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CD73 on tumor cells impairs anti-tumor T cell responses: a novel mechanism of tumor-induced immune suppression

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Abstract

CD73, originally defined as a lymphocyte differentiation antigen, is thought to function as a co-signaling molecule on T lymphocytes and an adhesion molecule that is required for lymphocyte binding to endothelium. We show here that CD73 is widely expressed on many tumor cell lines and is upregulated in cancerous tissues. Because the ecto-5′-nucleotidase activity of CD73 catalyzes AMP breakdown to immunosuppressive adenosine, we hypothesized that CD73-generated adenosine prevents tumor destruction by inhibiting antitumor immunity. We confirmed this hypothesis by showing that combining tumor CD73 knockdown and tumor-specific T cell transfer cured all tumor-bearing mice. In striking contrast, there was no therapeutic benefit of adoptive T-cell immunotherapy in mice bearing tumors without CD73 knockdown. Moreover, blockade of the A2A adenosine receptor with a selective antagonist also augmented the efficacy of adoptive T cell therapy. These findings identify a potential mechanism for CD73-mediated tumor immune evasion and point to a novel cancer immunotherapy strategy by targeting the enzymatic activity of tumor CD73.

Introduction

Accumulating evidence indicates that a dynamic cross-talk between a tumor and the immune system can control tumor growth (1,2). It is known that many tumors are potentially immunogenic, as supported by the presence of tumor-specific immune responses *in vivo*. However, spontaneous eradication of established tumors by endogenous immunity is often rare. A number of obstacles hinder the generation of effective tumor immunity. During tumor progression, tumor cells foster a tolerant microenvironment and the activation of multiple immunosuppressive mechanisms, which may act in concert to block effective immune responses (3). Obviously, a better understanding of the different aspects of tumor-induced immune suppression would help develop and refine novel immunotherapeutic strategies.

CD73, known as ecto-5′-nucleotidase (5′-NT, EC3.1.3.5) is a glycosyl-phosphatidylinositol-linked 70-kDa cell surface enzyme found in most tissues (4,5). CD73, originally defined as a lymphocyte differentiation antigen, is expressed on many cell types including subsets of lymphocytes (6), endothelial cells (7), and epithelial cells (8). Several *in vitro* earlier studies reported that CD73 functions as a co-signaling molecule on T lymphocytes (9-11) and as an

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adhesion molecule required for lymphocyte binding to the endothelium (12). Recently, it is believed that biological actions of CD73 are a consequence (at least in large part) of the regulated enzymatic phosphohydrolytic activity of extracellular nucleotides. This ecto-enzymatic cascade in tandem with CD39 (ecto-ATPase) generates adenosine from ATP/AMP often released from damaged or inflamed target cells into the extracellular environment (13, 14). Extracellular adenosine induces potent immunosuppressive effects, mainly mediated through four adenosine-binding G protein-coupled receptors: A1, A2A, A2B, and A3 (15). Notably, adenosine inhibits the activation and expansion of T cells primarily via the A2A adenosine receptor (A2AAR) (16,17). Modulation of inflammation by adenosinergic mechanisms has been characterized in various murine models including T cell–dependent autoimmune encephalomyelitis (18), colitis (19), and viral hepatitis (20), and in antitumor T cell immunity (21). In addition, it has been recently accepted that adenosine generated from $Foxp3^+CD4^+$ regulatory T cells (Tregs) through CD39/CD73 mediates immune suppression (22,23).

Interestingly, CD73 is highly expressed in many human solid tumors (24-29), and its elevated expression and activity are associated with tumor invasiveness and metastasis (30,31), and with shorter patient survival time (32), indicating CD73 is closely involved in cancer progression. Based on the immunomodulatory property of adenosine, we evaluated the role of CD73 in cancer immunity. Because A2AAR protected tumors from incoming anti-tumor T cells (21), we hypothesized that extracellular adenosine generated by CD73 on tumor cells would impair anti-tumor immunity. Indeed, we found that knockdown of CD73 on tumor cells by siRNA improved antitumor T cell responses including both activation and effector functions, completely restored efficacy of adoptive T cell therapy and led to long-term tumor-free survival of tumor-bearing mice. Therefore, targeting the enzymatic activity of tumor CD73 may be an important new approach to cancer immunotherapy.

Materials and Methods

Mice, cell lines and reagents

$Rag1^{-/-}$ mice and CD90.1 mice were purchased from the Jackson Laboratory, and C57BL/6 mice from NCI-Frederick. Dr. Hans Schreiber (University of Chicago) provided the OT-1 $Rag1^{-/-}$ mice and the human ovarian cancer line SKOV3. All animal experiments were approved by institutional animal use committees of the University of Texas Health Science Center at San Antonio. ID8 ovarian cells were provided by Dr. George Coukos (University of Pennsylvania). To obtain the ascites-derived ID8 cells, the ascites cells isolated from ID8-bearing mice (about 8 weeks after tumor injection) were plated in flasks and incubated overnight to allow attachment of cancer cells. After passage twice, the attached cells (ascites-derived ID8 cells) were examined for CD73 expression. Similar to regular ID8, ascites-derived ID8 cells are $CD326^+$ $CD45^-$, suggesting they are of epithelial origin (data not shown). To generate ID8 conditioned medium, 5×10^5 ID8 cells were cultured in 10 ml of complete medium in flasks for 2-3 days, and the supernatants were subsequently centrifuged and filtered for further experiments. All the cell lines were tested for mycoplasma infections, and maintained in complete medium composed of RPMI 1640 with 5% FBS. The OVA-derived peptide SIINFEKL (OVA-I) was synthesized by GenScript. All the Abs were obtained from eBioscience. The Alexa Fluor® 647 Annexin V apoptosis detection kit was from BioLegend. MTT, 5′-AMP, SCH58261 (A2AAR antagonist), α,β-methylene-ADP (APCP) and caffeine were purchased from Sigma. The 5′-N-Ethylcarboxamidoadenosine, (NECA) was from Tocris Bioscience.

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Generation of OVA vectors and cells expressing these genes

A complementary DNA (cDNA) encoding full length chicken ovalbumin (OVA) (gift of Dr. Dr. John Frelinger, University of Rochester Medical Center) was inserted into the retrovirus backbone vector MIGR1-IRES-eGFP. MIGR1-OVA-IRES-eGFP was transfected by electroporation into the PT67 cell line to produce retrovirus. ID8 cells were infected with MIGR1-OVA-IRES-eGFP and OVA-expressing cells were sorted twice based on GFP expression. OVA production was confirmed by ELISA (data not shown).

