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Research Article

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# CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice

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**CD73 is overexpressed in many types of human and mouse cancers and is implicated in the control of tumor progression. However, the specific contribution from tumor or host CD73 expression to tumor growth remains unknown to date. Here, we show that host CD73 promotes tumor growth in a T cell–dependent manner and that the optimal antitumor effect of CD73 blockade requires inhibiting both tumor and host CD73. Notably, enzymatic activity of CD73 on nonhematopoietic cells limited tumor-infiltrating T cells by controlling anti-tumor T cell homing to tumors in multiple mouse tumor models. In contrast, CD73 on hematopoietic cells (including CD4<sup>+</sup>CD25<sup>+</sup> Tregs) inhibited systemic antitumor T cell expansion and effector functions. Thus, CD73 on hematopoietic and nonhematopoietic cells has distinct adenosinergic effects in regulating systemic and local antitumor T cell responses. Importantly, pharmacological blockade of CD73 using its selective inhibitor or an anti-CD73 mAb inhibited tumor growth and completely restored efficacy of adoptive T cell therapy in mice. These findings suggest that both tumor and host CD73 cooperatively protect tumors from incoming antitumor T cells and show the potential of targeting CD73 as a cancer immunotherapy strategy.**

## Introduction

The resistance of many solid tumors to host immune responses has been in large part attributed to a spectrum of tumor-associated immune-suppressive mechanisms that have been well documented in both tumor-bearing mice and cancer patients (1–3). It is thought that tilting the balance from an immune-suppressive to an immune-active environment is necessary for effective cancer immunotherapy (1–3). Adenosine is a purine nucleoside found within solid tumors at elevated concentrations (4, 5) that may promote tumor growth by stimulating tumor angiogenesis (6, 7) and inhibiting antitumor immune responses (6–9). However, the mechanisms whereby adenosine accumulates in solid tumors and the potential effects resulting from this accumulation are not well understood.

CD73, also known as ecto-5'-nucleotidase (ecto-5'-NT, EC 3.1.3.5; refs. 10, 11), is a glycosylphosphatidylinositol-linked 70-kDa cell surface ectoenzyme found in many types of human and mouse cancers (6, 7, 9). We recently demonstrated that tumor-derived CD73 limits antitumor T cell immunity to promote tumor growth through its enzymatic activity in tandem with CD39 (ecto-ATPase) that produces extracellular adenosine (9, 12). Similarly, Stagg et al. showed that targeting CD73 using the anti-CD73 mAb TY/23 suppressed tumor growth and metastasis (13). Given that CD73 is expressed on many cell types, including subsets of lymphocytes (14), ECs (15), and epithelial cells (16), we hypothesize that both tumor and host CD73 protects tumors from incoming antitumor T cells. Indeed, it was previously found that CD73 is overexpressed on CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs (17), and the CD39-CD73 axis suppresses T cell function (18). However, the specific contribution from tumor or host CD73 expression to tumor growth remains unknown to date.

In the present study, we showed that host CD73 deficiency decreased tumor burden and increased mouse survival in a T cell–dependent manner and that inhibiting both tumor and host CD73 was required to achieve an optimal antitumor effect. We further dissected the distinct contribution of CD73 on both BM-derived and non-BM-derived host cells to systemic and local antitumor T cell immunity. Importantly, pharmacological blockade of CD73 using the selective inhibitor  $\alpha,\beta$ -methylene adenosine 5'-diphosphate (APCP) or an anti-CD73 mAb inhibited tumor growth and promoted efficacy of adoptive T cell therapy, which suggests that targeted CD73 therapy is an important and rational approach to cancer treatment.

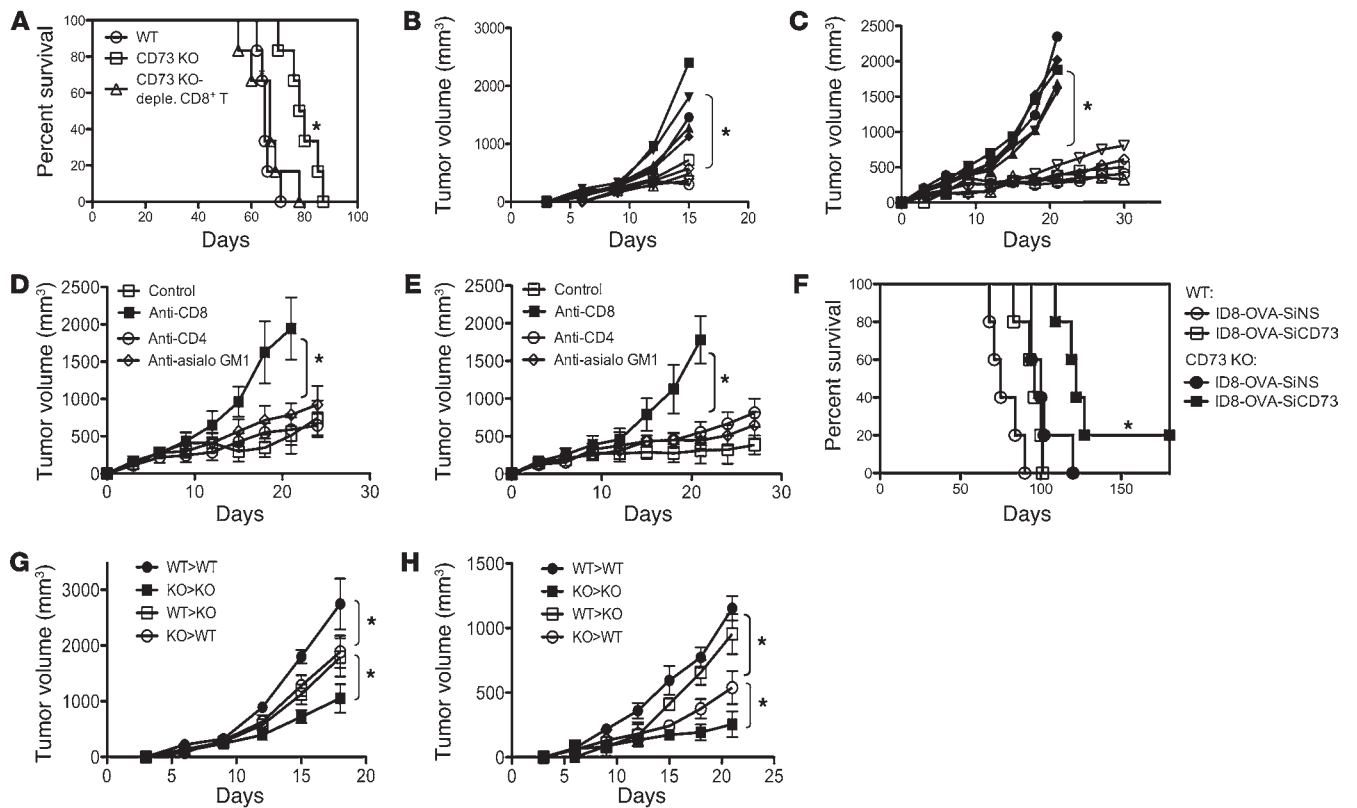
## Results

*Host CD73 promotes tumor growth.* We have previously shown that knockdown of CD73 expression on tumor cells increases mouse survival by improving antitumor T cell immunity (12). In this study, we investigated the role of host CD73 on antitumor immunity. Interestingly, there was a significant survival advantage in CD73 KO mice bearing peritoneal ID8 ovarian tumor compared with WT mice (WT, median 65 days; CD73 KO, median 80 days;  $P < 0.05$ ; Figure 1A). Furthermore, depletion of CD8 $\alpha^+$  cells from CD73 KO mice prior to tumor injection decreased their survival compared with the WT group (Figure 1A), which indicates that CD8 $^+$  T cells may play an important role in the inhibition of tumor progression in host CD73 deficiency.

A tumor-inhibiting advantage of host CD73 deficiency was also observed in s.c. B16F10 melanoma- or EL4 lymphoma-bearing mice (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI45559DS1). Similar to ID8 tumors, depletion of CD8 $\alpha^+$  cells abrogated the tumor-inhibiting advantage of host CD73 deficiency in B16F10-bearing mice (Supplemental Figure 1C). In addition, CD73 deficiency was more effective in inhibiting the growth of immunogenic EG7 (expressing

