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Original Article

Adora1 promotes colon cancer immune evasion via Irf1-PD-L1 signal axis

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Abstract: Immunotherapy targeting immune checkpoints such as programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) has emerged as a novel treatment option for various cancers, including colon cancer. However, immune evasion mechanisms can limit the efficacy of cancer immunotherapy. Understanding the regulatory mechanisms of PD-1/PD-L1 expression is therefore critical to enhancing immunotherapeutic outcomes. A previous study demonstrated that the adenosine A1 receptor (Adora1) regulates PD-L1 expression and tumor immune evasion in human melanoma; however, its role in colon cancer and associated immune escape remains poorly defined. To investigate this, we downregulated Adora1 expression in CT26 colon cancer cells using lentiviral transduction of Adora1-targeting shRNA. We assessed Adora1 and PD-L1 expression levels and evaluated cell proliferation in CT26 cells. *In vivo*, we inoculated CT26 cells into mice and monitored tumor growth, immune cell phenotypes, and T cell exhaustion within the tumors. Additionally, we evaluated T cell exhaustion *in vitro* by co-culturing T cells with CT26 cells. While Adora1 knockdown did not impact CT26 cell viability or proliferation *in vitro*, it significantly suppressed tumor growth *in vivo* ($P<0.0001$). Furthermore, Adora1 downregulation reduced T cell exhaustion (all $P=0.0025$) by decreasing PD-L1 expression in CT26 cells. Knockdown of Adora1 did not alter Atf3 expression but led to reduced Irf1 expression ($P=0.0268$), which contributed to the downregulation of PD-L1. Overall, these findings suggest that Adora1 downregulation inhibits immune escape in colon cancer by suppressing PD-L1 expression.

Keywords: Adora1, colon cancer, immune evasion, escape

Introduction

Colon cancer, also known as colorectal cancer, is one of the most common gastrointestinal malignancies and a leading cause of cancer-related death in Western developed countries [1]. In recent years, the incidence of colon cancer in China has risen rapidly, and it is expected to continue increasing in the coming years [2]. Therefore, there is an urgent need to develop more effective treatments for colon cancer.

Besides surgery, chemotherapy, and radiation therapy, targeted immunotherapy has revolutionized the treatment of several cancers, including colon cancer, and has emerged as a promising strategy to prolong patient survival [3, 4]. However, immune escape and disease relapse have been observed in a substantial subset of initial responders [5]. A deeper understanding of immune escape mechanisms is essential for improving the effectiveness of cancer immunotherapy. The immune checkpoint programmed death-1 (PD-1) is a critical immunosuppressive receptor expressed on various cells, including T cells, and its primary function is to inhibit effector T cell activity [6]. Programmed death-ligand 1 (PD-L1), the ligand of PD-1, is expressed by tumor cells and contributes to immune evasion and tumor progression [7]. In recent years, therapeutic strategies targeting the PD-1/PD-L1 interaction have shown significant efficacy across various tumor types [8]. However, less than 40% of patients respond to PD-1/PD-L1 inhibitors, and the underlying mechanisms driving resistance remain poorly understood [9]. Tumor PD-L1 expression has been recognized as a critical factor in predicting clinical response to PD-1/PD-L1 blockade therapy [10]. Therefore, eluci-

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dating the regulatory mechanisms controlling tumor PD-L1 expression is vital to enhance the therapeutic efficacy of anti-PD-1/PD-L1 treatments.

Adenosine is a nucleoside that has been shown to promote tumor progression [11]. Extracellular adenosine acts as a ligand by binding to four types of adenosine receptors: A1, A2A, A2B, and A3 [12]. The adenosine A1 receptor (Adora1) has been implicated in the pathogenesis of various cancers, exhibiting pleiotropic effects depending on the tumor type and context [13, 14]. Adora1 agonists have been reported to inhibit the proliferation of HepG2 liver carcinoma cells [15], while Adora1 antagonists have been shown to suppress proliferation in MCF-7 breast cancer cells [16]. A recent study demonstrated that Adora1 knockdown in melanoma cells inhibited cell proliferation, upregulated PD-L1 expression, promoted tumor immune evasion, and reduced antitumor efficacy [17]. However, the role of Adora1 in colon cancer, particularly in relation to cell proliferation and immune evasion, remains largely unexplored.

In the present study, we knocked down Adora1 expression in colon cancer cells and investigated its effects on tumor cell proliferation and immune evasion.

Materials and methods

Adora1 shRNA knockdown

The oligonucleotides for control shRNA (target sequence: 5'-CAACAAGATGAAGAGCACCAA-3'), Adora1 shRNA (target sequence: 5'-CCATTGCTCCTCATGGTTCTT-3'), and Irf1 shRNA (target sequence: 5'-AGATGGACATTATACCAGATA-3') were synthesized and cloned into the pLKO.1 lentiviral vector (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. The resulting pLKO.1 plasmids containing shRNA inserts were then co-transfected with the MISSION Lentiviral Packaging Mix into HEK293 cells to produce lentiviral particles, following the manufacturer's instructions.

To knock down Adora1, CT26 cells were transduced with the lentiviral particles at a multiplicity of infection of 100 in the presence of 5 μg/mL polybrene. Forty-eight hours post-transduction, the cells were selected using 1 μg/mL puromycin to generate stable transduced cell lines.

CT26 tumor cell inoculation

5×10^5 stably transduced CT26 cells were suspended in 200 μL of phosphate-buffered saline (PBS) and subcutaneously injected into the right flank of 6-8-week-old BALB/c mice. Tumor size and mouse survival rates were monitored at various time points following inoculation. At the end point, mice were sacrificed by CO_2 inhalation followed by tumor tissues harvesting. Tumor volume was calculated using the following formula: tumor volume = $L \times W^2$, where L = length of the tumor (the longest dimension), W = width of the tumor (the shortest dimension perpendicular to length). Animal studies were approved by School of Medicine, Huaqiao University (#A2023056).

Cell proliferation and viability assay

2×10^5 CT26 cells stably transduced with Adora1 shRNA (shAdora1 CT26 cells) and CT26 cells stably transduced with control shRNA (shControl CT26 cells) were seeded in the 6-well plates and cultured for six consecutive days. Cells were trypsinized every two days and counted to assess cell number. Cell viability was evaluated using CCK-8 assay. Briefly, 1×10^4 shAdora1 or shControl CT26 cells were seeded into 96-well plates. At designated time points, cells were incubated with CCK-8 reagent (100 μL/mL medium, diluted in culture medium) (Sigma, St Louis, MO) for 2 hours, and absorbance was measured at 450 nm using a microplate reader.

