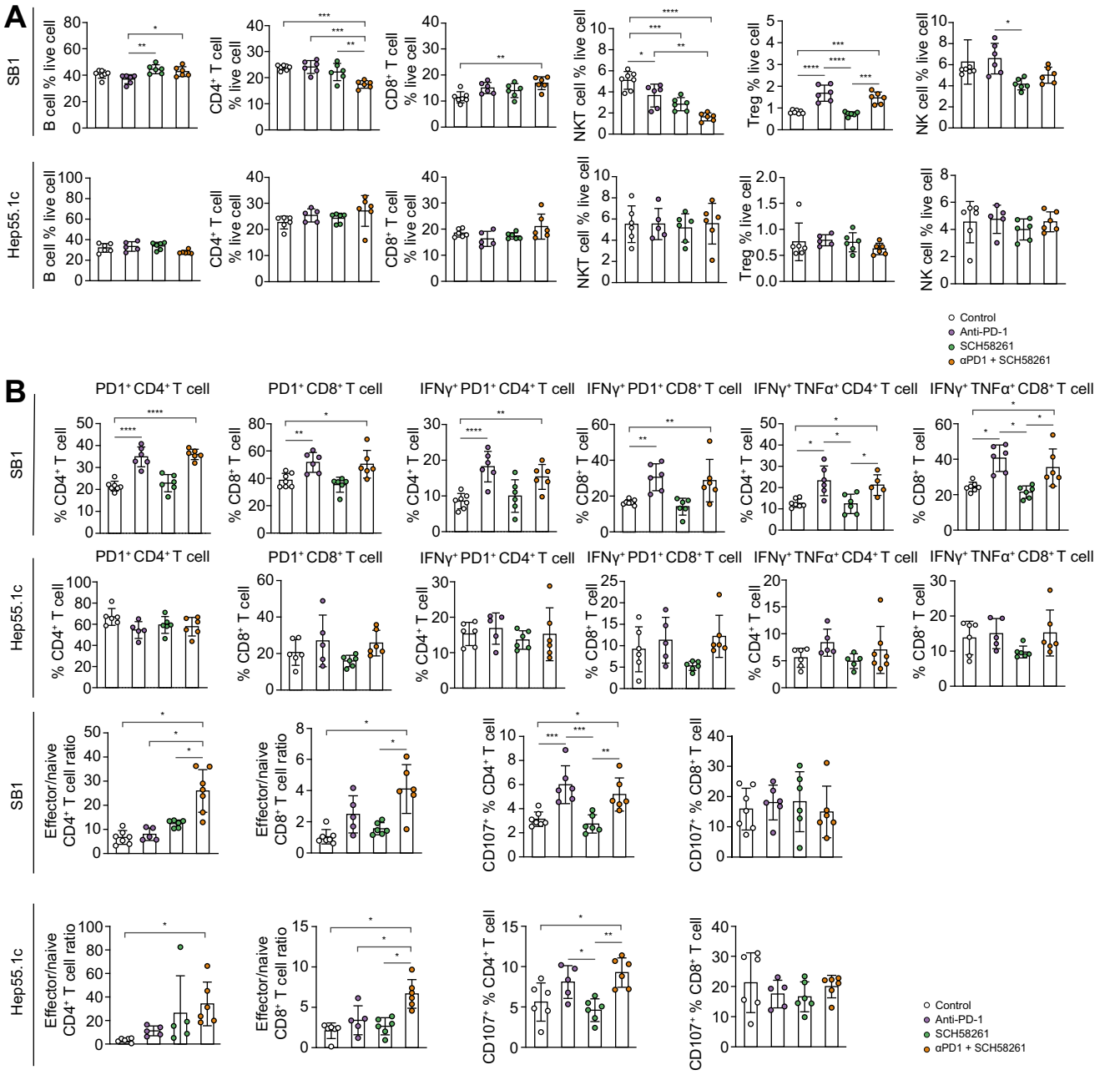


**Fig. 4. A2aR blockade + ICI therapy against liver cancer increase effector T cell frequency in the liver.** (A,B) SB1 or Hep55.1c cells were injected into the livers of C57BL/6 mice and treated with SCH58261, anti-PD1, or combination therapy from 5 days after injection. (A) The pan-immune cell population was analyzed with flow cytometry. The cytokine production and subsets of T cells were analyzed with flow cytometry. Manual gating for each cell subset is shown in (B); n = 6-7/group with experiments repeated twice. n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (A-B) Kruskal-Wallis and Dunn's *post hoc* test. NK, natural killer; Treg, regulatory T cell.