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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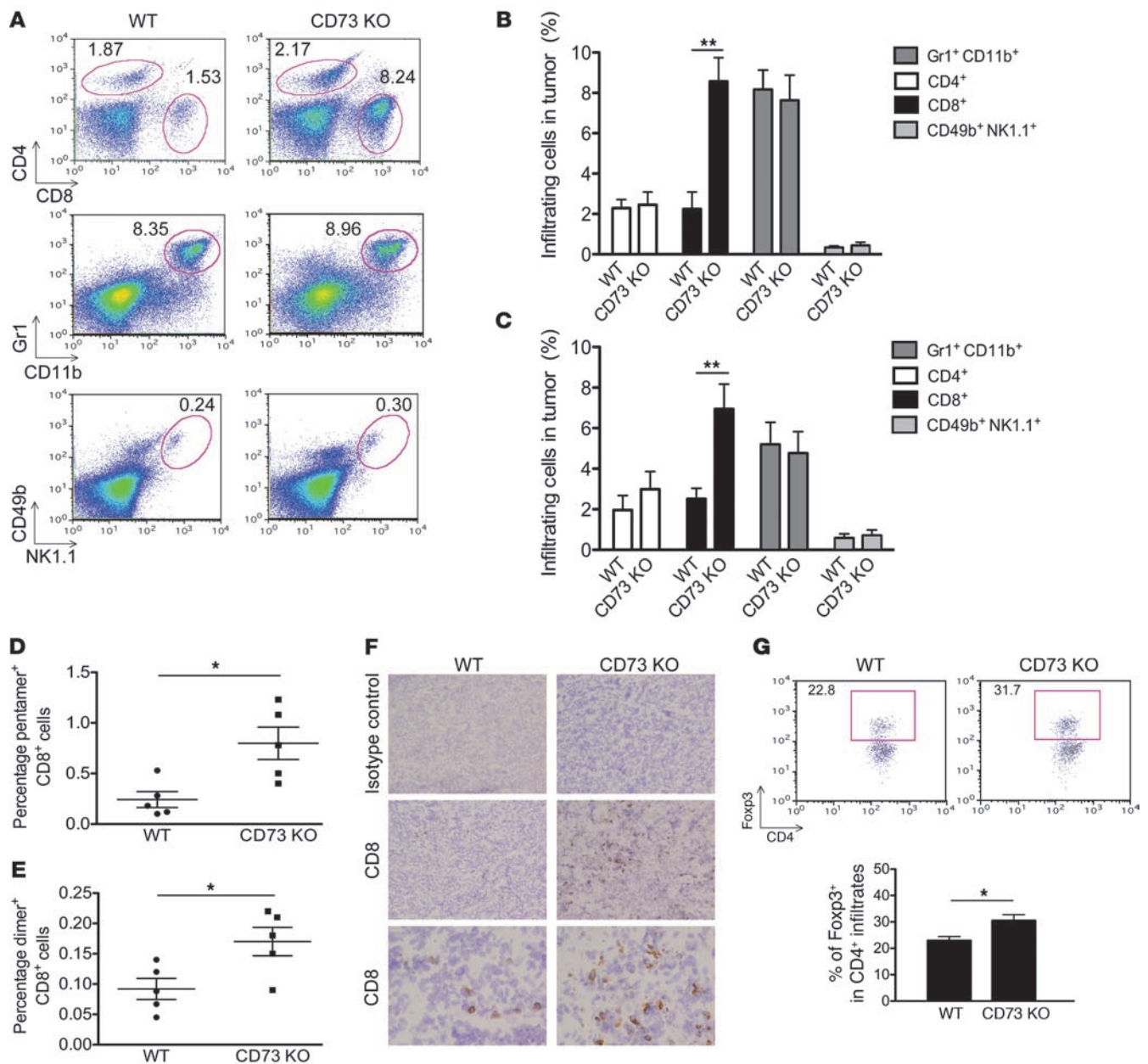
**Figure 1**  
 Tumor growth is inhibited in CD73 KO mice. (A) Increased survival of CD73 KO mice challenged with ID8 ovarian tumors is T cell dependent. Female C57BL/6 WT, CD73 KO, or CD73 KO mice depleted of CD8<sup>+</sup> T cells by anti-CD8 mAb 53.6.7 were inoculated i.p. with 1 × 10<sup>7</sup> ID8 cells (n = 6). (B and C) Suppression of tumor growth in CD73 KO mice. Mice (n = 3 or 5 per group) were inoculated with 10<sup>6</sup> EG7 (B) or B16-SIY (C) cells. White symbols, CD73 KO; black symbols, WT; each symbol represents 1 tumor-bearing mouse. (D and E) In CD73 KO mice (n = 5 per group), depletion of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or NK cells was achieved by twice-weekly i.p. injection of control Ig or anti-CD4, anti-CD8, or anti- $\alpha$ -asialo GM1 depleting Abs, respectively, beginning 1 day prior to EG7 (D) or B16-SIY (E) challenge. (F) Survival of WT or CD73 KO mice (n = 5–8 per group) after i.p. inoculation with 10<sup>7</sup> ID8-OVA-SiCD73 (CD73-silenced) or ID8-OVA-SiNS (CD73-nonsilenced control) tumor cells. (G and H) Tumor growth was enhanced by both BM-derived and non-BM-derived host cells expressing CD73. Chimeric mice (n = 5 per group), generated by BM reconstitution, were injected s.c. with 10<sup>6</sup> EG7 (G) or B16-SIY (H) cells. Data (mean  $\pm$  SD) are representative of 3 independent experiments (A and F), at least 10 independent experiments (B and C), or 2 (G and H) independent experiments. \*P < 0.05 vs. all other groups or as denoted by brackets.

OVA antigen) or B16-SIY (expressing SIY antigen) cells than parental tumor EL4 or B16F10 cells (compare Figure 1, B and C, with Supplemental Figure 1, A and B). Therefore, the efficacy of host CD73 deficiency in boosting antitumor immunity appears to depend in part on tumor immunogenicity.

To assess the roles of CD4<sup>+</sup> and NK cells in the tumor-inhibiting effects observed in CD73 KO mice, mice were inoculated with EG7 or B16-SIY cells and subsequently received depleting anti-CD4, anti-CD8 $\alpha$ , or anti- $\alpha$ -asialo GM1 against CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or NK cells, respectively. Notably, the tumor-inhibiting advantage of host CD73 deficiency was primarily dependent on CD8<sup>+</sup> cells, but independent of CD4<sup>+</sup> cells or NK cells (Figure 1, D and E). Moreover, the percentage of tumor-infiltrating B cells (CD3<sup>+</sup>B220<sup>+</sup>) was not significantly different in tumor-bearing WT versus CD73 KO mice (Supplemental Figure 1D), which suggests that they do not markedly contribute to the observed effects in our models.

We next studied how targeting CD73 on cancer cells, host cells, or both contributes to antitumor effects. To address this question, we used the following 3 experimental settings: (a) targeting CD73

on cancer cells alone, (b) targeting CD73 on host cells alone, and (c) targeting CD73 on both cancer cells and host cells. The primary tumor model was the OVA-expressing ovarian cancer cell line ID8-OVA, because we have successfully established cell lines stably transfected with CD73 siRNA (ID8-OVA-siCD73; CD73 silenced) or a control siRNA (ID8-OVA-siNS; CD73-nonsilenced). CD73 KO mice challenged with ID8-OVA-siCD73 survived significantly longer (median 122 days; range 109–180 days) than did CD73 KO mice with ID8-OVA-siNS (median 96 days; range 83–102 days) or WT mice with ID8-OVA-siCD73 (median 100 days; range 94–120 days; Figure 1F). Moreover, cytotoxic T lymphocytes from CD73 KO mice killed CD73-expressing (ID8-siNS) or CD73-deficient (ID8-siCD73) ID8 cells with equivalent efficiency in vitro (data not shown), which suggests that immunogenicity of CD73 is unlikely to play a substantial role in our experimental model. These data demonstrated that the optimal antitumor effect required targeting both tumor and host CD73. To determine which CD73<sup>+</sup> host cell populations contribute to this effect, BM chimeras (KO>WT, WT>KO, KO>KO, and WT>WT; ref. 19) were used to ablate the