Flow cytometry

Twenty days after CT26 tumor cell inoculation, xenograft tumors were harvested, and single-cell suspension were prepared. After washing with staining buffer (2% FBS in PBS), cells were stained with anti-CD11b (Biolegend, San Diego, CA), anti-CD3 (Biolegend), anti-CD45 (Biolegend), anti-CD8 (Biolegend), anti-Granzyme B (GZB) (Biolegend), anti-PD-1 (Biolegend), anti-Tim3 (Biolegend), anti-CD86 (Biolegend), anti-F4/80 (Biolegend), anti-CD206 (Biolegend), anti-CD25 (Biolegend), anti-Foxp3 (Biolegend), with the presence of anti-CD16/CD32 antibody (Biolegend) on ice for 30 min. After washing, the cells were analyzed using a BD LSR II Flow

Cytometer, and the results were processed with FlowJo software.

For certain experiments, stably transduced CT26 cells were harvested and stained with anti-PD-L1 antibody (BioLegend).

$CD8^+$ T cells isolation and culture

$CD8^+$ T cells were isolated from BALB/c mice. Single-cell suspensions were prepared from the spleen by crushing and filtering through a 70 μm nylon cell strainer (Sigma). Red blood cells were lysed using ACK lysis buffer. The $CD8^+$ T cells were purified using the $CD8a^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

Purified $CD8^+$ T cells were then seeded into plates pre-coated with 0.5 μg/mL anti-CD3 and 5 μg/mL anti-CD28.

Lactate dehydrogenase (LDH) cytotoxicity assay

T cell cytolytic activity against transduced CT26 cells was assessed using the LDH Cytotoxicity Detection Kit (Takara, Beijing, China). ShControl or shAdora1 CT26 cells were co-cultured with $CD8^+$ T cells at a ratio of 1:3 for 3 days. After incubation, the cell culture supernatant was collected and transferred to a new plate. LDH solution was then added to the supernatant, and absorbance was measured. Cytotoxicity was calculated using the formula described previously [18].

Transfection

The pCMV6-Adora1 expression plasmid was purchased from Origene (Beijing, China) and transfected into CT26 cells using Polyethylenimine (PEI), following the protocol described previously [19].

Western blot

Total proteins from transduced or transfected CT26 cells were extracted using radioimmunoprecipitation assay buffer (Thermo Fisher, USA) and subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer, polyvinylidene fluoride membranes were blocked with 5% non-fat milk. Primary antibodies, including anti-Adora1 (Abcam, Beijing, China), anti-PD-L1 (Abcam), anti-Atf3 (Abcam), anti-Irf1 (Abcam), and anti-GAPDH (Abcam), were incubated overnight at 4°C. The following day, horse radish peroxidase-conjugated secondary antibodies were incubated for 1 hour at room temperature. ECL Substrate (Thermo Fisher, USA) was used to visualize the reactive bands. Band intensity was quantified and analyzed using ImageJ.

RT-PCR

Total RNA from CT26 cells was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD). cDNA was then reverse transcribed using the PrimeScript RT Reagent Kit (Takara, China). Real-time quantitative PCR reactions were performed using TB Green Advantage qPCR Premix on the QuantStudio3 Real-Time PCR System. The primers used for real-time PCR were as follows: PD-L1 Forward: 5'-GCTCCAAAGGACTTGTACGTG-3', Reverse: 5'-TGATCTGAAGGGCAGCATTTC-3'; GAPDH Forward: 5'-AGGTCGGTGTGAACGGATT TG-3', Reverse: 5'-GGGGTCGTTGATGGCAACA-3'.

Statistical analysis

Data were presented as mean ± SD, and analyzed by GraphPad Prism software (version 8.0). *P* values of the comparison between two groups were calculated using two-tailed Student's t-test. *P* values of the comparison among three or more groups were calculated using one-way ANOVA followed with a Tukey post hoc test. And *P* values of the comparison between two groups along multiple time points were calculated using Repeated Measures ANOVA followed by a Bonferroni post hoc test. Statistical significance was considered when $P<0.05$.

Results

Knocking down Adora1 did not affect CT26 cell proliferation but repressed the tumor growth in mice

First, we evaluated the effect of knocking down Adora1 on CT26 cell proliferation and tumor growth in a xenograft model. As shown in **Figure 1A** and **1B**, CT26 cells transduced with lentivirus expressing Adora1 shRNA (shAdora1