Knockdown of CD73 expression by shRNA

The vector used to knockdown CD73 (Gene accession: NM_011851) expression was lentiviral pGIPZ, purchased from Open Biosystem. Two different pGIPZ constructs (RMM4431-98751230 and RMM4431-98762273) containing each shRNA sequence targeted to CD73 were selected. The pGIPZ non-silencing control vector from a Trans-Lentiviral™ GIPZ Packaging System (Open Biosysterm, TLP4615) was used as an expression control. To knockdown CD73, cells were transfected with the above vectors using an optimized Nucleofector Kit for Cell Lines purchased from Amaxa, a Lonza company. Twenty-four to 48 hours later, the transfection efficiency was evaluated by flow cytometry. To obtain stable clones, the transfected cells were selected with zeomycin and/or further sorted with a BD FACSAria Flow Cytometer gating on GFP^+ populations.

Measurement of CD73 activity

CD73 enzyme activity was evaluated by measuring the conversion of [^{14}C]IMP to [^{14}C]inosine as described previously (33). APCP was used as a specific inhibitor of CD73 (34). Thus, CD73 activity was calculated as the portion of the total IMP-hydrolyzing activity that was inhibited by APCP. The results were expressed as nmoles IMP hydrolyzed/hr/10^6 cells.

CFSE proliferation assay

To examine whether adenosine generated by CD73 on tumor cells suppresses T cell proliferation, 1×10^4 ID8 cells were incubated with conditioned medium in the absence or presence of 0.5 μM or 50 μM 5′-AMP alone, or 25 nM SCH58261 alone, or 5′-AMP plus SCH58261 for 4 h at 37°C. Splenocytes labeled with 5 μM CFSE were then incubated at 5×10^5 with conditioned medium from the above ID8 cultures, or regular complete medium as a control, and 1 μg/ml anti-CD3 mAb for 72 h. The CFSE dilution was measured by flow cytometry by gating on $CD8^+$ or $CD4^+$ cells. To demonstrate whether adenosine generated by tumor CD73 affects tumor antigen-specific T cell responses, ID8, ID8-OVA, ID8-OVA-SiNS or ID8-OVA-SiCD73 at 1×10^4 cells were incubated with conditioned medium as indicated above, or 10 μM NECA, or 10 μM APCP or APCP plus NECA for 4 h at 37°C. CFSE-labeled 5×10^5 OT-I splenocytes were subsequently added into the above tumor cell cultures. After 3 days, the floating cells were harvested and examined for CFSE dilution and intracellular IFN-γ staining by flow cytometry by gating on $CD8^+$ cells. For *in vivo* T cell proliferation assays, female C57BL/6 mice were inoculated i.p. with 1×10^7 ID8-OVA-SiNS or ID8-OVA-SiCD73 cells. Two weeks later, naïve CFSE-labeled OT-I CD90.1 splenocytes were injected i.p.. The proliferation of transferred cells in spleens, mesenteric lymph nodes (MLN) and peritoneal exudate cells (PEC) was monitored by gating on $CD8^+$ $CD90.1^+$ cells at d2, d4 and d6. The activation markers CD44 and CD69 or intracellular IFN-γ staining on these transferred cells ($CD8^+$ $CD90.1^+$) was also measured.

[^{3}H] incorporation assay

To study whether adenosine directly suppresses T cell proliferation, splenocytes were incubated at 5×10^5 cells with 1 μg/ml anti-CD3 mAb in X-VIVO 15 medium with or without adenosine at various concentrations (0.1-10 μM), and proliferation was measured after 72 h

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by [^{3}H] thymidine incorporation. To examine whether knockdown of CD73 expression promoted T cell proliferation, ID8SiNS and ID8SiCD73 at 1×10^4 cells were incubated with conditioned medium in the absence or presence of 0.5 μM or 50 μM 5′-AMP or 10 μM APCP alone or APCP plus AMP for 4 h at 37°C. Splenocytes were then incubated at 5×10^5 cells with conditioned medium from the above cultures and 1 μg/ml anti-CD3 mAb for 72 h. Eighteen hours before harvesting, cells were pulsed with [^{3}H] thymidine (1 μCi/well; Amersham Biosciences). [^{3}H] thymidine uptake was counted using a liquid scintillation counter and expressed as cpm.

In vivo killing assay

Analysis of tumor Ag-specific effector CTL activity *in vivo* was performed as previously described (35). Briefly, OVA-I peptide-pulsed CFSE^{high} and non-peptide-pulsed CFSE^{low} splenocytes were mixed at a ratio of 1:1 and a total of 2×10^7 cells were injected i.p. into recipient animals. PEC, MLN, and spleens were then harvested 24 h after adoptive transfer and CFSE fluorescence intensity was analyzed by flow cytometry. Gating on CFSE$^+$ cells, the percent killing was calculated as follows: 100 - ([(% OVA-I peptide-pulsed cells in OVA$^+$ tumor-bearing mice/% non-peptide-pulsed cells in OVA$^+$ tumor-bearing mice)/(% OVA-I peptide-pulsed cells in OVA$^-$ tumor-bearing mice /% non-peptide-pulsed cells in OVA$^-$ tumor-bearing mice)] x 100).

^{51}Cr release assay

OT-I CD8$^+$ T cells were stimulated with irradiated splenocytes and OVA-I peptide in the presence of IL-2 for 72 h. For APCP treatment, cancer cells were pre-incubated with 10 μM APCP for 4 h. The killing of ID8-GFP, ID8-OVA-SiNS, ID8-OVA-SiCD73 or APCP treated-ID8-OVA-SiNS cancer cells was determined using a standard ^{51}Cr release assays as previously described (36).

Cell apoptosis assay

ID8-OVA-SiNS or ID8-OVA-SiCD73 at 1×10^4 cells were incubated with conditioned medium in the absence or presence of 0.5 or 50 μM 5′-AMP alone, or 25 nM SCH58261 alone, or 5′-AMP plus SCH58261 for 4 h at 37°C. OT-I splenocytes were pre-activated with 1 μg/ml OVA-I peptide for 48 h. These activated cells were added into the above tumor cell cultures at 3×10^5. After 3 days in culture, the floating cells were harvested and examined for apoptosis by double staining with annexin V and propidium iodide and subjected to flow cytometry. To monitor the apoptosis of adoptively transferred T cells, female C57BL/6 mice were inoculated i.p. with 5×10^6 ID8-OVA-SiNS or ID8-OVA-SiCD73 cells. Three days later, pre-activated OT-I CD90.1 splenocytes were injected i.p.. PEC were collected and stained with annexin V at 24 h. Apoptotic cells were calculated as the percentage of annexin V$^+$ cells gated in the CD8$^+$ CD90.1$^+$ fraction.