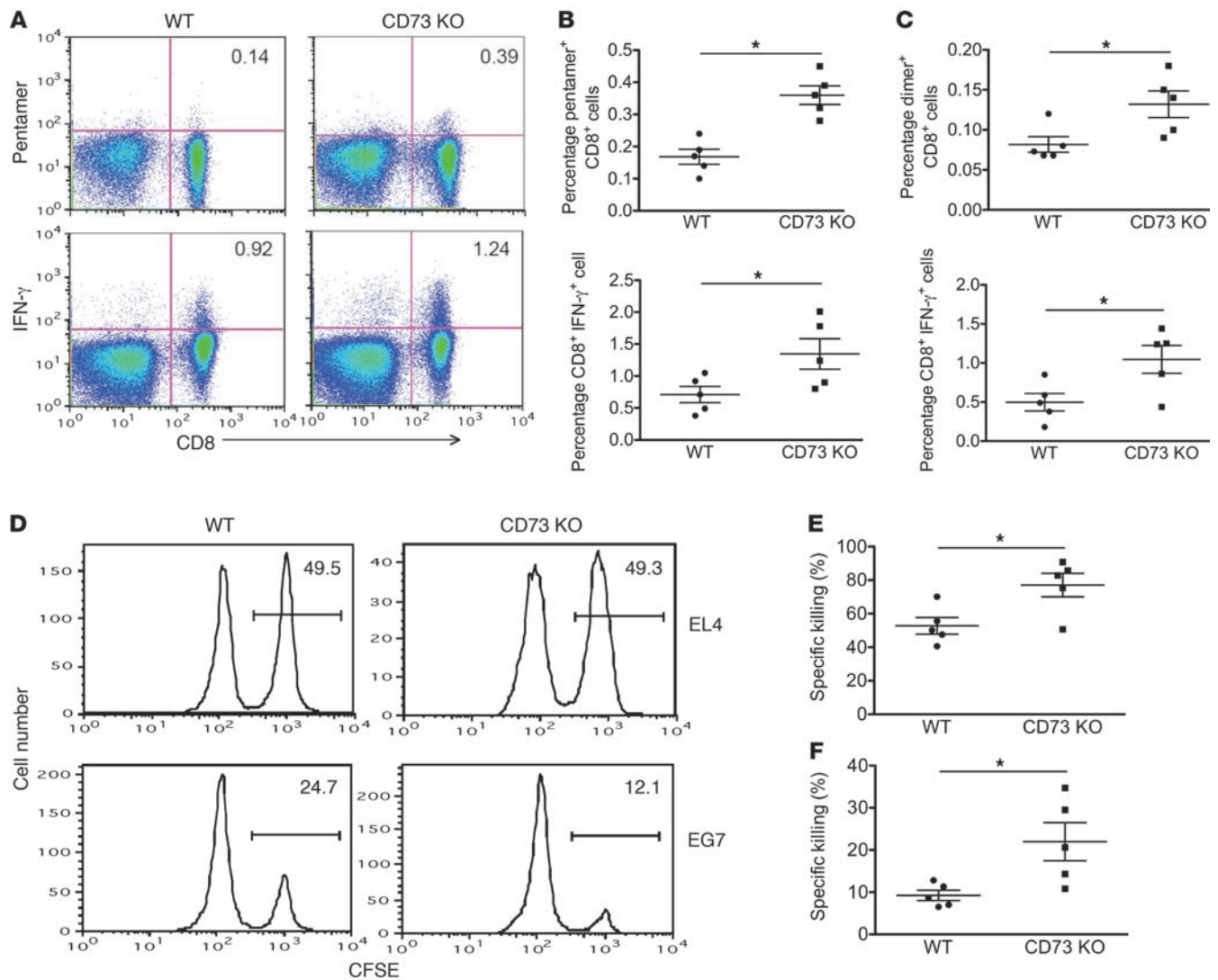


**Figure 2**

Tumor-infiltrating CD8<sup>+</sup> T cells accumulate preferentially in CD73 KO hosts. (A) Representative flow cytometric analysis of tumor-infiltrating immune cells from EG7-bearing WT or CD73 KO mice collected 14 days after tumor inoculation. (B and C) Percent CD4<sup>+</sup>TCRVβ<sup>+</sup>, CD8<sup>+</sup>TCRVβ<sup>+</sup>, Gr1<sup>+</sup>CD11b<sup>+</sup>, and CD49b<sup>+</sup>NK1.1<sup>+</sup> cells in tumor infiltrates of WT or CD73 KO mice collected 14 days after inoculation with EG7 (B) or B16-SIY (C) tumor cells (n = 5). (D) Frequency of pentamer<sup>+</sup>CD8<sup>+</sup> cells specific for the OVA epitope SIINFEKL in infiltrates from mice in B, as determined by flow cytometry (n = 5). (E) Frequency of dimer<sup>+</sup>CD8<sup>+</sup> cells specific for the epitope SIYRYRYGL in infiltrates from mice in C, as determined by flow cytometry (n = 5). (F) Representative immunohistochemical staining using anti-CD8 mAb on EG7 tumor tissue sections obtained from A. Original magnification, ×10 (top and middle); ×40 (bottom). (G) Representative dot plots of Foxp3 expression in B16-SIY tumor-infiltrating CD4<sup>+</sup> cells. Percent Foxp3<sup>+</sup> cells is indicated within plots and summarized (n = 3). Data (mean ± SD) are representative of 3 independent experiments. \*P < 0.05; \*\*P < 0.01.

expression of CD73 selectively in hematopoietic and nonhematopoietic cells to further dissect the mechanisms of host CD73 in the control of tumor growth. We found that CD73 deficiency on both hematopoietic and nonhematopoietic cells was most effective in limiting the growth of EG7 or B16-SIY (Figure 1, G and H). CD73 deficiency in the hematopoietic or nonhematopoietic com-

partment in EG7 (Figure 1G) and deficiency in the hematopoietic compartment alone in B16-SIY (Figure 1H) conferred an antitumor advantage compared with CD73 sufficiency or deficiency in both hematopoietic and nonhematopoietic compartments. This discrepancy between EG7 and B16-SIY could be attributable to the intrinsic differences in tumor models among other factors.

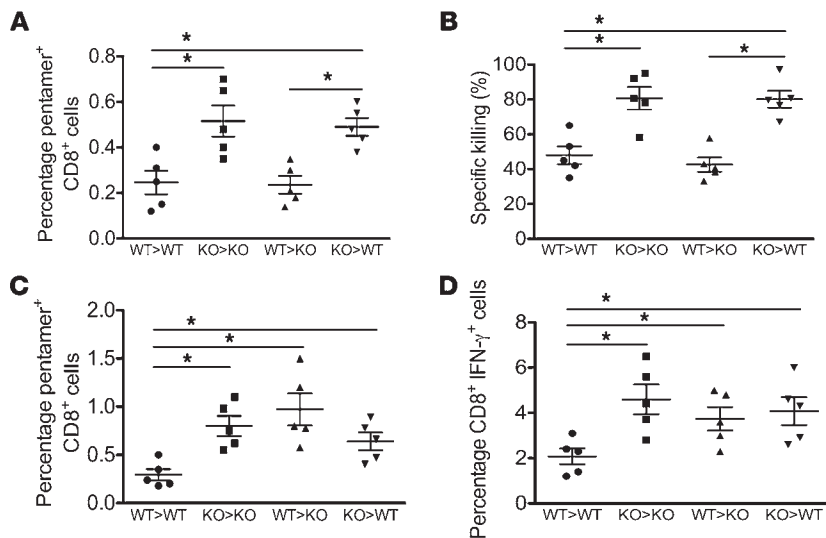


**Figure 3**

Host CD73 impairs endogenous antitumor T cell immunity. (A) Representative flow cytometric analyses of antitumor T cells in spleen from EG7-bearing WT or CD73 KO mice 14 days after tumor inoculation. (B) The H-2K<sup>b</sup>-OVA/SIINFEKL pentamer was used to track OVA-specific CD8<sup>+</sup> T cells. Pentamer<sup>+</sup>CD8<sup>+</sup> and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cell frequency in response to SIINFEKL peptide is summarized ( $n = 5$ ). (C) The H-2K<sup>b</sup>-SIYRYRYGL-IgG dimer was used to track SIY-specific CD8<sup>+</sup> T cells in spleen from B16SIY-tumor bearing WT or CD73 KO mice 14 days after tumor inoculation. Dimer<sup>+</sup>CD8<sup>+</sup> and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cell frequency in response to SIYRYRYGL peptide is summarized ( $n = 5$ ). (D) Representative flow cytometric analyses of in vivo antigen-specific killing capacity of antitumor T cells from EL4- or EG7-bearing WT and CD73 KO mice. Equal numbers ( $10^7$  cells each) of CFSE<sup>hi</sup> SIINFEKL peptide-pulsed and CFSE<sup>lo</sup> non-peptide-pulsed WT splenocytes were adoptively transferred into tumor-bearing mice. Numbers denote percentage of SIINFEKL peptide-pulsed target cell killing in spleen, calculated as described in Methods. (E) Percent killing for EG7-bearing mice ( $n = 5$ ). (F) Percent killing of adoptively transferred SIYRYRYGL peptide-pulsed splenocytes from parallel experiments involving B16-SIY-bearing mice ( $n = 5$ ). Data (mean  $\pm$  SD) are representative of 3 independent experiments. \* $P < 0.05$ .

*Distribution of tumor-infiltrating immune cells.* We hypothesized that the observed tumor resistance of CD73 KO mice was primarily mediated by CD8<sup>+</sup> T cells. To determine whether this was the case, we characterized the immune cells infiltrating in tumor tissues. At 12 days after tumor inoculation, we found no significant alterations in the percentages of CD4<sup>+</sup>, NK (CD49b<sup>+</sup>NK1.1<sup>+</sup>), or myeloid-derived suppressor (Gr1<sup>+</sup>CD11b<sup>+</sup>) cells in local infiltrates of EG7 or B16-SIY tumors in CD73 KO versus WT mice (Figure 2, A–C). In contrast, remarkably more CD8<sup>+</sup> leukocytes and tumor antigen-specific CD8<sup>+</sup> T cell accumulation were found

in tumors in CD73 KO versus WT mice by flow cytometry and immunohistochemistry (Figure 2, A–F), which may be explained in part by the fact that host CD73 deficiency preferentially induced more proliferative antigen-specific CD8<sup>+</sup> T cells (Supplemental Figure 2). These data suggest that loss of CD73 expression in mice results in increased infiltration of CD8<sup>+</sup> T cells into the tumor tissue that may contribute to the inhibition of immunogenic tumor growth. Interestingly, there were significantly more phenotypic CD4<sup>+</sup>Foxp3<sup>+</sup> Treg infiltrates in B16-SIY tumors in CD73 KO than in WT mice (Figure 2G).

**Figure 4**

CD73 has distinct roles in hematopoietic and non-hematopoietic cells in regulating antitumor T cell responses. The H-2K<sup>b</sup>-OVA/SIINFELK pentamer was used to stain OVA-specific CD8<sup>+</sup> T cells in spleens of EG7 tumor-bearing WT and CD73 KO chimeric mice 14 days after tumor inoculation. (A) Frequency of pentamer<sup>+</sup>CD8<sup>+</sup> cells ( $n = 5$ ). (B) Percent in vivo OVA-specific killing ( $n = 5$ ). (C and D) Frequency of pentamer<sup>+</sup>CD8<sup>+</sup> (C) and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (D) cells in tumor infiltrates, as determined by flow cytometry ( $n = 5$ ). Data are representative of 2 independent experiments. \* $P < 0.05$ .

*CD73 expression on nonhematopoietic and hematopoietic cells regulates local and systemic antitumor T cell immunity.* The tumor model we tested using the surrogate antigens OVA or SIY allowed us to assess the in vivo tumor antigen-specific T cell immune response. Pentamer staining showed that a greater number of OVA-reactive CD8<sup>+</sup> T cells and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells were detected in EG7-bearing CD73 KO mice than in WT mice (Figure 3, A and B). Similarly, dimer staining showed that a greater number of SIY-reactive CD8<sup>+</sup> T cells and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells were detected in spleens of B16-SIY-bearing CD73 KO mice than WT mice (Figure 3C).