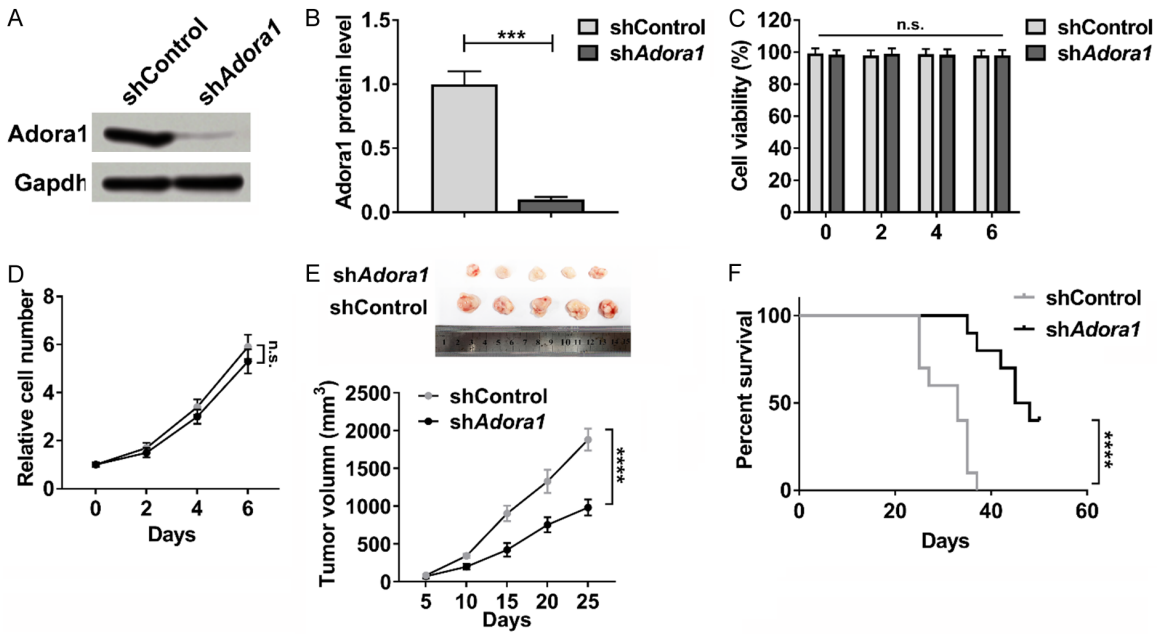

Figure 1. Deletion of Adora1 in CT26 cells represses the tumor growth in mice. (A) The protein level of Adora1 in shControl and shAdora1 CT26 cells was determined by immunoblotting. (B) The statistical result of (A) (n=3). (C, D) shControl and shAdora1 CT26 cells were cultured for 6 days. Cells viability (C) and proliferation (D) were determined (n=6). (E, F) shControl and shAdora1 CT26 cells (5×10^5) were subcutaneously injected into the backs of mice to establish tumors. (E) Representative tumor images at day 25, and tumor volumes in the experimental groups (n=5). (F) Survival curves were estimated (n=10).

CT26 cells) showed a significant reduction in endogenous Adora1 compared to CT26 cells transduced with lentivirus expressing control shRNA (shControl CT26 cells). Quantification revealed a significant decrease in the protein level of Adora1 in shAdora1 CT26 cells (**Figure 1B**, P=0.0009). We detected similar cell viability (**Figure 1C**), cell proliferation (**Figure 1D**) between shControl CT26 cells and sh*Adora1* CT26 cells, but reduced cell migration (Figure S1A, P=0.0453) and invasion (Figure S1B, P=0.0499) in sh*Adora1* CT26 cells, indicating knocking down Adora1 decreased cells migration and invasion, but did not affect cell growth *in vitro*. In contrast, mice inoculated with shAdora1 CT26 cells exhibited significantly smaller tumors compared to those inoculated with shControl CT26 cells (**Figure 1E**, P<0.0001). Consistently, mice inoculated with shAdora1 CT26 cells demonstrated a significantly higher survival rate compared to those inoculated with shControl CT26 cells (**Figure 1F**, P<0.0001). Collectively, these results suggest that knocking down Adora1 suppressed tumor growth in mice.

Knocking down Adora1 in CT26 tumor mitigates T cell exhaustion in xenograft tumors

Since downregulation of Adora1 did not affect the proliferation of CT26 cells *in vitro* but suppressed tumor growth *in vivo*, we proceeded to investigate the underlying mechanism by profiling the immune cells in xenograft tumors (Figure S2A). We found that there was no significantly difference of the percentage of $CD11b^+/CD45^+$ monocyte (**Figure 2A**), M1 macrophages (Figure S2B), M2 macrophages (Figure S2C), $CD3^+$ T cells (**Figure 2B**), Treg cells (Figure S2D) and $CD8^+$ T cells (**Figure 2C**) in xenograft tumor between shControl and shAdora1 CT26 tumors. In contrast, shAdora1 CT26 tumors exhibited a significantly higher percentage of $GZB^+/CD8^+$ T cells (**Figure 2D** and **2E**, P=0.0079), a decreased percentage of $PD\text{-}1^+/CD8^+$ T cells (**Figure 2F** and **2G**, P=0.0037), and a reduced percentage of $Tim3^+/CD8^+$ T cells (**Figure 2H** and **2I**, P=0.0145) compared to shControl tumors. Upregulation of Tim3 and PD-1, along with downregulation of GZB, are typical markers of T cell exhaustion [20]. These results indicated that