$CD8^+$ T cells from C57BL/6 mice and BALB/c mice after orthotopic injection of three different liver tumor cell lines. A2aR expression was higher on hepatic $CD8^+$ T cells derived from liver tumor-bearing mice and on hepatic $CD4^+$ T cells from SB1 tumor-bearing mice. However, A2aR expression did not change on $CD4^+$ and $CD8^+$ T cells from BALB/c mice after injection with CT26 colon cancer cells (Fig. 2A). These results suggest that A2aR expression on T cells increases in the liver of tumor-bearing mice.

### The A2aR expression increases upon treatment with ICI therapy

Next, we investigated A2aR expression on T cells in mice treated with ICI. SB1 cells were injected into the liver of mice and treated with anti-PD-1 therapy (Fig. 2B). A2aR expression on hepatic $CD4^+$ and $CD8^+$ T cells increased in tumor-bearing mice after anti-PD1 therapy (Fig. 2C). Similarly, the expression of CD39 on $CD8^+$ T cells increased in liver tumor-bearing mice after treatment

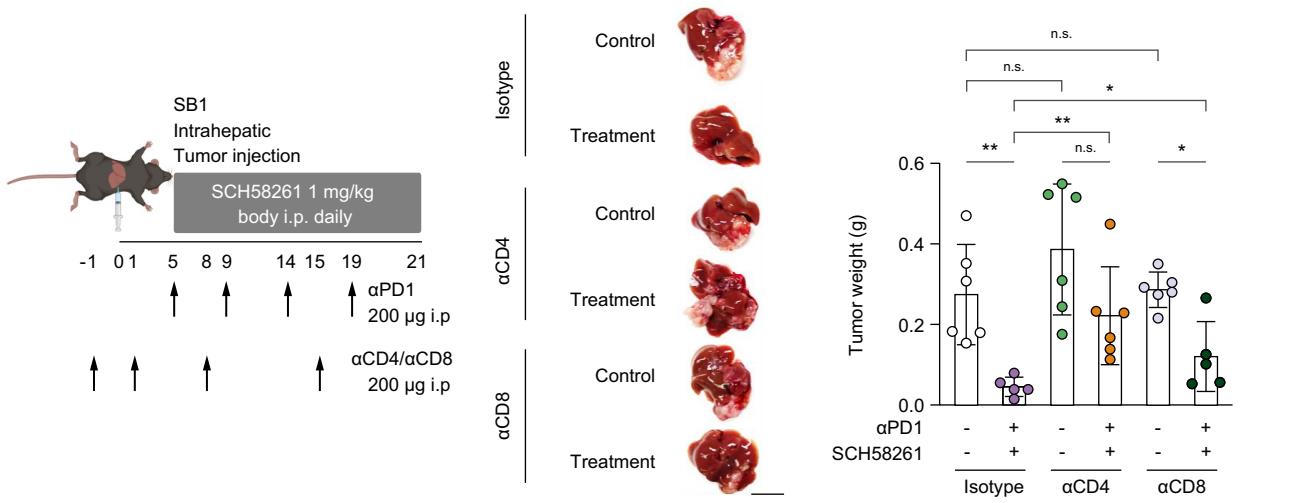


**Fig. 5. CD4$^+$T cells are required for effective A2aR blockade + ICI therapy against liver cancer.** SB1 cells were injected into the livers of C57BL/6 mice and treated with combination therapy with or without anti-CD4 antibody or anti-CD8 antibody. The representative liver image and tumor weight are shown. Scale bar:1 cm; n = 5-6/group. n.s., $p$ >0.05; *$p$ <0.05; **$p$ <0.01; ***$p$ <0.001. Kruskal-Wallis and Dunn's *post hoc* test. ICI, immune checkpoint inhibitor.

(Fig. 2D). In contrast, A2aR expression on CD4$^+$ and CD8$^+$ T cells did not change when C57BL/6 naïve, tumor-free mice were treated with anti-PD-1 (Fig. S3).