We next examined the effector cytolytic function of tumor antigen-specific T cells. Target cell lysis in vivo was remarkably improved in draining LNs (DLNs; data not shown) and spleens of EG7-bearing CD73 KO versus WT mice (Figure 3, D and E). Similarly, enhanced target killing was also observed in spleens of B16-SIY-bearing CD73 KO mice compared with that in tumor-bearing WT mice (Figure 3F), which suggests that host CD73 deficiency helps retain T cell cytolytic activity.

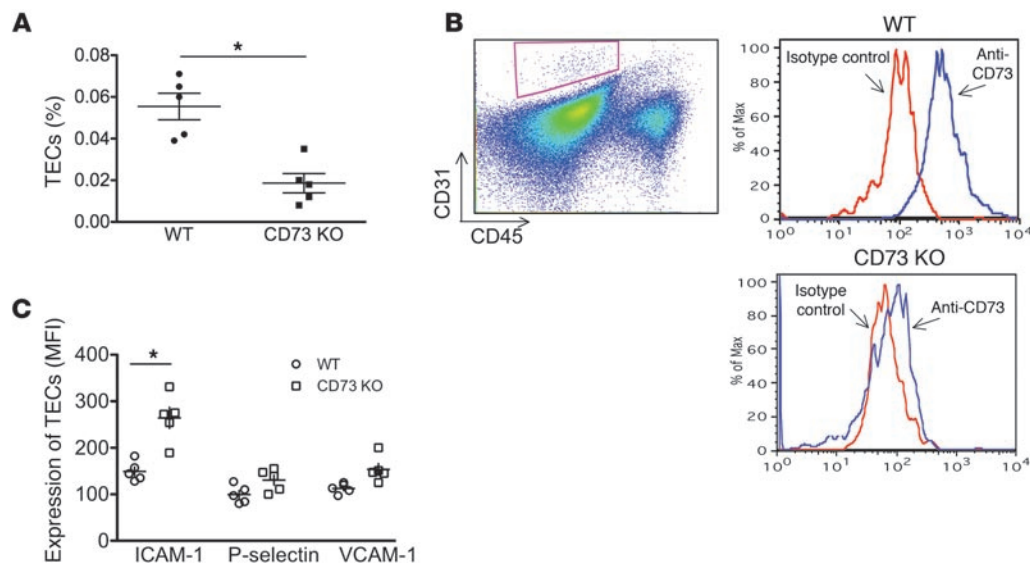
Because CD73 deficiency on both hematopoietic and nonhematopoietic cells was required to limit tumor growth effectively (Figure 1, E and F), we examined the roles of CD73 on hematopoietic and non-hematopoietic cell compartments in regulating antitumor T cell immunity. OVA-reactive CD8<sup>+</sup> T cell populations were significantly increased in spleens of chimeric mice lacking CD73 on BM-derived cells (KO>KO and KO>WT; Figure 4A). However, mice with CD73-sufficient BM had fewer antigen-specific CD8<sup>+</sup> T cells, regardless of whether CD73 was expressed on nonhematopoietic cells (WT>WT and WT>KO). Similarly, enhanced in vivo OVA-specific killing was observed in spleens of KO>KO and KO>WT mice compared with that in WT>WT and WT>KO chimeras (Figure 4B). These data show that CD73 expression on hematopoietic cells, and not on nonhematopoietic cells, significantly influenced systemic antitumor CD8<sup>+</sup> T cell immunity. Interestingly, CD73 deficiency in just 1 compartment conferred an equal accumulation advantage of OVA-reactive CD8<sup>+</sup> T cells and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells in tumor tissues (Figure 4, C and D), which suggests that CD73 expression on nonhematopoietic cells primarily influenced tumor-reactive CD8<sup>+</sup> T cells at the tumor site. We conclude that host CD73 impairs endogenous tumor antigen-specific T cell function and CD73 has distinct roles in hematopoietic and nonhematopoietic cells in limiting antitumor T cell immunity.

*EC CD73 regulates T cell endothelial adhesion and migration.* To test a role for CD73 in T cell-endothelium interactions, we used APCP, a specific CD73 inhibitor, and 5'-N-ethylcarboxamidoadenosine (NECA), an adenosine analog and general adenosine receptor agonist. Activated T cells adhered promptly to TNF- $\alpha$ -treated bEnd.3 ECs. Exposure of ECs to the CD73 enzymatic substrate 5'-AMP or NECA attenuated T cell adhesion. APCP neutralized the effect of 5'-AMP and restored adhesion of T cells (Supplemental Figure 3A). Similar results were obtained in an assay to assess transmigration of activated T cells across an endothelial layer (Supplemental Figure 3B). Thus, we conclude that CD73 prevents T cell adhesion to – and transmigration through – endothelium via its enzymatic activity, which generates extracellular adenosine.

To test whether adenosine affects endothelial molecules implicated in T cell adhesion and migration, we measured expression levels of ICAM-1, VCAM-1, and P-selectin in TNF- $\alpha$ -treated ECs by flow cytometry. Both NECA and 5'-AMP downregulated ICAM-1, VCAM-1, and P-selectin expression. APCP alone had no effect on the expression of these molecules, but prevented their downregulation in the presence of 5'-AMP (Supplemental Figure 3C). No change was detected in the expression level of CD31 (Supplemental Figure 3C).

Fewer CD31<sup>+</sup>CD45<sup>-</sup> tumor ECs (TECs) were detected in tumor tissues of B16-SIY-bearing CD73 KO mice than WT mice (Figure 5A). Importantly, TECs expressed CD73 (Figure 5B), which suggests that adenosine generated by CD73 on TECs may regulate tumor-reactive T cell adhesion and migration. Indeed, TECs showed enhanced levels of ICAM-1 expression in CD73 KO versus WT mice (Figure 5C).

*Blockade of CD73 in vivo augments T cell homing to tumors.* Because EC CD73-generated adenosine limited T cell adhesion and migration (Figure 5), we next tested whether host CD73 deficiency increased tumor antigen-specific (OVA-specific) T cell homing to tumors and thereby facilitated antitumor T cell immunity. As expected, a significantly larger number of adoptively transferred OVA-specific OT-I T cells homed to EG7 tumors and DLNs in CD73 KO versus WT mice, but not to spleens, lung, or liver of either (Figure 6, A and B, and data not shown). Similar results were obtained in B16-SIY tumors (Figure 6C and data not shown). In contrast, nearly equal numbers of adoptively transferred OT-I cells were detected



**Figure 5** Endothelial CD73 limits T cell adhesion and migration. **(A)** Percent CD31<sup>+</sup>CD45<sup>-</sup> TECs in B16-SIY tumors ( $n = 5$ ). **(B)** TECs in B16-SIY tumors expressed CD73. **(C)** Expression of the adhesion molecules ICAM-1, VCAM-1, and P-selectin on TECs from B16-SIY-bearing mice ( $n = 5$ ). Data are representative of 2 independent experiments. \* $P < 0.05$ .

in spleens and LNs of tumor-free WT and CD73 KO mice (data not shown). These results were also confirmed by adoptive transfer of nontransgenic tumor-reactive T cells (Supplemental Figure 4A). Moreover, consistent with our previous study (20), there was no significant difference in the number of transferred WT or CD73 KO OT-I T cells in EG7-bearing mice (Supplemental Figure 4B), which suggests that CD73 on T cells was not involved in T cell homing to tumors.

We next tested whether blockade of CD73 *in vivo* leads to enhanced accumulation of tumor-reactive T cells in tumors. Consistent with prior data, inhibition of CD73 enzymatic activity using APCP significantly increased antigen-specific T cell homing to EG7 and B16-SIY (Figure 6, D and E). In further support of a role for adenosine metabolism in these effects, NECA significantly reduced T cell homing to tumors. Similar reductions of T cell homing were observed in tumor-bearing mice treated with the adenosine A2 receptor (A2AR and A2BR) agonist CV1808, but not with the A2AR-specific agonist CGS 21680 (Figure 6, D and E), which suggests that CD73-generated adenosine regulates T cell homing primarily through A2BR. APCP treatment did not influence antigen-specific T cell activation or proliferation 24 hours after T cell transfer (data not shown), which suggests that it is unlikely that activation or proliferation markedly influences our T cell homing assessments. Staining transferred tumor-specific T cells that migrated into tumor tissues of WT and CD73 KO mice with annexin V and mAbs to CXCR4 and CCR4 suggested that it is less likely that survival status or expression levels of the above homing molecules contributed to the increased T cell homing observed in tumor-bearing CD73 KO mice (Supplemental Figure 5).