Analysis of cells by FACS

All the samples except for tumor cells were initially incubated with 2.4G2 to block antibody binding to Fc receptors. Single cell suspensions were stained with 1 μg of relevant Abs and then washed twice with cold PBS. Intracellular IFN-γ staining was performed as previously described (35). Samples were analyzed on a LSR-II and data were analyzed with FlowJo software.

Tumor challenge and treatments

Cultured cancer cells were trypsinized and washed once with DMEM. Cancer cells (1×10^7) in suspension were injected i.p. into the indicated mice. One week later, tumor-bearing mice (n=5-8 per group) were left untreated or adoptively transferred with 1×10^7 OT-I T cells i.p.

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Meanwhile, tumor-bearing mice were treated with SCH58261 (5 mg/kg, i.p.) or caffeine given as drinking water (0.1% wt/vol) at d7 and adoptively transferred with 1×10^7 OT-I T cells i.p. at d8. Mice treated with SCH58261 or caffeine alone were used as controls. Mice were treated with SCH58261 three times weekly until mice gained >30% body weight (indication of ascites formation). For APCP treatment, one week after tumor challenge, mice were administer APCP 20 mg/kg i.v. once daily for one week followed by twice weekly. Survival of the mice was measured.

Statistical analysis

The statistical differences between the survival of groups of mice were calculated according to the log-rank test. The statistical significance of other measurements in different groups was determined by Student's *t* test. Probability values >0.05 were considered non-significant.

Results

CD73 expression on tumor cells negatively modulates T cell responses *in vitro*

To determine the roles of CD73 in cancer, we first examined CD73 expression on various cancer cells by flow cytometry. As shown in Supple. Fig. 1, CD73 was detected at different levels on 9 of 9 cancer cell lines, indicating that many types of cancers can express CD73. We found that epithelial ovarian cancer ID8 also expressed CD73 (Fig. 1a). Notably, CD73 expression (Fig. 1a) and activity (Supple. Fig. 3b) were further upregulated on ascites-derived ID8 cells and MC38 colon cancer cells cultured from a biopsy of this tumor (data not shown), suggesting that high levels of CD73 expression are likely induced in the local tumor microenvironment. This concept is supported by demonstrating elevated CD73 expression on cultured ID8 cell treated *in vitro* with malignant ascites from ID8-bearing mice (Supple. Fig. 2).

Extracellular adenosine downregulates immune responses by inhibiting T cells. Consistent with a previous study (22), we found that in serum-free medium adenosine, even at a low concentration of 1 μM, directly suppressed anti-CD3 mAb-induced T cell proliferation (Fig. 1b). Because ID8 cells express CD73, we hypothesized that extracellular adenosine generated by CD73 on ID8 cells would negatively regulate T cell responses. We found that conditioned medium from ID8 cancer cells slightly suppressed anti-CD3 Ab-induced CD4 and CD8 T cell proliferation (Fig. 1c). Moreover, proliferation of both T cell populations was remarkably inhibited when ID8 cells were treated with AMP. It has been demonstrated that CD73 hydrolyzes extracellular AMP into bioactive adenosine, which mediates immune suppression of T cells through the A2AAR receptor (22,23). To test the hypothesis that extracellular adenosine generated by CD73 on ID8 cells suppresses T cell response through A2AAR, ID8 cells were treated with AMP and the supernatants were added to T cell cultures with or without the A2AAR antagonist SCH58261 (Fig. 1c). Blocking A2AAR with SCH58261 significantly abrogated AMP induced suppression of T cell proliferation. This suppressive effect is not mediated directly by AMP, because direct addition of AMP to T cell cultures failed to influence T cell proliferation (data not shown). To confirm whether CD73 contributes to adenosine-mediated immune suppression, we used siRNA to knock down CD73 expression on ID8 cells (Fig. 1a). The efficiency of CD73 knockdown was >95% as shown by immunofluorescence and the measurement of CD73 enzymatic activity (Fig. 1a). As shown in Fig. 1d, knockdown of CD73 expression on ID8 cells promoted T cell proliferation even in the presence of AMP. AMP did not inhibit T cell proliferation in the presence of the CD73 selective inhibitor APCP, indicating AMP itself is not immunosuppressive, but must be converted to adenosine by CD73's ecto-5′-NT enzyme activity. In addition, SKOV3 human ovarian cancer cells highly expressed functional CD73 (Supple. Fig. 3), suggesting that our findings may apply to human ovarian cancers.

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To assess tumor CD73 mediated inhibitory effects on antigen-specific T cell responses, we generated CD73-silenced or control non-silenced ID8 cells expressing OVA (ID8-OVA-SiNS or ID8-OVA-SiCD73). ID8-OVA-SiNS cells did not stimulate T cell proliferation (Fig. 2a) or IFN-γ production (Fig. 2b) unless treated with SCH58261, implying that there is an endogenous source of adenosine in these co-cultures that inhibits T cell responses. By contrast, massive T cell division and IFN-γ secretion even in the presence of AMP were observed when T cells were incubated with ID8-OVA-SiCD73 cells (Fig. 2a and b). To further support tumor CD73-generated adenosine as the suppressive mechanism, we treated ID8-OVA cells with APCP, NECA (a general adenosine receptor agonist) or APCP plus NECA. As expected, APCP treatment rescued T cell proliferation, and this effect was abrogated in the presence of NECA (Fig. 2c).

We next examined whether CD73-mediated adenosinergic signaling affects cancer cell killing by specific CTL. As shown in Supple. Fig. 4a, cancer cells untreated with OT-I cells grew to complete confluence. ID8-OVA and ID8-OVA-SiNS resisted killing by OT-I CTL and also grew to complete confluence. However, blocking A2AAR with SCH58261 resulted in partial lysis of cancer cells, indicating that adenosine helps cancer cells acquire resistance to growth inhibition by antigen-specific effector T cells. Strikingly, ID8-OVA-SiCD73 cell growth was largely inhibited by OT-I CTL. Furthermore, this growth inhibition of cancer cells remained effective even with addition of AMP. The number of remaining viable cancer cells treated with or without OT-1 cells was quantified by a MTT assay (Supple. Fig. 4b). The diminished number of ID8-OVA-SiCD73 cells was not due to lower rates of cancer cell proliferation because there was no *in vitro* growth and adhesion advantage when CD73 on ID8 cells was silenced (Supple. Fig. 5). In a more quantitative ^{51}Cr release assay, the ability of OT-1 CTL to kill ID8-OVA-SiNS and ID8-OVA-SiCD73 cells was compared. CD73 knockdown rendered tumor cells more susceptible to T cell killing. The suppression of killing that occurred when ID8-OVA-SiNS cells were targets required CD73 enzymatic activity because APCP treatment restored killing to the levels seen with ID8-OVA-SiCD73 cells (Fig. 2d). Therefore, we conclude that tumor CD73 is required for generation of adenosine that compromises cancer cell killing by CTL.