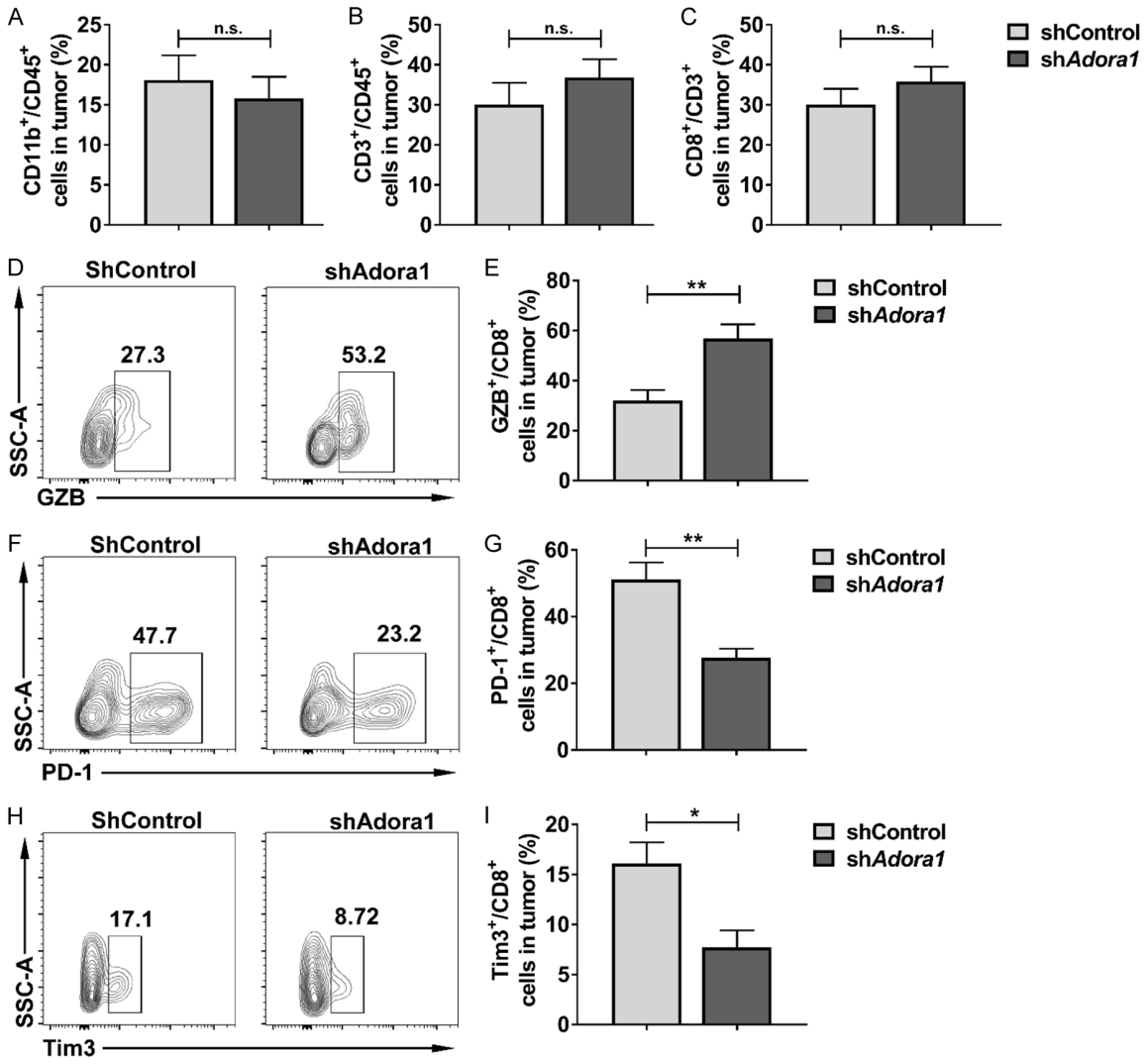

Figure 2. Knockdown of Adora1 in CT26 tumor mitigates T cell exhaustion in xenograft tumors. Tumors were harvested at day 20 post inoculation for FACS analysis. The percentage of $CD11b^+/CD45^+$ cells (A), $CD3^+/CD45^+$ cells (B), $CD8^+/CD3^+$ cells (C), $GZB^+/CD8^+$ cells (D and E), PD-$1^+/CD8^+$ cells (F and G), and $Tim3^+/CD8^+$ cells (H and I) were analyzed by FACS. n=5.

downregulation of Adora1 in CT26 tumor mitigated T cell exhaustion in xenograft tumors.

Knocking down Adora1 in CT26 cells suppressed T cell exhaustion in vitro

We next evaluated the effects of Adora1 downregulation in CT26 cells on $CD8^+$ T cell function and exhaustion *in vitro*. $CD8^+$ T cells were co-cultured with either shControl CT26 cells or shAdora1 CT26 cells. We found that $CD8^+$ T cells co-cultured with shAdora1 CT26 cells exhibited significantly enhanced cytotoxicity compared to those co-cultured with shControl CT26 cells (P=0.0084), indicating that downregulation of Adora1 in CT26 cells enhanced $CD8^+$ T cell cytotoxicity toward CT26 cells (**Figure 3A**).

Furthermore, downregulation of Adora1 in CT26 cells significantly increased the percentage of $GZB^+/CD8^+$ T cells (**Figure 3B**, P=0.0081), decreased the percentage of PD-$1^+/CD8^+$ T cells (**Figure 3C**, P=0.0025), and reduced the percentage of $Tim3^+/CD8^+$ T cells (**Figure 3D**, P=0.0013) in the co-cultured $CD8^+$ T cell population. Collectively, these data demonstrate that downregulation of Adora1 in CT26 cells inhibits T cell exhaustion *in vitro*.

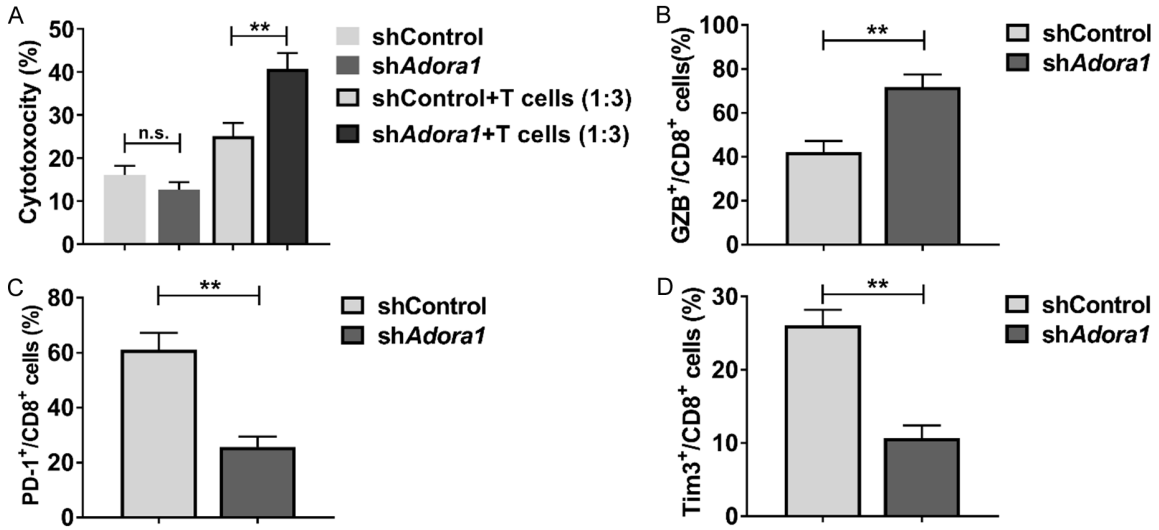

Figure 3. Knockdown of Adora1 in CT26 cell inhibits T cell exhaustion *in vitro*. (A) shControl and shAdora1 CT26 cells were co-cultured with $CD8^+$ T cells at an E: T ratio of 1:3 for 3 days. Cytotoxicity was evaluated with the LDH-releasing assay. (B-D) shControl and shAdora1 CT26 cells were co-cultured with CD8 T cells at a ratio of 1:3 for 3 days, and $GZB^+/CD8^+$ cells (B), $PD\text{-}1^+/CD8^+$ cells (C), and $Tim3^+/CD8^+$ (D) were analyzed by FACS. n=4.