### The combination of SCH58261 and anti-PD1 therapy is effective in mice with orthotopic liver tumors

Based on the observation that A2aR expression increased in T cells in tumor-bearing mice, we investigated the effect of a potent and selective A2aR antagonist, SCH58261,[31–33] in tumor bearing mice. First, we excluded a direct effect of SCH58261 on tumor cells *in vitro*. Incubation of different tumor cells with the A2aR inhibitor affected cell viability only at micromolar concentrations of SCH58261, indicating a lack of a direct effect on tumor cell proliferation (Fig. S4). Next, we tested SCH58261 treatment in mice with intrahepatic SB1, Hep55.1c, or B16–F10 tumors. Mice were treated 5 days after cell line injection for a total of 15 days. Similar to our *in vitro* studies, SCH58261 monotherapy had no effect on tumor growth (Fig. 3A). Based on the observation that A2aR expression on T cells changed upon anti-PD1 treatment in mice with orthotopically growing tumors (Fig. 2C), we tested the combination of SCH58261 plus anti-PD1 treatment. Combined SCH58261 plus anti-PD1 had a stronger effect on growth of intrahepatic tumors than individual treatment alone in both tumor models (Fig. 3B). Next, we asked if this effect was also found in mice with subcutaneous tumors. SB1 cells were injected subcutaneously into C57BL/6 mice and treated with SCH58261 plus an anti-PD1 antibody (Fig. 3C). Neither single agent treatments nor the combination of SCH58261 plus an anti-PD1 antibody significantly affected growth of subcutaneous tumors (Fig. 3C). Collectively, these results suggest that A2aR blockade can have synergistic effects with anti-PD1 therapy in murine models of orthotopic liver cancer.

### SCH58261 has a synergistic effect with immune checkpoint therapy modulated by CD4$^+$ T cells

To better understand the mechanism of SCH58261 and anti-PD1 treatment's efficacy, we studied immune cells isolated from tumor-bearing mice after treatment with the combination of SCH58261 and an anti-PD1 antibody. Both SB1 and Hep55.1c intrahepatic tumors were reduced in size after treatment with SCH58261 plus anti-PD1 (Fig. 3B), however, there were no common quantitative differences in B cells, CD4$^+$ and CD8$^+$ T cells, NKT cells, regulatory T cells and NK cells found in RIL175 and Hep55.1c tumor models (Fig. 4A). Next, we studied CD107 expression and cytokine release by T cell to assess T-cell function. CD107$^+$ CD4$^+$ T cells in the liver increased upon ICI treatment in both tumor models, but SCH58261 treatment had no additional effect. Similar results were observed when we looked at IFN$\gamma^+$TNF$\alpha^+$ CD4$^+$ and CD8$^+$ T cells (Fig. 4B). Finally, we analyzed the ratio of effector:naïve T cells. The ratio of CD44$^+$CD62L$^{neg}$ effector T cells and CD44$^{neg}$CD62L$^+$ naïve T cells were calculated both for hepatic CD4$^+$ and CD8$^+$ T cells. The effector CD4$^+$ T cell and CD8$^+$ T cell population significantly increased in the combination therapy group in the liver of both orthotopic models but did not increase in mice which had received anti-PD1 or SCH58261 monotherapy (Fig. 4B). Interestingly, this increase in the ratio of effector:naïve T cells after anti-PD1 plus SCH58621 treatment was not observed in tumor-infiltrating lymphocytes from subcutaneous tumors (Fig. S5). Finally, we performed CD4$^+$ T cell and CD8$^+$ T-cell depletion to verify which cell type was needed to exert anti-tumor immunity in mice treated with the combination. While both depletion of CD8$^+$ T cells and CD4$^+$ T cells ameliorated the effect of combination therapy, depletion of CD4$^+$ T cells had a stronger effect on tumor growth (Fig. 5). Based on these results, we suggest that SCH58261 increases effector T cells and activates T cells synergistically with anti-PD1 in a CD4$^+$ T cell-dependent manner.

## Discussion

This study demonstrates that the adenosine synthesis proteins, CD39 and CD73, and receptor (A2aR) are overexpressed in T cells from human PBMCs after immunotherapy. Hepatic A2aR-expressing T cells increased in tumor-bearing mice and after anti-PD1 treatment. A2aR inhibitor and anti-PD1 treatment

revealed potent anti-tumor effects together in two murine liver cancer models.