CD73 expression on tumor cells promotes T cell apoptosis

It has been reported that extracellular adenosine can trigger apoptosis in thymocytes and peripheral T cells (37,38). We thus tested tumor CD73 mediated adenosinergic effects on the viability of antigen-specific T cells. As shown in Fig. 3a, knockdown of tumor CD73 promoted T cell survival *in vitro*. Many more T cells underwent apoptosis in the presence of AMP when incubated with ID8-OVA-SiNS, and adding of A2AAR inhibitor SCH58261 lessened T cell death, suggesting that tumor CD73-derived adenosine supports T cell apoptosis. This notion is further strengthened by the fact that the use of NECA directly induced more apoptosis of activated T cells (Fig. 3b).

We next determined whether T cell apoptosis *in vitro* correlates with activated T cell depletion *in vivo*. Twenty-four hours after pre-activated OT-I splenocytes were injected into tumor-bearing mice, there were significantly more transferred OT-I cells ($CD8^+CD90.1^+$) in mice harboring ID8-OVA-SiCD73 than in mice harboring ID8-OVA-SiNS (Fig. 3c). In addition, around 2-fold more transferred OT-I cells underwent significant apoptosis in mice harboring ID8-OVA-SiNS cancer cells than in mice harboring ID8-OVA-SiCD73 cancer cells (Fig. 3d). Thus, CD73 expressed by ID8 tumors inhibits the survival of tumor-specific T cells.

Tumor CD73 impairs antitumor T cell responses *in vivo*

We investigated whether CD73 negatively regulates antitumor T cell responses *in vivo*. As shown in Fig. 4a (upper panels) and Fig. 4b, as early as d2, more adoptively transferred OT-1

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T cells divided in ID8-OVA-SiCD73 tumor-bearing mice as compared with those in ID8-OVA-SiNS tumor-bearing mice. Similar results were observed at d4 and d6, indicating T cells proliferated faster when tumor CD73 was silenced on tumor cells. This proliferation is tumor antigen specific because OT-I T cells failed to divide in control ID8-GFP tumor-bearing mice lacking OVA antigen expression. Interestingly, much less significant T cell proliferation was found in PEC than in draining mesenteric lymph nodes (MLN) or spleen from ID8-OVA-SiNS tumor-bearing mice, especially at d4 and d6 (Fig. 4b), suggesting local tumor microenvironmental factors may preferentially limit T cell proliferation in the peritoneal cavity. By contrast, T cells proliferated nearly equivalently in PEC, MLN and spleen from ID8-OVA-SiCD73 tumor-bearing mice (Fig. 4b). Moreover, divided T cells highly expressed the activation markers CD69 and CD44, and produced IFN-γ at d2 (Fig. 4a lower panels), d4 and d6 (data not shown), indicating adoptively transferred T cells were effectively activated. We next examined the effector cytolytic function of adoptively transferred antigen-specific T cells. Target cell lysis *in vivo* was almost unimpaired in MLN and spleen of ID8-OVA-SiNS tumor-bearing mice compared to that in ID8-OVA-SiCD73 tumor-bearing mice. In contrast, target killing was remarkably suppressed in PEC from ID8-OVA-SiNS compared to ID8-OVA-SiCD73 tumor-bearing mice, suggesting that knockdown of CD73 on tumor cells helps retain T cell cytolytic activity (Fig. 4c and d). Taken together, we conclude that in the local tumor microenvironment of the peritoneum of mice bearing CD73-expressing tumors, antigen-specific T cell responses including both activation and effector functions were suppressed, and could be improved by knockdown of tumor CD73.

Knockdown of CD73 expression increases overall survival and decreases tumor burden

We asked whether knockdown of CD73 expression on tumor cells prolongs survival of mice challenged with ID8 tumor cells. The median survival was 77 days (range 65-82) for ID8SiNS-bearing mice and 95 days (range 80-105) for ID8SiCD73-bearing mice (p=0.013) (Fig. 5a). Further, ID8SiNS-bearing mice gained significant weight from ascites earlier than ID8SiCD73-bearing mice (data not shown). A similar survival advantage was observed when ID8-bearing mice were treated by the CD73 inhibitor APCP (Fig. 5b). This pharmacological benefit is not likely due to targeting tumor CD73 alone because the activity of host CD73 (e.g. on Tregs) could be equally inhibited. There was also a significant difference in survival between ID8-OVA-SiNS-bearing mice (median 73 days; range 70-86) and ID8-OVA-SiCD73-bearing mice (median 96 days; range 83-160 days) (p=0.006) (Fig. 5c). To test the hypothesis that knockdown of tumor CD73 increases overall survival because tumor CD73 negatively regulates T cell responses, ID8-SiNS or ID8-SiCD73 cells were inoculated i.p. into T cell deficient Rag1$^{-/-}$ mice. As expected, there was no significant difference in survival of mice bearing the two types of tumors (p=0.119; Fig. 5d). The survival advantage observed (Fig. 5a) is most likely independent of Tregs because tumor CD73 knockdown did not alter the infiltration and activity of local Tregs (Supple. Fig. 6).

Blockade of adenosinergic effects improves adoptive T cell therapy

ID8-GFP-bearing and ID8-OVA-bearing mice had nearly equivalent survival (median 65 vs. 60 days, p=0.085), and ID8-OVA-bearing mice treated with OVA-specific OT-I T cells only survived to 60 days (range 47-66 days) (Fig. 6a), indicating that T cell therapy is not effective for this tumor. By striking contrast, all ID8-OVA-SiCD73-bearing mice treated with OT-I T cells were tumor-free through 160 days when they were sacrificed (Fig. 6b). Equally remarkable, these T cell-treated mice had no visible peritoneal tumor nodules and no measurable ascites at the end of the study (data not shown). Confirming the role of tumor CD73 in inhibiting tumor-specific anti-tumor immunity, 90% of ID8-OVA-SiNS-bearing mice treated with OT-I T cells died within 100 days (range 95-107 days) (Fig. 6b). We therefore conclude that knockdown of CD73 on tumor cells restores efficacy of adoptive T cell therapy. In confirmation, we tested a second OVA-expressing tumor model (EG7, Supple. Fig. 7a) and

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again showed improved proliferation and activation of adoptively transferred OT-1 T cells in EG7-SiCD73 tumor-bearing mice (Supple. Fig. 7b). T cell therapy also induced significant regression of EG7-SiCD73 but not control EG7-SiNS tumors (Supple. Fig. 7c).