Knocking down Adora1 inhibited PD-L1 expression in CT26 cells

As the PD-1/PD-L1 pathway is a major regulator of T cell exhaustion [21], we next investigated the effect of Adora1 on PD-L1 expression in CT26 tumor cells. We overexpressed or knocked down Adora1 in CT26 cells and assessed PD-L1 expression. As shown in **Figure 4A**, shAdora1 CT26 cells exhibited a significantly decreased mRNA level of PD-L1 compared to shControl CT26 cells (P=0.0006). CT26 cells transfected with the Adora1-expressing plasmid (Adora1-OE CT26 cells) showed a significant increase in PD-L1 mRNA expression (P=0.0009).

Correspondingly, we observed a significant downregulation of PD-L1 protein in shAdora1 CT26 cells (P=0.033) and a significant increase in PD-L1 protein in Adora1-OE CT26 cells (P=0.0002) by western blot (**Figure 4B** and **4C**) and flow cytometry (**Figure 4D** and **4E**, P=0.0128, P=0.0003). We further analyzed PD-L1 expression in $CD45^-$ cells from xenograft tumors and found a significantly decreased percentage of $PD\text{-}L1^+/CD45^-$ cells in shAdora1 CT26 tumors (**Figure 4F** and **4G**, P=0.0014). Taken together, these results demonstrate that downregulation of Adora1 leads to decreased PD-L1 expression in CT26 cells.

Adora1 regulates PD-L1 expression via Irf1

Finally, we investigated the mechanism underlying Adora1-mediated regulation of PD-L1 expression. A previous study demonstrated the negative regulation of PD-L1 expression by Adora1 through ATF3 in melanoma [17].

We explored whether knocking down or overexpressing Adora1 affected Atf3 expression in CT26 colon carcinoma cells. As shown in **Figure 5A** and **5B**, shAdora1 CT26 cells and Adora1-OE CT26 cells exhibited similar protein levels of Atf3 to shControl CT26 cells, indicating that Adora1 did not regulate Atf3 expression in CT26 cells.

In contrast, we observed a significant decrease in Irf1, a positive regulator of PD-L1 [22], in shAdora1 CT26 cells (P=0.0268), and a significant increase in Irf1 in Adora1-OE CT26 cells (P=0.0002) (**Figure 5A** and **5C**). We further evaluated the effects of Irf1 on PD-L1 expression. Knocking down Irf1 in CT26 and Adora1-OE CT26 cells significantly reduced endogenous PD-L1 expression (**Figure 5D** and **5E**) (P<0.0001). Collectively, these results demonstrate that Adora1 regulates PD-L1 expression via Irf1.

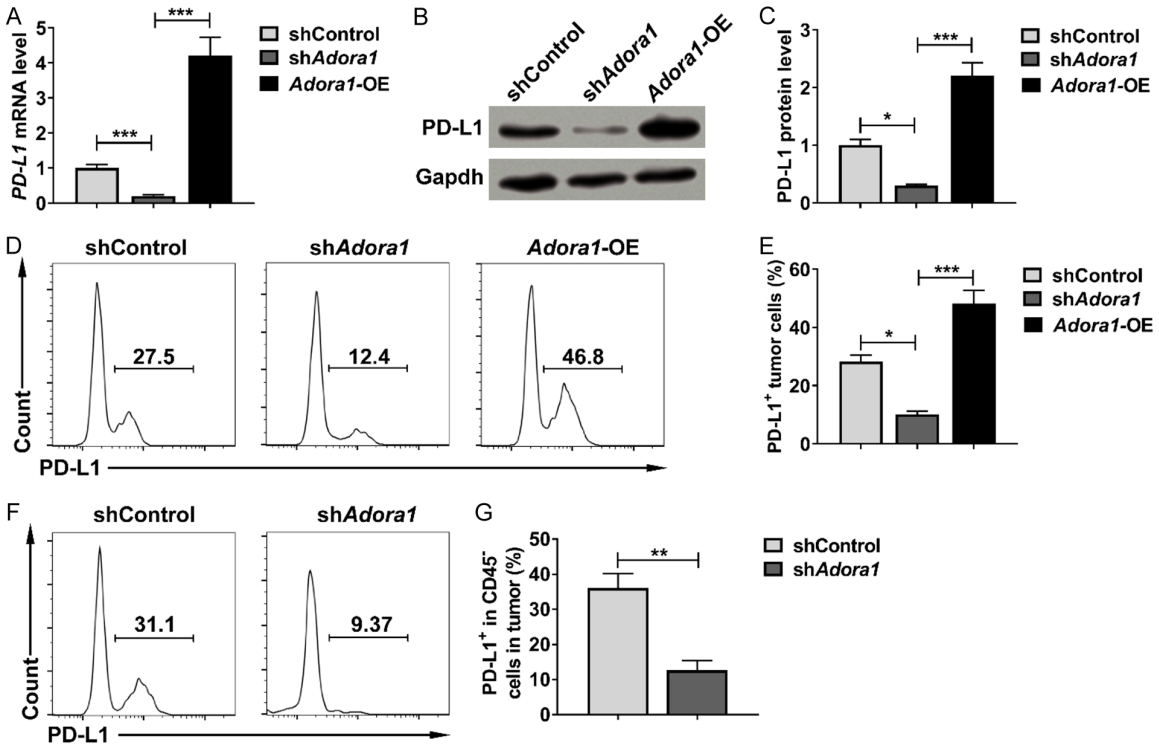

Figure 4. Knockdown of Adora1 decreases the expression of PD-L1 in CT26 tumor cells. (A-E) shControl, shAdora1 and Adora1 overexpressing (Adora1-OE) CT26 cells were harvested for examining mRNA level (A), protein level (B and C), and membrane-bound protein level (D and E) of PD-L1 by RT-qPCR, western blot, and FACS, respectively. n=3. (F, G) shControl and shAdora1 CT26 tumors were harvested at day 20 for analyzing. The expression level of PD-L1 in non-immune cells ($CD45^-$). n=5.