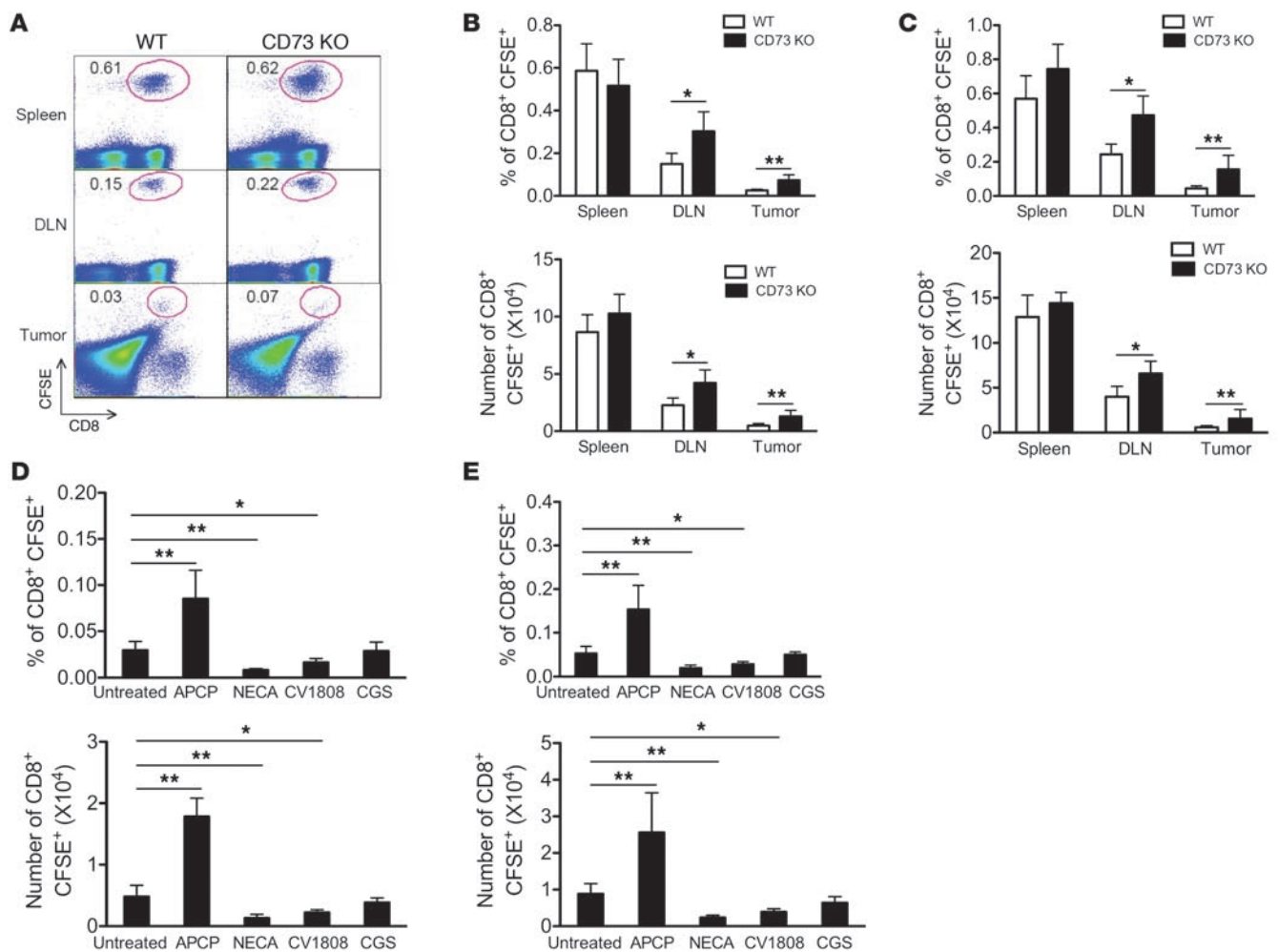
*CD4<sup>+</sup> Tregs inhibit T cell-mediated antitumor effects in a CD73-dependent manner.* Recent evidence has suggested the involvement of CD39 and CD73 in the functional activity of Tregs through the production of adenosine (17, 18, 21). In agreement with these suggestions, we demonstrated that CD73 KO Tregs from naive mice were less able than WT Tregs to suppress IFN- $\gamma$  production

*in vitro* in the presence of AMP (Supplemental Figure 6A). Currently, little is known about the role of CD73-generated adenosine in controlling antitumor immunity by tumor Tregs. To determine the activity of CD73 KO Tregs against antitumor immunity, we first examined the phenotypes of Tregs in tumor-bearing mice. Notably, there was no significant alteration in the expression of various Treg-associated markers, including CD39, GITR, CTLA-4, and Foxp3, on CD4<sup>+</sup>CD25<sup>+</sup> T cells from spleens of tumor-bearing WT and CD73 KO mice (Supplemental Figure 6B).

We next examined tumor antigen-specific T cells in the presence or absence of tumor Tregs from CD73 KO or WT mice. Interestingly, substantially more antigen-specific CD8<sup>+</sup> T cells producing IFN- $\gamma$  accumulated in both spleens and DLNs in mice receiving tumor CD73 KO Tregs than in those receiving WT Tregs, but less than in those without the transfer of tumor Tregs. As expected, in the presence of WT tumor Tregs, the number and IFN- $\gamma$  production of tumor-reactive T cells was greatly inhibited (Figure 7, A and B). These data accord with the notion that CD73 on CD4<sup>+</sup> Tregs negatively regulates antitumor T cell immunity.

Previous studies demonstrated that Tregs inhibit tumor growth by directly impairing antitumor T cells upon transfer of CD25-depleted T cells into T cell-deficient tumor-bearing *Rag1*<sup>-/-</sup> mice (22). Using the same model, WT or CD73 KO T cells with or without CD25 depletion were adoptively transferred into B16-SIY-bearing *Rag1*<sup>-/-</sup> mice (Figure 7C). WT T cells alone failed to control tumor growth effectively unless CD25<sup>+</sup> cells were depleted. However, tumor growth was greatly inhibited by CD73 KO T cell transfer alone. Moreover, CD25<sup>+</sup> cell-depleted CD73 KO T cells were more effective in controlling tumor growth, which suggests that CD73 KO CD25<sup>+</sup> T cells retain partial suppression of the antitumor T cell responses.

To test a role for CD73 by Tregs on endogenous antitumor effects, we compared the effectiveness of Treg depletion on tumor growth between WT and CD73 KO mice using denileukin diftotox (DT; ref. 23), a fusion protein of IL-2 and diphtheria toxin.

**Figure 6**

Blockade of CD73 in vivo augments T cell homing to tumors. Activated OT-I CD90.1 cells were labeled with CFSE and adoptively transferred into EG7-tumor bearing WT or CD73 KO mice. (A) Representative flow cytometric analyses of OT-I cells in the spleen, DLN, and EG7 tumor 24 hours after transfer. (B) Frequency and absolute number of CFSE<sup>+</sup>CD8<sup>+</sup> OT-I cells among different tissues from EG7-bearing mice 24 hours after T cell transfer ( $n = 5$ ). (C) Frequency and absolute number of CFSE<sup>+</sup>CD8<sup>+</sup> 2C cells among different tissues from B16-SIY-bearing mice 24 hours after T cell transfer ( $n = 5$ ). (D) Frequency and absolute number of CFSE<sup>+</sup>CD8<sup>+</sup> OT-I cells among tumor tissues from EG7-bearing WT mice treated as indicated and assessed 24 hours after T cell transfer ( $n = 5$ ). (E) Frequency and absolute number of CFSE<sup>+</sup>CD8<sup>+</sup> 2C cells among tumor tissues from B16-SIY-bearing WT mice treated as indicated and assessed 24 hours after T cell transfer ( $n = 5$ ). Data (mean  $\pm$  SD) are representative of 3 independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

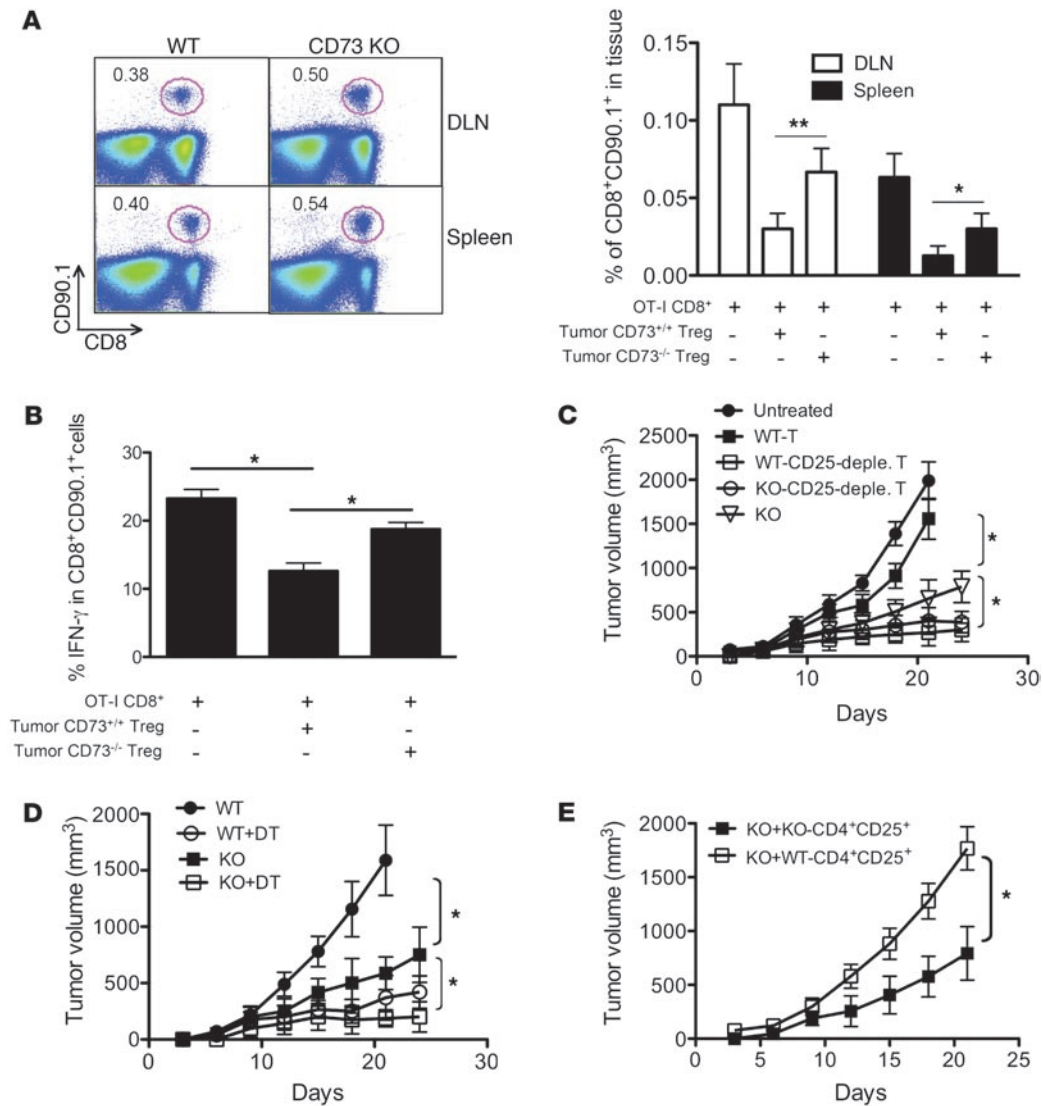
Consistent with prior reports, the greatest deletion of Tregs relative to untreated mice was seen on day 1 after injection, and Treg numbers almost normalized by day 7 (Supplemental Figure 7). Importantly, DT treatment greatly inhibited tumor growth in WT mice, indicative of a role for Treg-mediated suppression of anti-tumor immunity. In contrast, DT treatment was less effective in tumor-bearing CD73 KO mice compared with WT mice (Figure 7D). Moreover, adoptive transfer of WT Tregs, but not CD73 KO Tregs, reversed the tumor-inhibiting advantage of host CD73 deficiency, consistent again with the direct role of CD73 on Tregs in tumor growth (Figure 7E).