To test if pharmacological inhibition of A2AAR rendered antitumor T cells resistant to inhibition by tumor microenvironmental adenosine, we studied the effects of SCH58261, a specific A2AAR antagonist (Fig. 6c) or of caffeine (a general adenosine receptor antagonist which at physiologically relevant concentrations preferentially antagonizes the A2AAR (21)) (Fig. 6d). There was no significant increased survival of ID8-OVA-bearing mice treated with SCH58261 alone compared with that of untreated tumor-bearing mice (median survival, 70 vs. 76 days, p=0.221). However, the combination of SCH58261 and adoptive T cell therapy significantly improved survival in tumor-bearing mice. As expected, T cell therapy alone failed to provide any therapeutic benefit (median survival, 70 days). Likewise, the survival of mice treated with T cells and caffeine was superior to mice treated caffeine alone or T cells alone. Collectively, these data support our thesis that blockade of the tumor CD73-adenosine-A2AAR pathway rescues tumor specific immunity and enhances the efficacy of adoptive T-cell therapy.

Discussion

Here we present a new mechanism of tumor-induced immunosuppression: CD73 expressed on tumors negatively modulates tumor antigen-specific T cell immunity. Extracellular adenosine derived from AMP/ATP is generated primarily through the combined action of CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1 which converts ATP and ADP to adenosine) and ecto-5′-nucleotidase (CD73) found on the surface of a variety of cell types. Although upregulation of CD73 expression was found in many human tumors, the function of tumor CD73 remains unclear to date. Recently, the contribution of CD73-mediated generation of extracellular adenosine to host defense systems has been widely explored because adenosine is a well-known anti-inflammatory mediator (39). Interestingly, more recent studies led to the identification of the A2AAR-mediated so-called “adenosinergic pathway” as critical in physiological regulation of immune responses *in vivo* (40,41). Thus, CD73 overexpression on cancer cells prompted us to examine its role in immune modulation in cancer.

Cancer cells having CD39 (data not shown) and CD73 ecto-nucleotidases on the cell surface possess the capacity both to generate immunosuppressive adenosine and to clear the extracellular proinflammatory factor ATP. Among its distinct anti-inflammatory roles, it is believed that adenosine modulates T cell responses primarily by binding to A2AAR on T cells. A2AAR engagement suppresses T cell proliferation, inflammatory cytokine secretion, and reduces surface expression of cytokine receptors by elevating the intracellular levels of cyclic (c)AMP through adenylyl cyclase stimulation (42). Indeed, increased intracellular cAMP triggers protein kinase A-mediated phosphorylation and activation of carboxy-terminal Src kinase (Csk), which in turn abrogates TCR signaling and IFN-γ production by inhibiting Lck (43). Signaling through the A2AAR and/or A2BAR have been reported to inhibit various aspects of $CD8^+$ T cell responses involved in antitumor T cell immunity (21) including activation and proliferation (16), lethal hit delivery (44), Fas ligand up-regulation (45), and secretion of effector cytokines such as IFN-γ (46). Therefore, adenosinergic signaling in cancer is most likely immunosuppressive (47,48).

We highlight the major role of tumor CD73-generated adenosine that prevents tumor destruction from antitumor T cells and raise the feasibility of new strategies to overcome this tumor-induced immunosuppressant by genetic ablation or pharmacological inhibition of CD73 activity. Our findings suggest future studies to establish whether CD73 can serve as a primary trigger of tumor-protecting immunosuppressive molecules. Our data are in further agreement with the recent view stated by Ohta *et al.* (21) that targeting the adenosine-A2AAR pathway

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is a cancer immunotherapy strategy to prevent inhibition of antitumor T cells in the tumor microenvironment. Hypoxia is associated with accumulation of extracellular adenosine that may inhibit antitumor $CD8^+$ T cells by increasing their immunosuppressive intracellular cAMP levels (49). More importantly, we believe that extracellular adenosine accumulated in tumor microenvironment is likely in large part produced by CD39/CD73 expressed on tumor cells. Targeting A2AAR either by siRNA or pharmacological antagonists has limitations because activation of the other adenosine receptors in addition to A2AR may account for $CD8^+$ T cell failure to destroy tumor (48,50). Indeed, only about 60% of mice with genetically ablated A2AR reject tumors (21). Furthermore, global inhibition of A2AAR may have unwanted side effects. Moreover, CD73 has been directly involved in cancer cell growth and invasion (31). Thus, targeting the enzymatic activity of tumor CD73 appears to have more therapeutic benefits for the tumor-bearing host. We expect that blocking A2AAR signals on T cells and targeting CD73 on tumor cells could improve therapeutic efficacy beyond that achievable with either alone. Whether additional benefits would be obtained by inhibiting the expression of CD39 remains to be explored.

Of note, inhibiting CD73 alone fails to cure cancer despite increasing host survival and inhibiting tumor growth. This is likely due to the insufficient number of effector antitumor T cells in the tumor microenvironment that are not only unable to control the cancer but which are also readily influenced by other immunosuppressive mechanisms. Interestingly, inhibiting CD73 remarkably improves the therapeutic antitumor effectors of transferred tumor-specific T cells. Therefore, the optimal strategy to counteract immunosuppressive adenosinergic effects of CD73 in the tumor microenvironment is complementary to other approaches directed at improving the development and function of antitumor T cells such as adoptive T cell therapy and dendritic cell vaccines.

In summary, our data show that extracellular adenosine generated by CD73 on tumor cells negatively regulates both the activation phase and effector phase of the antitumor T cell response and promotes T cell apoptosis. We propose that CD73 on tumor cells could be a therapeutic target for the prevention of tumor-induced immunosuppression although future studies investigating the role of CD73 in endogenous tumors may be needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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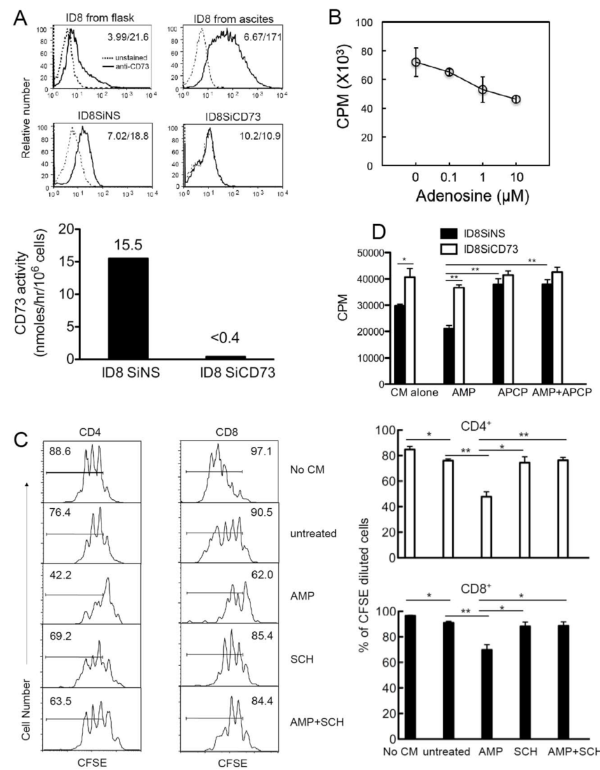