One interesting result from our study was the observation that A2aR expression on T cells following anti-PD1 antibody therapy only increased in tumor-bearing mice. Furthermore, A2aR antagonist monotherapy did not show anti-tumor effects, in line with a study by Willingham and colleagues, which reported that single-agent A2aR inhibition lacked an anti-tumor effect without ICI treatment.[34] Ohta and colleagues also showed that single-agent A2aR inhibition did not have an anti-tumor effect in the absence of tumor-specific T-cell transfer,[13] while a recent clinical trial showed that an A2aR antagonist lacked anti-tumor effects without tumor-specific T-cell infiltration or enhancement of adenosine signaling.[14] Based on these results, we propose that expansion of A2aR-expressing effector T cells by ICI treatment might be necessary for A2aR inhibition to exhibit anti-tumor effects.

Overall, the expression of genes involved in adenosine signaling was rather low in HCC. This may be because we used bulk RNA sequencing datasets from tumor tissue, which may have contained only a limited number of immune cells, which is a potential limitation of this study.

The efficacy of A2aR antagonists for murine cancer therapy has been described in renal cancer by Fong *et al.*, wherein they describe $CD8^+$ T-cell recruitment following A2aR inhibitor treatment. They also conducted a clinical trial showing that treatment efficacy was related to the adenosine-related gene expression profile before treatment.[14] The combination of an A2aR antagonist and immunotherapy has been shown to have a synergistic effect on a subcutaneous colon cancer model by activating CD8 cells and blocking Treg activity in the tumor.[35] Furthermore, the inhibition of CD73 and A2aR reduced tumor initiation, growth, and metastasis in a melanoma model by recruiting effector lymphocytes and increasing IFN$\gamma$ expression.[22]

Herein, we describe the A2aR expression on hepatic T cells when an intrahepatic tumor is present and following anti-PD1 treatment. The combination effect with anti-PD1 therapy was only seen in the liver orthotopic model while $CD4^+$ T-cell depletion attenuated this effect. Overall, this data suggests that in liver tumors where anti-PD1 therapy or other ICIs are ineffective, the addition of A2aR blockade may disinhibit adenosine-mediated T-cell immunosuppression. On the other hand, A2aR agonists have been reported to increase Treg proliferation and immunosuppressive function,[36] though an A2aR inhibitor did not affect the number of Tregs in the liver of the orthotopic liver tumor model (Fig. 4A). This suggests that A2aR inhibition directly disinhibits and expands effector T cells in murine liver tumors by attenuating the adenosine-A2AR-cAMP axis.

Currently, there are limited treatment options for hepatobiliary cancers and many cancers are not responsive to immunotherapy. We show that targeting the adenosine pathway is mechanistically promising for clinical application. Intriguingly, an A2aR antagonist is already used for the treatment of Parkinson's disease, indicating a promising safety profile for early clinical trials.[37]

In conclusion, A2aR is highly expressed in human liver cancer and immune therapy increases the A2aR expression of T cells. We demonstrate that an A2aR antagonist enhanced the anti-tumor effect of anti-PD1 therapy in tumor-bearing mice, highlighting a putative therapeutic target for clinical application in hepatobiliary cancers and potentially cancers that have metastasized to the liver.

## Abbreviations

A2aR, adenosine A2a receptor; CCA, cholangiocarcinoma; CTLA4, cytotoxic T-lymphocyte antigen 4; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PD1, programmed death 1; TACE, transarterial chemoembolization; Tregs, regulatory T cells.

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## Conflict of interest

The authors declare no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

Conceptualization: YM, JDM, CM, TFG. Methodology: YM, JDM, CM, TFG, BR, KCB, SW, JCM, CF, XC. Investigation: YM, JDM. Visualization: YM, JDM, TFG. Funding acquisition: TFG, MC. Project administration: YM, JDM, CM, TFG. Supervision: TFG, MC. Writing – original draft: YM, TFG. Writing – review & editing: YM, JDM, CM, KCB, BR, RB, BLG, SW, JCM, CF, XC, TFG.

## Data availability statement

Data analyzed in the study are available from the corresponding author by request.

## Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100959.

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