We did not expect CD73 KO effector T cells to have defects in antitumor responses. This notion was supported by our observation that CD73 KO T cells (CD4<sup>+</sup> or CD8<sup>+</sup>) were nearly equal in response to anti-CD3/CD28 beads compared with WT T cells in vitro (Sup-

plemental Figure 8A). More importantly, there was no significant difference in antigen-specific cell proliferation and IFN- $\gamma$  production between transferred OT-II WT T cells and OT-II CD73 KO T cells after OVA immunization (Supplemental Figure 8B). Adoptive transfer of CD25-depleted CD73 KO T cells inhibited tumor growth with efficiency equivalent to that of CD25-depleted WT T cells (Figure 7C). Together, these data indicate that CD73 makes a less substantial contribution to effector T cell function. However, it is possible that CD73 expression on effector T cells could contribute to adenosine production in the tumor microenvironment.

*Pharmacological blockade of CD73 activity inhibits tumor growth and augments the efficacy of adoptive T cell therapy.* Given that CD73 expression on both cancer cells and host cells impairs anti-tumor immunity, inhibition of CD73 may represent a feasible and effective anticancer treatment. Consistent with a role for



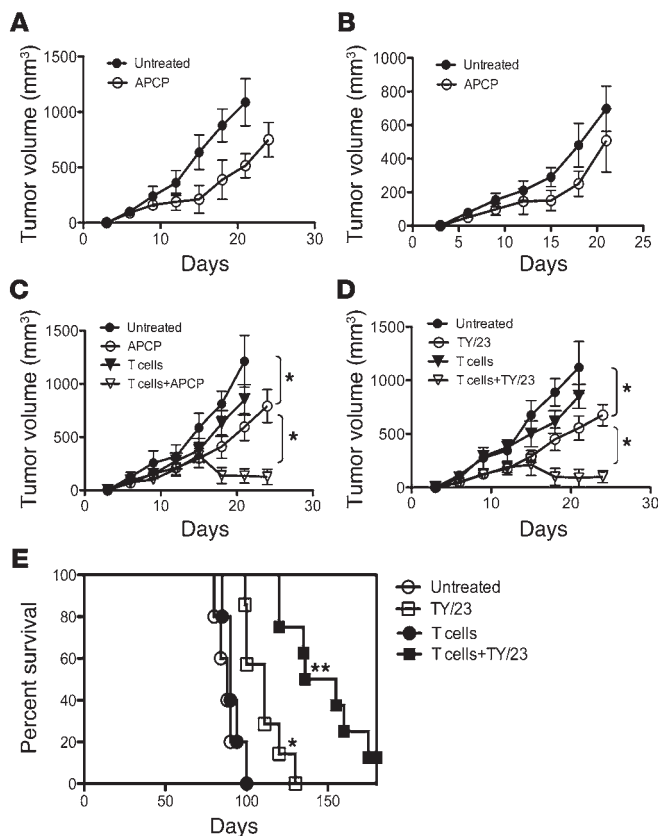


**Figure 7**

CD73 expression by CD4<sup>+</sup>CD25<sup>+</sup> Tregs inhibits antitumor T cell immunity and facilitates tumor growth. (A and B) Purified OT-I CD8<sup>+</sup>CD90.1<sup>+</sup> T cells ( $2 \times 10^6$ ) were injected i.v. into WT mice ( $n = 3$  per group) with or without  $10^6$  splenic CD4<sup>+</sup>CD25<sup>+</sup> Tregs derived from EG7-bearing WT or CD73 KO mice. The next day,  $10^6$  EG7 cells were injected s.c. into the T cell recipients. 3 days later, percent transferred T cells in spleen and DLN (A) and percent IFN- $\gamma$ <sup>+</sup> cells among the transferred cells in spleen (B) were determined by flow cytometry ( $n = 3$ ). (C) For CD25<sup>+</sup> T cell depletion, splenic T cells were further purified by negative selection using magnetic bead-conjugated anti-CD25 Ab. *Rag1*<sup>-/-</sup> mice ( $n = 5$  per group) were challenged with  $10^6$  B16-SIY cells 24 hours after receiving  $10^7$  total T cells or CD25-depleted T cells from naive WT or CD73 KO mice. (D) Treg depletion using DT inhibited tumor growth. WT or CD73 KO mice ( $n = 5$  per group) were challenged with  $10^6$  B16-SIY cells. 3 days later, mice were treated with DT (2  $\mu$ g/mouse twice weekly). (E) Adoptive transfer of WT Tregs, but not CD73 KO Tregs, reversed the tumor-inhibiting advantage of host CD73 deficiency. CD4<sup>+</sup>CD25<sup>+</sup> Tregs derived from WT or CD73 KO mice were injected i.v. into CD73 KO mice. The next day,  $10^6$  B16-SIY cells were challenged. Data (mean  $\pm$  SD) are representative of 3 (A and B) or 2 (C–E) independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

host as well as tumor CD73 in augmented tumor growth, pharmacological inhibition of CD73 activity using the small-molecule inhibitor APCP suppressed tumor growth effectively in WT mice and less effectively in CD73 KO mice (Figure 8, A and B). We next tested whether CD73 blockade improves the efficacy of adoptive T cell therapy. In the B16-SIY model, transfer of high-avidity tumor-specific T cells (SIY-specific transgenic 2C T cells) failed to control tumor growth (Figure 8, C and D).

In contrast, combining APCP treatment with adoptive T cell therapy resulted in tumor regression, which was more effective than APCP treatment alone (Figure 8C). Notably, we found no histological evidence of inflammation by H&E staining (data not shown) or increased number of CD45<sup>+</sup> or CD3<sup>+</sup> cells in the livers or kidneys of mice treated with APCP plus T cells relative to untreated mice (Supplemental Figure 9). Similar results were observed in mice treated with the anti-CD73 mAb TY/23

**Figure 8**

Pharmacological blockade of CD73 activity inhibits tumor growth and augments the efficacy of adoptive T cell therapy. **(A and B)** WT **(A)** or CD73 KO **(B)** mice ( $n = 5$  per group) were challenged s.c. with  $10^6$  B16-S1Y cells. 1 week later, mice were treated with APCP (20 mg/kg i.v.) once daily for 1 week, then twice weekly, and tumor volume was measured. **(C and D)** WT mice ( $n = 5$  per group) were s.c. challenged with  $10^6$  B16-S1Y cells. 10 days later, mice were treated with either APCP as described above **(C)** or 100  $\mu$ g TY/23 **(D)** 2 times weekly and adoptively transferred with  $5 \times 10^6$  2C T cells 13 days after tumor challenge as indicated. Tumor volumes were measured at the indicated times. **(E)** Female WT mice were inoculated i.p. with  $10^7$  ID8 cells. 1 week later, tumor-bearing mice were left untreated or transferred with  $10^7$  ID8-reactive T cells i.p. and/or treated with TY/23 as described above. Survival of the mice ( $n = 5-8$  per group) was measured. Data are representative of 2 independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

(Figure 8D). For further confirmation, we tested the peritoneal ID8 tumor model and found that the median 146-day survival of mice treated with both T cells (nontransgenic tumor-reactive T cells) and TY/23 was superior to that in mice treated with TY/23 alone or with T cells alone (111 and 90 days, respectively;  $P < 0.01$ ; Figure 8E). Collectively, these data support our thesis that CD73 blockade by either small-molecule inhibitors or Abs rescues tumor-specific immunity and enhances the efficacy of adoptive T cell therapy.