Figure 1. Adenosine generated by tumor CD73 suppresses T cell proliferation

(a) CD73 expression on various ID8 ovarian cancer cells was analyzed by flow cytometry (dashed line, unstained/ solid line, stained). The efficiency of CD73 knockdown was confirmed by the measurement of CD73 enzymatic activity. **(b)** Adenosine directly suppressed T cell proliferation. Data are mean ± SD of triplicate wells. **(c)** Adenosine generated by tumor CD73 suppressed T cell proliferation. ID8 cells were incubated with conditioned medium as indicated. CFSE-labeled splenocytes were then incubated with the above conditioned medium or regular complete medium (No CM) in the presence of 1 μg/ml anti-CD3 mAb for 72 h. The proliferation (CFSE dilution) was evaluated by flow cytometry by gating on $CD8^+$ or $CD4^+$ cells. **(d)** Knockdown of CD73 expression promoted T cell proliferation. ID8SiNS and ID8SiCD73 cells were incubated with conditioned medium as indicated. Splenocytes were then incubated with the above conditioned medium in the presence of 1 μg/ml anti-CD3 mAb for 72 h. Data are mean ± SD of triplicate wells. Data are representative of three independent experiments (**c,d**). *, $p<0.05$; **, $p<0.01$.

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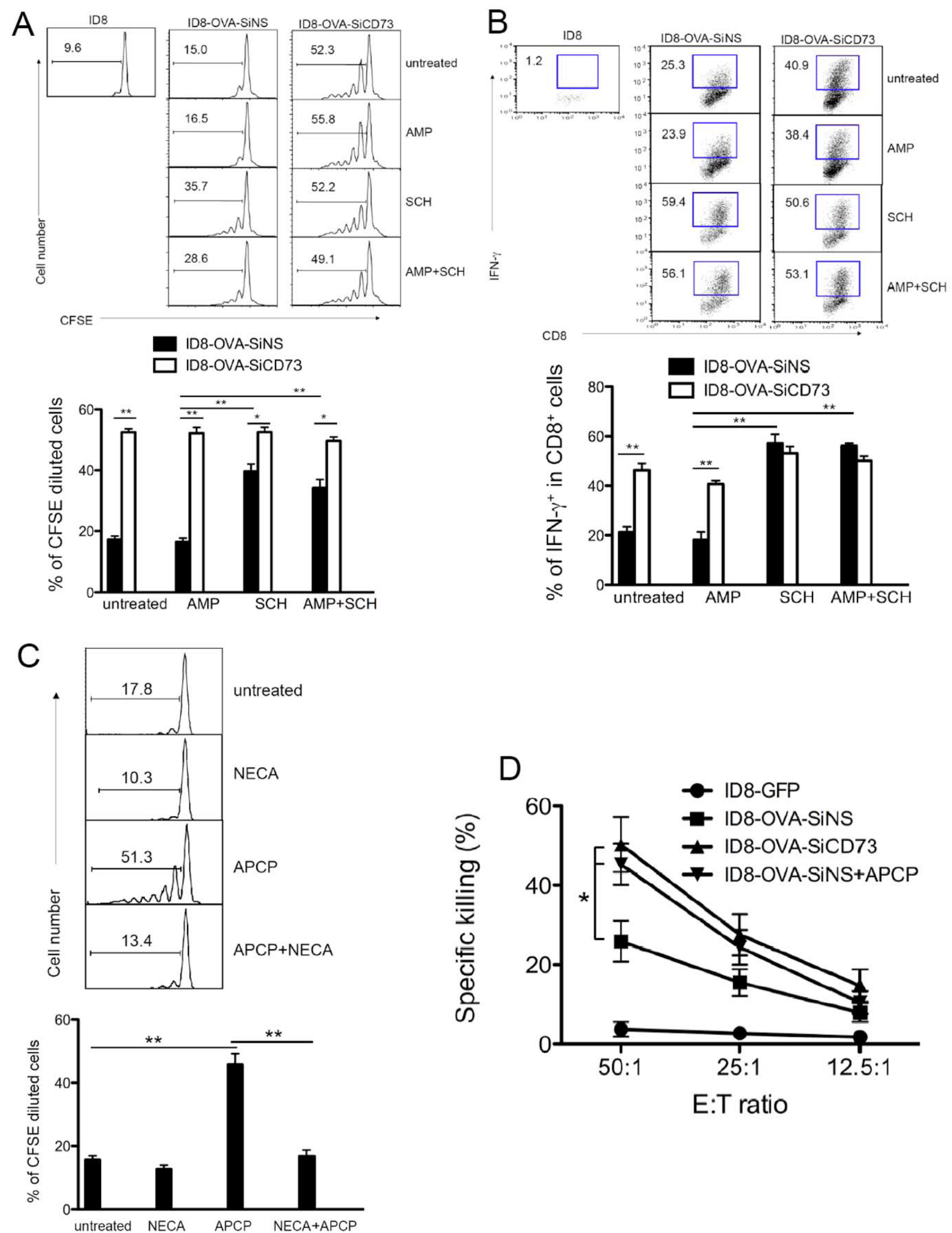

Figure 2. Tumor CD73 impairs antigen specific T cell responses

ID8-OVA-SiNS or ID8-OVA-SiCD73 cells were incubated with conditioned medium as indicated. After 3 days in culture, the floating cells were harvested and examined for the CFSE dilution **(a)** and intracellular IFN-γ staining (**b**) by flow cytometry by gating on $CD8^+$ cells. Cells incubated with conditioned medium alone were used as control (untreated). **(c)** ID8-OVA cells were incubated with conditioned medium as indicated. OT-I splenocytes were added into the above tumor cell cultures. After 3 days in culture, the floating cells were harvested and examined for the CFSE dilution by gating on $CD8^+$ cells. (**d**) Knockdown of CD73 expression promotes tumor cell killing by OT-I CTL *in vitro*. The killing of ID8-GFP, ID8-OVA-SiNS, ID8-OVA-SiCD73 and APCP-treated ID8-OVA-SiNS by OT-I CTL was measured in a

standard ^{51}Cr release assay. Data are representative of three independent experiments (**a,b,c**) or two independent experiments (**d**). *, $p<0.05$; **, $p<0.01$.