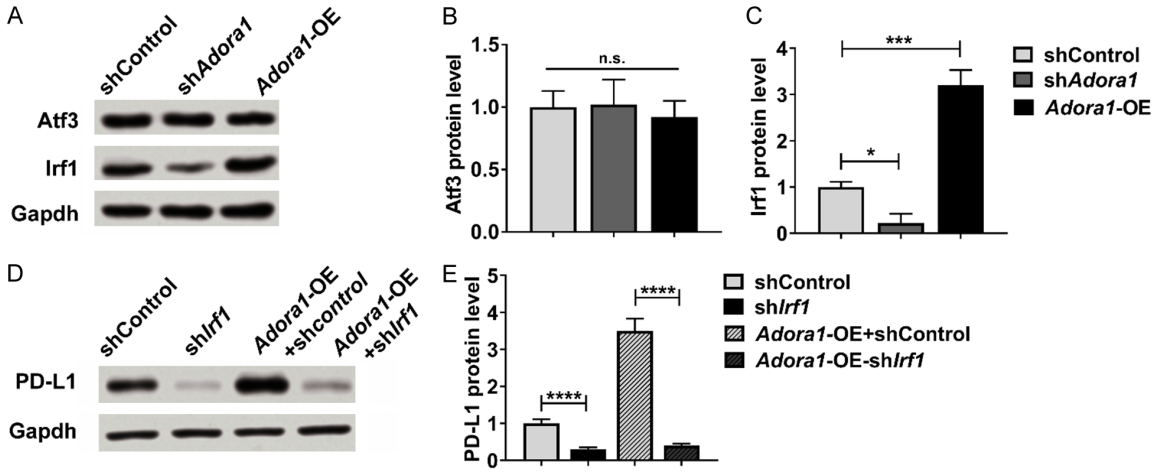

Figure 5. Adora1 regulates PD-L1 expression via Irf1. (A-C) shControl, shAdora1 and Adora1-OE CT26 cells were harvested for examining protein level of Atf3 (A and B) and Irf1 (A and C) by western blot. (D, E) Irf1 was knocked down in CT26 and Adora1-OE CT26 cells by shRNA. CT26 cells were harvested for examining the protein level of PD-L1 by western blot. n=3.

Discussion

In the present study, we demonstrated that downregulation of Adora1 in colon cancer cells suppressed tumor PD-L1 expression via Irf1, leading to the inhibition of T cell exhaustion and enhanced anti-tumor efficacy. Given that Adora1 upregulates PD-L1 expression and pro-

motes immune evasion in colon cancer, Adora1 may serve as a potential therapeutic target for the treatment of colon cancer.

Blocking the immune checkpoint PD-1 or PD-L1 using neutralizing antibodies has been shown to be beneficial for several cancer patients [23]. However, the overall response rate to PD-1/PD-L1 therapy has not met expectations [24]. PD-L1 expression is known to be regulated within the tumor microenvironment through multiple mechanisms, which contribute to tumor immune escape [25, 26]. Therefore, targeting the regulation of PD-L1 is also critical for improving the efficacy of anti-PD-L1 therapy.

In the present study, we demonstrated that downregulation of Adora1 by siRNA suppressed the expression of PD-L1 in colon cancer CT26 cells, which ultimately resulted in the prevention of T cell exhaustion in CT26 tumors. Adora1 has been implicated in various cancers. It has been reported that Adora1 is significantly upregulated in MDA-MB-468 and CW-2 cancer cells [27], as well as in human colorectal adenocarcinoma tissues [28]. The upregulation of Adora1 in cancer cells suggests potential functional roles of Adora1 in cancer progression. However, the precise roles of Adora1 in cancers remain poorly understood, and previous studies on Adora1 using agonists and antagonists in different cancer models have yielded controversial results.

For example, Adora1 agonists inhibited cell proliferation in astrocytoma [29] and melanoma cells [30]. In contrast, Adora1 agonists promoted cell proliferation, while Adora1 antagonists suppressed cell proliferation in breast cancer cells [31, 32]. Using the siRNA approach, Liu and colleagues knocked down endogenous Adora1 in human melanoma cells and found that Adora1 deletion suppressed cell growth [17]. In contrast, we demonstrated that deletion of Adora1 in colon cancer CT26 cells did not affect cell proliferation or viability *in vitro*. These differing effects of Adora1 deletion may be attributed to the differences in the cell types used, further emphasizing the specificity of each cancer model.

In the same study, Liu and colleagues further demonstrated that deletion of Adora1 resulted in increased expression of PD-L1 in human melanoma cells, inactivation of co-cultured T cells *in vitro*, and suppression of anti-tumor immunity *in vivo*. The negative regulation of PD-L1 by Adora1 was mediated through ATF3 [17]. Interestingly, we found that deletion of Adora1 led to decreased expression of PD-L1 in colon cancer cells, which prevented T cell exhaustion and suppressed tumor immune escape. Furthermore, the regulation of PD-L1 by Adora1 in colon cancer cells did not depend on ATF3, as deletion of Adora1 did not affect ATF3 expression in CT26 cells, but instead was mediated through Irf1.

It remains unclear why deletion of Adora1 had opposing effects on PD-L1 expression in human melanoma versus colon cancer CT26 cells. This could be attributed to differences in the regulatory pathways involved. We further demonstrated that deletion of Adora1 regulated the expression of Irf1, a positive regulator of PD-L1, but did not affect ATF3 expression. Since different cancer cells were used, Adora1 deletion might influence distinct signaling pathways. Interestingly, we observed that deletion of Adora1 resulted in the downregulation of Irf1, which is known to positively regulate PD-L1 expression. It would be of great interest to investigate the mechanism by which Irf1 expression is regulated by Adora1. Upon binding with adenosine, Adora1 mediates the activation of the PI3K/AKT signaling pathway [33]. Whether the PI3K/AKT pathway participates in Adora1-mediated regulation of Irf1 remains to be determined.

Our study has several limitations. First, it is crucial to strengthen the clinical relevance of Adora1 by validating the correlation between Adora1 expression levels and clinical outcomes in publicly available immunotherapy cohorts. Second, since the liver is the primary site of colorectal cancer (CRC) metastasis and CRC liver metastasis is highly resistant to immunotherapy, it would be valuable to investigate whether Adora1 expression levels are consistent between the primary tumor and liver metastasis.

Conclusion

We demonstrated that downregulation of Adora1 led to a suppression of PD-L1 expression and inhibited tumor immune escape in colon cancer, suggesting that Adora1 could

serve as a potential therapeutic target for the treatment of colon cancer.

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Disclosure of conflict of interest

None.

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References

[1] Underwood PW and Pawlik TM. Precision medicine for metastatic colorectal cancer: where do we stand? Cancers (Basel) 2024; 16: 3870.

[2] Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I and Jemal A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2024; 74: 229-263.