## Discussion

We previously showed that CD73 on tumor cells functions as an ecto-5'-nucleotidase to promote tumor growth by impairing antitumor T cell immunity (12). We now demonstrate that CD73 on host cells, through its enzymatic activity, also protects tumors from antitumor T cells, which suggests that CD73 expression on both cancer cells and host cells cooperatively mediates tumor immune evasion. Other CD73 functions in this regard, such as intracellular signaling (10), may need further investigation. Our published (12) and present data support the concept that integrated components of the tumor environment generate extracellular adenosine, contributing to the immune-suppressive microenvironment that facilitates tumor growth.

Using BM chimeric mice, we found that optimal antitumor effects require CD73 expression on both BM-derived and non-BM-derived host cell populations. To dissect the contribution of host CD73 to tumor immunity further, we focused on 2 major host cell populations: non-BM-derived ECs and BM-derived  $CD4^+CD25^+$  Tregs that express high levels of CD73. We demonstrated that CD73 on hematopoietic cells (including  $CD4^+CD25^+$

Tregs) suppressed systemic antitumor T cell expansion and effector function, whereas CD73 derived from nonhematopoietic cells (presumably ECs) primarily affected local antitumor T cell immunity by limiting T cell homing to tumors. Therefore, CD73 has distinct roles in hematopoietic and nonhematopoietic cells in regulating antitumor T cell responses. We cannot exclude the possibility that CD73 on other nonhematopoietic cells or other immune cell populations (e.g., certain subsets of B cells and DCs; refs. 14, 24, 25) contributes to tumor progression. Moreover, host CD73-mediated adenosinergic effects on other cell populations (e.g., DCs; refs. 26, 27) and on NK-like innate cells (NKp46<sup>+</sup> lymphoid tissue-inducer cells; ref. 28), rather than antitumor T cells, need further investigation.

Of the ectoenzymes involved in leukocyte extravasation, CD73 and its function in leukocyte trafficking has been studied extensively (29). CD73 has been proposed to modulate lymphocyte-EC interactions as an adhesion molecule (30, 31). Furthermore, CD73 plays important roles in vivo in maintaining the integrity of the vascular endothelium during hypoxia (32-34); in mediating efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis (35); and in regulating leukocyte adhesion to the endothelium during cardiac ischemia-reperfusion (36), atherosclerosis (37), cardiac allograft vasculopathy (38), and neointimal hyperplasia (39). In agreement, we recently demonstrated that CD73-generated adenosine regulates the ability of lymphocytes to migrate across high endothelial venules after LPS injection, thus limiting their access to inflamed LNs (20). Moreover, our present in vitro results of adhesion and transmigration assays confirmed that endothelial CD73 suppresses transendothelial leukocyte



trafficking through its enzymatic activity. Because CD73 deficiency in the host enhances the accumulation of tumor-reactive T cells in the tumor microenvironment, we hypothesize that this enhanced infiltration is due, at least in part, to increased tumor-reactive T cell migration in the absence of CD73-generated adenosine. In support of this idea, we found that host-derived TECs highly expressed CD73; more importantly, host CD73 deficiency and pharmacological inhibition of CD73 using APCP enhanced the homing of adoptively transferred antigen-specific T cells to DLNs and tumors with upregulation of endothelial adhesion molecules. Furthermore, adenosine analog NECA and adenosine A2 receptor agonist CV1808, but not A2AR-specific agonist CGS 21680, remarkably reduced T cell homing. Thus, we conclude that endothelial CD73-generated adenosine limits the access of tumor-reactive T cells to tumors primarily via the activation of A2BR. It is possible that activation of A2BR enhances the endothelial barrier and hampers adhesion of leukocytes to the endothelium through cAMP-dependent intracellular signaling (40–42), which in turn may increase the expression and activity of CD73 and further prevent adhesion of leukocytes (43). It is less likely that increased survival or expression of homing molecules of infiltrating antitumor T cells contributes to the increased tumor T cell homing in CD73 KO mice.

There was no significant difference in the homing of WT versus CD73 KO T cells, consistent with our previous results (20), which suggests that CD73 on T cells is not involved in T cell homing to tumors. Indeed, CD73 is differently regulated in lymphocytes and ECs (44). It is possible that endothelial CD73 stimulates pathological angiogenesis to promote tumor growth, similar to CD39 (45), as adenosine is known to upregulate the expression of VEGF. It is also possible that CD73-generated adenosine inhibits the expansion of tumor-infiltrating T cells.

The involvement of CD39 and CD73 in the functional activity of Tregs via adenosine production has been recently highlighted (46, 47). Thus, we assessed the role of CD73-generated adenosine in controlling antitumor immunity by tumor Tregs. Of note, there were more Treg infiltrates in tumors in CD73 KO than in WT mice. The reason behind this increase is unclear. It is likely that host CD73 deficiency accelerates the migration of Tregs in addition to T effector cells, as discussed above. Alternatively, CD73 KO Tregs could mount a stronger proliferative response to tumor antigen stimulation than WT Tregs. Importantly, we showed that CD73 on tumor Tregs impaired antitumor T cell expansion and IFN- $\gamma$  production in vivo. From experiments involving adoptive transfer of Tregs and depletion of Tregs using anti-CD25 mAb or DT, we conclude that CD73 expression by Tregs protects tumors from antitumor T cell immunity. Similarly, a very recent elegant study showed that CD39 expression on Tregs modulates NK cell reactivity against tumor cells (48). Other work has reported increased CD73/CD39 expression and activity on Tregs infiltrating human follicular lymphoma (49) and patients with cancers of the head and neck (50, 51). Moreover, Th17 cell development was suppressed through the adenosinergic pathway by ovarian tumor-associated Tregs highly expressing CD39 (52), which could be a previously unappreciated mechanism that tumors hijack to evade the immune system (53). Taken together, these data suggest an intriguing adenosinergic mechanism by which CD73/CD39-mediated immune suppression by Tregs contributes to tumor progression.

Both tumor and host CD73-generated adenosine prevented tumor destruction by antitumor T cells, raising the feasibility of new strategies to overcome this immunosuppression by inhibi-

tion of CD73 activity. Among 4 different adenosine receptors, A1, A2A, A2B, and A3, the cAMP-elevating signaling through A2AR or A2BR in T cells results in inhibition of antitumor T cell function (4), which could explain the observation of slowed tumor growth in *A2AR<sup>-/-</sup>* (4) or *A2BR<sup>-/-</sup>* mice (54). We are currently testing whether A2AR and/or A2BR is responsible for suppressing antitumor T cells by endogenous CD73-generated adenosine.

APCP is a well-established inhibitor of the enzyme activity of CD73 and has been used for this purpose since the 1970s. We found that APCP did not alter cAMP levels in cultured cells (our unpublished observations), which suggests that it does not interact with P1 receptors. We acknowledge the possibility that APCP could, in principle, interact with purinergic receptors. However, our findings of consistent results for 3 different methods of CD73 ablation (siRNA gene targeting, CD73 KO mice, and APCP treatment) in both current and previously published (12) *in vitro* and *in vivo* studies give us confidence that APCP is not working via off-target effects in our models. NECA is indeed a general adenosine receptor agonist and should therefore mimic the action of CD73 to produce adenosine, which can then act at any available adenosine receptor.

The ultimate goal of our research is to establish a potential and feasible strategy of targeted CD73 therapy for cancer that can be readily translated into the clinical arena. Thus, it is important to know whether CD73 functions as an immunosuppressor in human cancer patients. Indeed, we found that CD73 was highly expressed in melanoma lesions compared with halo nevi (common benign skin lesions). Moreover, CD73 expression was associated with poor immune status (lack of perforin secretion) of tumor-infiltrating CD8<sup>+</sup> T cells (data not shown), which indicates that CD73 is likely closely involved in human cancer immunity. Similar results were obtained in human ovarian cancer (data not shown). Thus, targeted CD73 therapy is not limited to specific types of solid tumors but is of more general significance.

In summary, detailed analysis of CD73 expression on cancer cells and/or host cells regulating antitumor immunity may help improve our understanding of immunosuppressive mechanisms in the tumor microenvironment that promote tumor evasion. Inhibition of CD73 could be a therapeutic adjuvant to improve cancer immunotherapy.

## Methods

**Mice, cell lines, and reagents.** C57BL/6 *Rag1<sup>-/-</sup>* mice and CD90.1 mice were purchased from The Jackson Laboratory, and C57BL/6 mice were from NCI-Frederick. CD73 KO mice (*CD73<sup>-/-</sup>*) were generated as described previously (32). H. Schreiber (University of Chicago, Chicago, Illinois, USA) provided the OT-1 *Rag1<sup>-/-</sup>* mice, 2C transgenic mice, SIYRYGL peptides, and EL4, EG7, B16F10, and B16-SIY cell lines. All animal experiments were approved by institutional animal use committees of the University of Texas Health Science Center. ID8, ID8-OVA-SiNS, and ID8-OVA-SiCD73 ovarian cells were described previously (12). The mouse EC line bEND.3 was obtained from the American Type Culture Collection. The OVA-derived peptide SIINFEKL (OVA-I) was synthesized by GenScript. DT (DAB389IL-2), a fusion protein of IL-2 and diphtheria toxin was described previously (23) and obtained from Eisai Pharmaceuticals. All the mAbs were obtained from eBioscience and BioLegend. The Alexa Fluor 647 Annexin V apoptosis detection kit was from BioLegend. 5'-AMP, CGS 21680 (selective A2AR agonist), and APCP were purchased from Sigma-Aldrich. The adenosine analog and general AR agonist NECA and the adenosine A2 receptor agonist CV1808 were from Tocris Bioscience.