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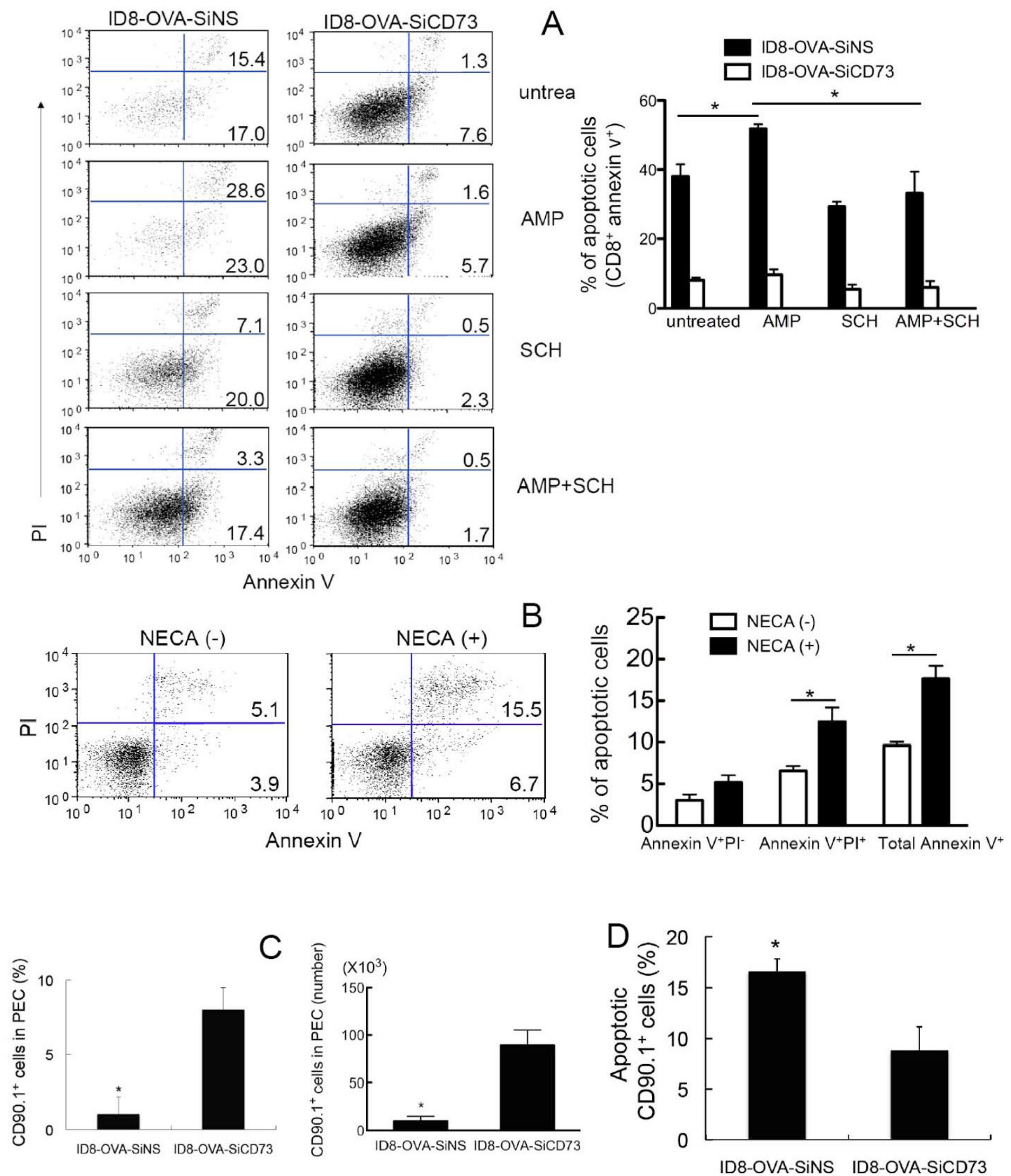

Figure 3. Tumor CD73 promotes apoptosis of antigen-specific T cells

(a) ID8-OVA-SiNS or ID8-OVA-SiCD73 were incubated with conditioned medium as indicated. Pre-activated OT-I splenocytes were added into the above tumor cell cultures, or only incubated with or without NECA **(b).** After 3 days in culture, the floating cells were harvested and examined for apoptosis by double staining with annexin V and propidium iodide (PI) and subjected to flow cytometry by gating on $CD8^+$ cells. **(c)** Female C57BL/6 mice were inoculated i.p. with $5{\times}10^6$ ID8-OVA or ID8-OVA-SiCD73 cells. Three days later, pre-activated OT-I CD90.1 splenocytes were injected i.p. into each tumor-bearing mouse. Peritoneal exudate cells (PEC) were collected and counted at 24 h. The percentage and number of transferred cells ($CD90.1^+$ $CD8^+$) was shown. **(d)** Apoptotic cells were calculated as the

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percentage of annexin V^+ cells gated in the $CD90.1^+$ $CD8^+$ fraction. Data represented as mean ± SD of cells from 3 mice of each group. Data are representative of three independent experiments (**a,b**) or two independent experiments (**c,d**). *, $p<0.05$.

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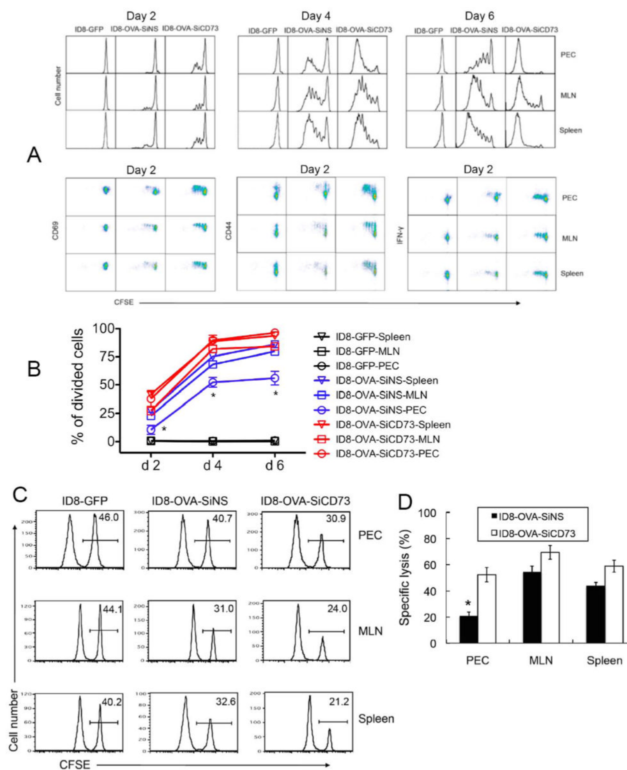