[3] Xie YH, Chen YX and Fang JY. Comprehensive review of targeted therapy for colorectal cancer. Signal Transduct Target Ther 2020; 5: 22.

[4] Ruff SM, Brown ZJ and Pawlik TM. A review of targeted therapy and immune checkpoint inhibitors for metastatic colorectal cancer. Surg Oncol 2023; 51: 101993.

[5] Iorgulescu JB, Braun D, Oliveira G, Keskin DB and Wu CJ. Acquired mechanisms of immune escape in cancer following immunotherapy. Genome Med 2018; 10: 87.

[6] Budimir N, Thomas GD, Dolina JS and Salek-Ardakani S. Reversing T-cell exhaustion in cancer: lessons learned from PD-1/PD-L1 immune checkpoint blockade. Cancer Immunol Res 2022; 10: 146-153.

[7] Pu Y and Ji Q. Tumor-associated macrophages regulate PD-1/PD-L1 immunosuppression. Front Immunol 2022; 13: 874589.

[8] Zou W, Wolchok JD and Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: mechanisms, response biomarkers, and combinations. Sci Transl Med 2016; 8: 328rv4.

[9] Pitt JM, Vetizou M, Daillere R, Roberti MP, Yamazaki T, Routy B, Lepage P, Boneca IG, Chamaillard M, Kroemer G and Zitvogel L. Resistance mechanisms to immune-checkpoint blockade in cancer: tumor-intrinsic and -extrinsic factors. Immunity 2016; 44: 1255-1269.

[10] Sharma P and Allison JP. The future of immune checkpoint therapy. Science 2015; 348: 56-61.

[11] Arab S and Hadjati J. Adenosine blockage in tumor microenvironment and improvement of cancer immunotherapy. Immune Netw 2019; 19: e23.

[12] Borea PA, Gessi S, Merighi S, Vincenzi F and Varani K. Pharmacology of adenosine receptors: the state of the art. Physiol Rev 2018; 98: 1591-1625.

[13] Allard B, Allard D, Buisseret L and Stagg J. The adenosine pathway in immuno-oncology. Nat Rev Clin Oncol 2020; 17: 611-629.

[14] Xia C, Yin S, To KKW and Fu L. CD39/CD73/A2AR pathway and cancer immunotherapy. Mol Cancer 2023; 22: 44.

[15] Hosseinzadeh H, Jaafari MR and Shamsara J. Selective inhibitory effect of adenosine A1 receptor agonists on the proliferation of human tumor cell lines. Iran Biomed J 2008; 12: 203-208.

[16] Lin Z, Yin P, Reierstad S, O'Halloran M, Coon VJ, Pearson EK, Mutlu GM and Bulun SE. Adenosine A1 receptor, a target and regulator of estrogen receptoralpha action, mediates the proliferative effects of estradiol in breast cancer. Oncogene 2010; 29: 1114-1122.

[17] Liu H, Kuang X, Zhang Y, Ye Y, Li J, Liang L, Xie Z, Weng L, Guo J, Li H, Ma F, Chen X, Zhao S, Su J, Yang N, Fang F, Xie Y, Tao J, Zhang J, Chen M, Peng C, Sun L, Zhang X, Liu J, Han L, Xu X, Hung MC and Chen X. ADORA1 inhibition promotes tumor immune evasion by regulating the ATF3-PD-L1 axis. Cancer Cell 2020; 37: 324-339, e8.

[18] Nguyen HH, Kim T, Song SY, Park S, Cho HH, Jung SH, Ahn JS, Kim HJ, Lee JJ, Kim HO, Cho JH and Yang DH. Naive CD8 (+) T cell derived tumor-specific cytotoxic effectors as a potential remedy for overcoming TGF-beta immunosuppression in the tumor microenvironment. Sci Rep 2016; 6: 28208.

[19] Longo PA, Kavran JM, Kim MS and Leahy DJ. Transient mammalian cell transfection with polyethylenimine (PEI). Methods Enzymol 2013; 529: 227-240.

[20] Yi JS, Cox MA and Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology 2010; 129: 474-481.

[21] Lee J, Ahn E, Kissick HT and Ahmed R. Reinvigorating exhausted T Cells by blockade of the

PD-1 pathway. For Immunopathol Dis Therap 2015; 6: 7-17.
[22] Moon JW, Kong SK, Kim BS, Kim HJ, Lim H, Noh K, Kim Y, Choi JW, Lee JH and Kim YS. IFNgamma induces PD-L1 overexpression by JAK2/STAT1/IRF-1 signaling in EBV-positive gastric carcinoma. Sci Rep 2017; 7: 17810.
[23] Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK and Iyer AK. PD-1 and PD-L1 Checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. Front Pharmacol 2017; 8: 561.
[24] Yarchoan M, Hopkins A and Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. N Engl J Med 2017; 377: 2500-2501.
[25] Kalantari Khandani N, Ghahremanloo A and Hashemy SI. Role of tumor microenvironment in the regulation of PD-L1: a novel role in resistance to cancer immunotherapy. J Cell Physiol 2020; 235: 6496-6506.
[26] Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, Wu X, Ma J, Zhou M, Li X, Li Y, Li G, Xiong W, Guo C and Zeng Z. Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape. Mol Cancer 2019; 18: 10.
[27] Dai T, Li N, Han F, Zhang H, Zhang Y and Liu Q. AMP-guided tumour-specific nanoparticle delivery via adenosine A1 receptor. Biomaterials 2016; 83: 37-50.
[28] Khoo HE, Ho CL, Chhatwal VJ, Chan ST, Ngoi SS and Moochhala SM. Differential expression of adenosine A1 receptors in colorectal cancer and related mucosa. Cancer Lett 1996; 106: 17-21.
[29] Sai K, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, Saito M, Yamamura T and Nishizaki T. A(1) adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neurotoxicology 2006; 27: 458-467.
[30] Daniele S, Zappelli E, Natali L, Martini C and Trincavelli ML. Modulation of A1 and A2B adenosine receptor activity: a new strategy to sensitise glioblastoma stem cells to chemotherapy. Cell Death Dis 2014; 5: e1539.
[31] Dastjerdi MN, Valiani A, Mardani M and Ra MZ. Adenosine A1 receptor modifies P53 expression and apoptosis in breast cancer cell line Mcf-7. Bratisl Lek Listy 2016; 117: 242-246.
[32] Dastjerdi MN, Rarani MZ, Valiani A and Mahmoudieh M. The effect of adenosine A1 receptor agonist and antagonist on p53 and caspase 3, 8, and 9 expression and apoptosis rate in MCF-7 breast cancer cell line. Res Pharm Sci 2016; 11: 303-310.
[33] Liu H and Xia Y. Beneficial and detrimental role of adenosine signaling in diseases and therapy. J Appl Physiol (1985) 2015; 119: 1173-1182.