**Analysis of cells by flow cytometry.** All samples except for tumor cells were initially incubated with 2.4G2 to block Ab binding to Fc receptors. Single-cell suspensions were stained with 1  $\mu$ g of relevant mAbs and then washed twice with cold PBS. K<sup>b</sup>/OVA pentamer (Proimmune) staining was performed as we previously described (55). K<sup>b</sup>/SIY dimer staining and intracellular IFN- $\gamma$  staining were performed as previously described (19). Samples were analyzed on a LSRII, and data were analyzed with FlowJo software.

**BM transplantation.** BM chimeric mice were generated as described previously (19). Briefly, CD73 KO mice and WT mice were exposed to 10 Gy total-body irradiation. BM cells from the femur and tibia of matched CD73 KO mice and WT mice were harvested under sterile conditions. Irradiated recipient mice received 10<sup>7</sup> BM cells i.v. The success of BM transplantation was verified by flow cytometry analysis of immune cell populations (data not shown). Mice were housed for 8 weeks after BM transplantation before experimentation.

**Immunohistochemistry.** Standard indirect immunoperoxidase staining with modification was performed on 5- $\mu$ m cryosections using rat anti-mouse CD8a mAb (Biolegend) by our Cancer Therapy and Research Center pathology core facility.

**Adhesion and transmigration assay.** T cell adhesion and transmigration assays were performed as described previously (37, 38), with some modifications. bEND.3 ECs grown at approximately 90% confluence in 6-well plates were treated with 5'-AMP (100  $\mu$ M), NECA (10  $\mu$ M), and APCP (50  $\mu$ M) for 24 hours, and then with or without TNF- $\alpha$  (20 ng/ml; PeproTech) for 4 hours. Expression of ICAM-1, VCAM-1, P-selectin, or CD31 on these untreated or treated ECs was determined by flow cytometry and expressed as MFI. Splenic T cells were activated with anti-CD3 mAb (0.5  $\mu$ g/ml) for 24 hours and then labeled with CFSE (Invitrogen). These activated T cells were added to the plate and cocultured with ECs for 1 hour. After intensive washing, the adherent T cells were detached by trypsin-EDTA, and CFSE<sup>+</sup> cells were counted with an LSRII flow cytometer as the flow-through cell number over a constant time period (90 s). For transmigration assays, ECs were seeded onto a collagen-coated transwell insert (5  $\mu$ m pore size, polycarbonate membrane, 24-well format; Corning Costar) and cultured for 1 or 2 days to make a confluent monolayer under similar treatment conditions. Activated T cells (2  $\times$  10<sup>5</sup>) were then applied to the EC monolayer. After 24 hours of incubation, the migrated cells were collected from the lower wells and counted with an LSRII flow cytometer as the flow-through cell number over a constant time period (90 s) for CFSE<sup>+</sup> cells.

**In vivo killing assay.** Analysis of tumor antigen-specific effector CTL activity in vivo was performed as previously described (19). Briefly, OVA-I (SIINFEKL) or SIY (SIYRYLYGL) peptide-pulsed CFSE<sup>hi</sup> and non-peptide-pulsed CFSE<sup>lo</sup> splenocytes were mixed at a 1:1 ratio, and a total of 2  $\times$  10<sup>7</sup> cells was injected i.p. into recipient animals. DLNs (i.e., inguinal LNs) and spleen were then harvested 24 hours after adoptive transfer, and CFSE fluorescence intensity was analyzed by flow cytometry. Gating on CFSE<sup>+</sup> cells, killing was calculated as (% OVA-I/SIY peptide-pulsed cells in OVA<sup>+</sup>/SIY<sup>+</sup> tumor-bearing mice)/(% non-peptide-pulsed cells in OVA<sup>+</sup>/SIY<sup>+</sup> tumor-bearing mice)/(% OVA-I/SIY peptide-pulsed cells in OVA<sup>-</sup>/SIY<sup>-</sup> tumor-bearing mice)/(% non-peptide-pulsed cells in OVA<sup>-</sup>/SIY<sup>-</sup> tumor-bearing mice) and expressed as a percentage.

**In vivo T cell homing assay.** OT-I CD90.1 or 2C transgenic splenocytes were stimulated with OVA-I peptides or SIY peptides for 24 hours, respectively. For nontransgenic T cell activation, splenocytes from EG7-bearing mice or B16-SIY-bearing mice were restimulated with OVA-I peptides or SIY peptides for 72 hours, respectively. T cell homing to tumors of similar sizes (150–300 mm<sup>3</sup>) in WT and CD73 KO mice was assessed as follows: CFSE-labeled naive or activated CD8<sup>+</sup> T cells (10<sup>7</sup> per mouse) were subsequently injected i.v. into EG7- or B16-SIY-bearing mice receiving APCP (20 mg/kg i.p.), CV1808 (1 mg/kg i.p.), CGS 21680 (1 mg/kg i.p.), NECA (0.5 mg/kg i.p.), or equivalent control solvent

DMSO 4 or 24 hours prior to T cell transfer. The percentage and number of CFSE<sup>+</sup>CD8<sup>+</sup> cells in spleen, DLN, tumor tissues, lung, liver, and kidney were determined 24 hours after T cell transfer by flow cytometry.

**T cell purification and adoptive transfer.** To examine the role of CD73 on tumor Tregs, splenic CD4<sup>+</sup>CD25<sup>+</sup> Tregs were selected with a CD4<sup>+</sup>CD25<sup>+</sup> Treg isolation kit (StemCell Technologies). Splenic CD8<sup>+</sup> T cells from OT-I CD90.1 mice were selected with a CD8<sup>+</sup> T cell enrichment kit (StemCell Technologies). Periodic flow cytometric analysis routinely showed greater than 95% purity. 2  $\times$  10<sup>6</sup> purified OT-I CD8<sup>+</sup>CD90.1<sup>+</sup> T cells were injected i.v. into WT mice with or without 10<sup>6</sup> CD4<sup>+</sup>CD25<sup>+</sup> Tregs from EG7-bearing WT or CD73 KO mice. The next day, 10<sup>6</sup> EG7 tumor cells were injected s.c. into the T cell-transferred mice. 3 days later, the percentages of transferred T cells (CD8<sup>+</sup>CD90.1<sup>+</sup>) and transferred IFN- $\gamma$ <sup>+</sup> T cells in spleens and DLNs were determined by flow cytometry.

**Tumor challenge and treatments.** Cultured cancer cells were trypsinized and washed once with DMEM. In the ID8 ovarian cancer model, cancer cells (1  $\times$  10<sup>7</sup>) in suspension were injected i.p. In the s.c. melanoma B16-SIY or lymphoma EG7 model, cancer cells (1  $\times$  10<sup>6</sup>) in suspension were injected s.c. For T cell therapy, OT-I CD90.1 or 2C transgenic splenocytes were stimulated with OVA-I peptides or SIY peptides for 24 hours and with IL-2 (10 ng/ml) for a further 48 hours. To generate ID8-reactive T cells (55), splenic CD3<sup>+</sup> T cells purified by the Pan T cell kit (StemCell Technologies) from ID8-bearing CD73 KO mice were incubated with BM-derived DCs pulsed with UV-irradiated ID8 cells at a 10:1 T cell/DC ratio for 6 days. Mice were treated at day 10 with 20 mg/kg APCP i.v. once daily for 1 week followed by twice weekly, or with 100  $\mu$ g anti-CD73 mAb TY/23 twice weekly; at day 13, the above activated transgenic T cells (5  $\times$  10<sup>6</sup> per mouse) or tumor-reactive T cells (10<sup>7</sup> per mouse) were adoptively transferred i.v. To examine the impact of Tregs on T cell therapy, T cells from spleens of C57BL/6 mice were purified by negative selection using the Pan T cell enrichment kit (StemCell Technologies) according to the manufacturer's protocol. For CD25<sup>+</sup> T cell depletion, purified total splenic T cells were further purified by negative selection using magnetic bead-conjugated anti-CD25 Ab from StemCell Technologies. CD25 depletion was confirmed to eliminate greater than 95% of CD25<sup>+</sup> T cells from total T cell populations. *Rag1*<sup>-/-</sup> mice were challenged with 10<sup>6</sup> B16-SIY cells 24 hours after receiving adoptive transfer of either 10<sup>7</sup> total T cells or CD25-depleted T cells from WT or CD73 KO mice. Depletion of endogenous Tregs using DT was performed as described previously (23, 56); WT or CD73 KO mice were challenged with 10<sup>6</sup> B16-SIY cells. 3 days later, mice were treated i.p. with DT at 2  $\mu$ g per mouse twice weekly. For adoptive transfer of Tregs (56), splenic CD4<sup>+</sup>CD25<sup>+</sup> Tregs (2  $\times$  10<sup>6</sup> per mouse) from WT or CD73 KO mice were injected i.v., and 10<sup>6</sup> B16-SIY cells were given the next day. Depletion of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or NK cells was achieved by twice-weekly i.p. injection of depleting mAb clone GK1.5 (anti-CD4, 200  $\mu$ g), clone 53.6.7 (anti-CD8 $\alpha$ , 200  $\mu$ g), or anti-asialo GM1 (200  $\mu$ g; Wako Pure Chemical) starting 1 day prior to tumor challenge. Flow cytometry confirmed depletion efficiency of target cells for 3 days after injections. The size of tumor was determined at 3-day intervals. Tumor volumes were measured along 3 orthogonal axes (*a*, *b*, and *c*) and calculated as  $abc/2$ . Survival of the mice was monitored.

**Statistics.** Differences in 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 2-tailed Student's *t* test or the nonparametric Mann-Whitney *U* test as appropriate. *P* values greater than 0.05 were considered nonsignificant.

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