Figure 4. Knockdown of CD73 expression improves anti-tumor T cell responses

(a) The proliferation of transferred CFSE-labeled OT-I CD90.1 cells in spleens, mesenteric lymph nodes (MLN) and PEC from the indicated tumor-bearing mice was measured by gating on $CD8^+$ $CD90.1^+$ cells at d2, d4 and d6. The activation marker CD44, CD69 or intracellular IFN-γ staining on these transferred cells ($CD8^+$ $CD90.1^+$) was also measured at d2, d4 and d6 (data not shown). **(b)** Percentage of divided cells among the $CD8^+$ $CD90.1^+$ cells, as assessed by CFSE dilution, was measured and plotted. Data represented as mean ± SD of cells from 3 mice of each group. *, $p<0.05$, compared to ID8-OVA-SiCD73-PEC. **(c)** Tumor-bearing mice were injected i.p. with 1×10^7 OT-1 splenocytes. 5 days later, OVA-I-pulsed (CFSE-Hi) or no peptide-pulsed (CFSE-Lo) target cells from C57BL/6 mice were i.p. transferred into these tumor-bearing mice. Spleens, MLN and PEC were harvested 24 h later and analyzed for CFSE fluorescence. The number in each histogram indicated the percentage of CFSE-Hi population in all $CFSE^+$ cells. Panel **(d)** showed the compiled data of percentage of killing. *, $p<0.05$, n=6. Data are representative of two **(a,b,c,d)** independent experiments.

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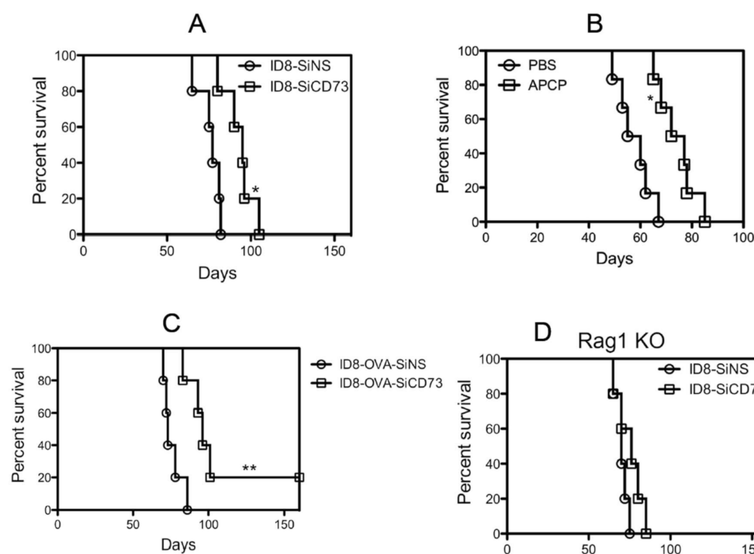

Figure 5. Knockdown of CD73 expression increases overall survival of tumor-bearing mice
Female C57BL/6 wild type mice (**a**) or Rag $1^{-/-}$ mice (**d**) were inoculated i.p. with 1×10^7 ID8SiNS or ID8SiCD73 cells. (**b**) Female C57BL/6 mice were treated with APCP one week after ID8 tumor challenge. (**c**) Female C57BL/6 mice were inoculated i.p. with 1×10^7 ID8-OVA-SiNS or ID8-OVA-SiCD73 cells, and survival of the mice (5-8 each group) was measured. *, $p<0.05$; **, $p<0.01$. Data are representative of two (**b,d**) independent experiments or three independent experiments (**a,c**).

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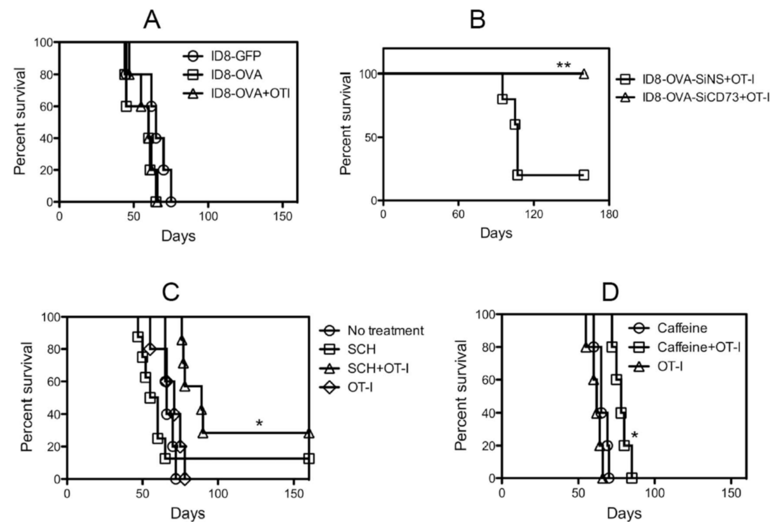

Figure 6. Blockade of adenosinergic pathway augments adoptive T cell immunotherapy

(**a**) Female C57BL/6 mice were inoculated i.p. with 1×10^7 ID8-GFP or ID8-OVA cells. One week later, ID8-OVA tumor-bearing mice were left untreated or i.p. transferred with 1×10^7 OT-I T cells. (**b**) Female C57BL/6 mice were inoculated i.p. with 1×10^7 ID8-OVA-SiNS or ID8-OVA-SiCD73 cells. One week later, 1×10^7 OT-I T cells were i.p. infused into those tumor-bearing mice. **, $p<0.01$. **(c, d)** Female C57BL/6 mice were inoculated i.p. with 1×10^7 ID8-OVA cells. One week later, tumor-bearing mice were left untreated or i.p. transferred with 1×10^7 OT-I T cells. Meanwhile, tumor-bearing mice were treated with SCH58261 (**c**) or caffeine (**d**) given as drinking water (0.1% wt/vol) at d7 and i.p infused with 1×10^7 OT-I T cells at d8. Mice treated with SCH58261 (**c**) or caffeine (**d**) alone were used as control. Mice were treated with SCH58261 three times weekly. Survival of the mice (5-8 each group) was measured. *, $p<0.05$; **, $p<0.01$. Data are representative of 3 **(a,b,c)** independent experiments.