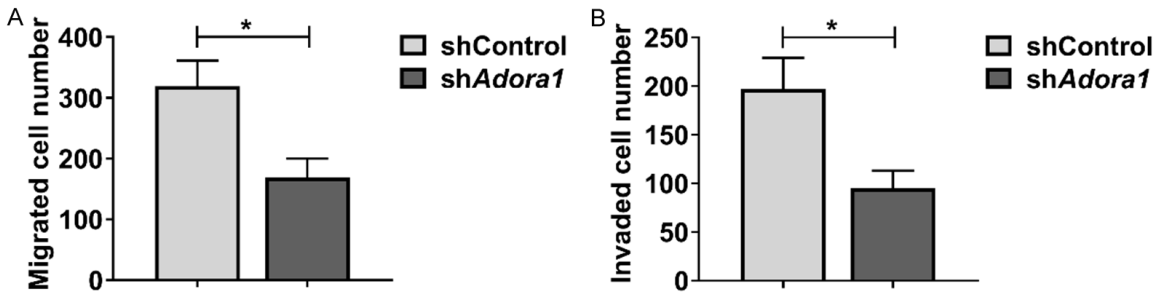

Figure S1. Knockdown of Adora1 decreased cells migration and invasion. (A, B) The migrated (A) and invaded (B) cells number of shControl and sh*Adora1* CT26 cells was determined by migration and invasion assay. n=3.

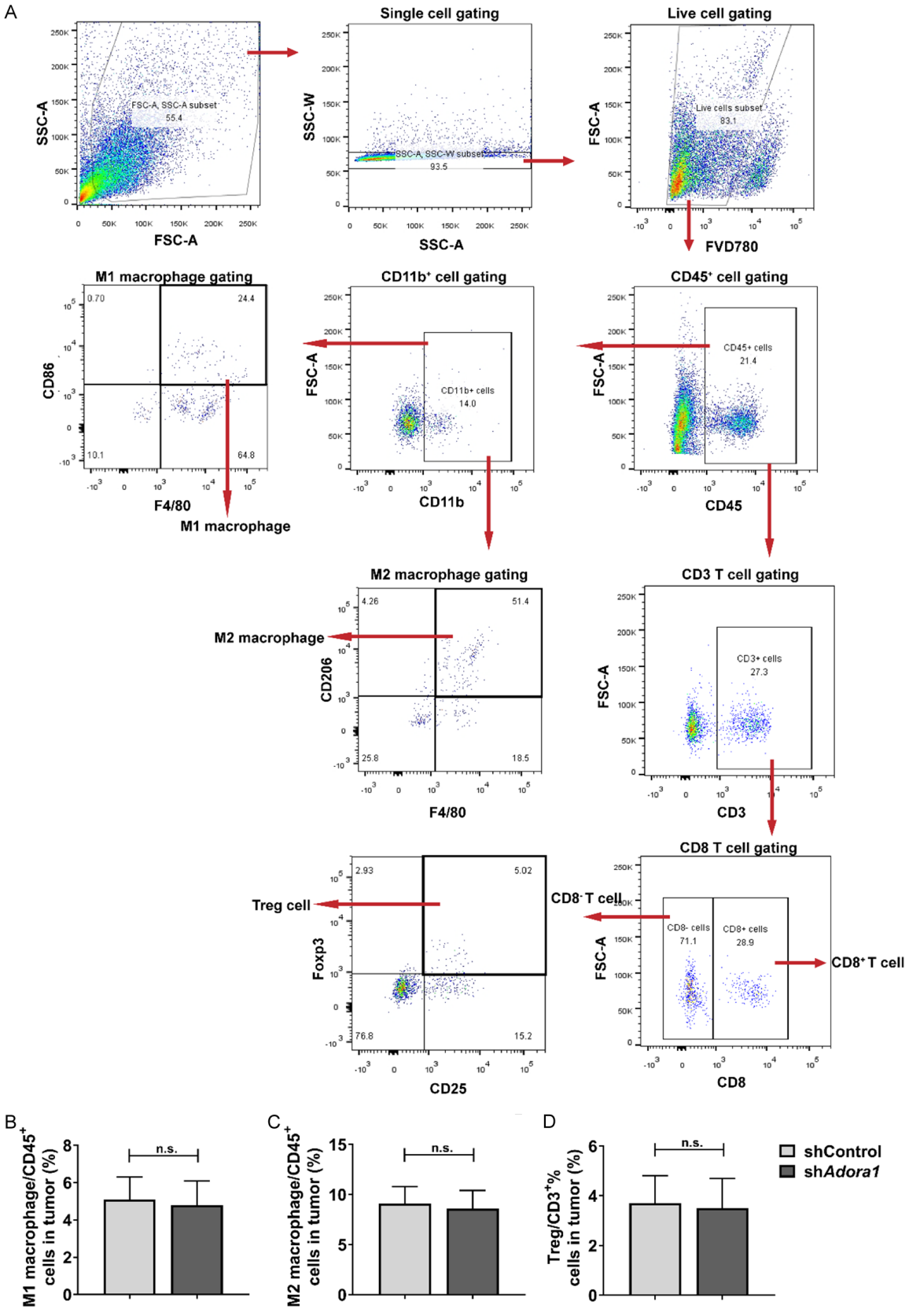

Figure S2. Downregulating Adora1 did not impact M1/2 macrophages and Tregs. A. The gating strategy for CT26 tumor tissue. B-D. shControl and sh*Adora1* CT26 tumors were harvested at day 20 for FACS analysis. The percentage of M1 macrophages ($CD11b^+F4/80^+CD86^+$), M2 macrophages ($CD11b^+F4/80^+CD206^+$) in $CD45^+$ cells, Treg cells ($CD8^-CD25^+Foxp3^+$) in CD3 T cells were analyzed by FACS. n=5.