Am J Cancer Res 2024;14(2):585-600
www.ajcr.us /ISSN:2156-6976/ajcr0153898

Original Article

CD39$^+$ tumor infiltrating T cells from colorectal cancers exhibit dysfunctional phenotype

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Received October 13, 2023; Accepted January 18, 2024; Epub February 15, 2024; Published February 28, 2024

Abstract: Recent studies revealed that CD39 was highly expressed in tumor-specific CD4$^+$ tumor infiltrating lymphocytes (TILs). However, the divergent function of CD39$^+$ T cells remains to be elucidated in colorectal cancer (CRC). In this study, T cells from CRC patients and tumor-bearing mice were isolated to evaluate the function of CD39 in T cells. We found that CD39 was elevated in intratumoral T cells from CRC patients, and negatively correlated with cytokine secretion capacity. T cell activation induced CD39 expression, and CD39$^+$ T cells produced more IFN-γ in response to CRC tumor antigens. In addition, CD39$^+$ T cells in the spleens of tumor-bearing mice exhibited a stronger anti-tumor activity in vitro than CD39$^-$ T cells, but there was no significant difference in the anti-tumor activities between CD39$^-$ TILs and CD39$^+$ TILs. Moreover, we found that CD39$^+$ T cells expressed higher checkpoint molecules and contained a higher proportion of Treg cells than CD39$^-$ T cells, suggesting that CD39$^+$ T cells may be correlated with an immunosuppressive phenotype. And CD39 expression on T cells could convert pro-inflammatory eATP to immunosuppressive eADO. However, both T cells from the vaccinated-wild-type mice and CD39$^{-/-}$ mice could recognize and eliminate tumor cells in vitro, and adoptive transfer of these T cells resulted in tumor growth inhibition in tumor-bearing mice. In conclusion, our study revealed the divergent functions of CD39$^+$ T cells, which were reactive to tumor antigen but exhibited a dysfunctional phenotype.

Keywords: CD39, TILs, PBLs, colorectal cancer, neoantigen

Introduction

CD39 is an ectonucleoside triphosphate diphosphohydrolase that catalyzes the hydrolysis of β- and γ-phosphate residues of ADP or ATP to AMP, and plays a crucial role in regulating the concentration of extracellular ATP (eATP), which limits P2-mediated purinergic signaling of ATP [1, 2]. Together with ecto-5'-nucleotidase CD73, AMP can be further converted to immunosuppressive extracellular adenosine (eADO). Thus, CD39 and CD73 serve as the immunological switch that turns immune-stimulatory eATP to immunosuppressive eADO, therefore mediating anti-tumor immunity. In solid tumors, a large amount of eATP is released to the tumor microenvironment (TME), and dying cells release ATP into the extracellular matrix due to damaged cell membranes, contributing to the major source of eATP within the TME [3].

The therapeutic potential of CD39 in cancer treatment was inspired by several prior studies, which indicated that the volume of engrafted syngeneic tumors was dramatically reduced in CD39-deficient mice [4, 5]. Tumor growth and liver metastasis were increased in transgenic mice overexpressing CD39 in comparison to wild-type or CD39-deficient mice [6]. Moreover, pharmacological blockade of CD39 by ectonucleotidase POM-1 enhanced anti-tumor immune response and suppressed tumor growth and metastasis [7]. Antibodies against CD39 and CD73 combined with chemotherapeutic drugs

https://doi.org/10.62347/IZEN3736

boosted anti-tumor immunity in a syngeneic mouse model [8]. Currently, two CD39 antagonists TTX-030 and IPH5201, have moved into phase I clinical trials [9]. It has been reported that CD39 deficiency or blockade restores the activation of effector T cells and facilitates the migration of dendritic cells, monocytes, and macrophages within the TME [8].

CD39 is extensively expressed in various cancers, vascular endothelial cells, fibroblasts, and infiltrated immune cells [10, 11]. Remarkably, T cells infiltrated in tumor tissues (TILs) expressed higher levels of CD39 compared to T cells in peripheral blood or lymphoid organs [12]. In 2018, a milestone study revealed that bystander $CD8^+CD39^-$ T cells abundantly infiltrated in tumor tissue while tumor-specific T cells recognizing neoantigens were predominantly enriched in the subset of $CD8^+CD39^+$ TILs [13]. Several independent groups have reported similar results, which suggested that $CD8^+CD39^+CD103^+$ TILs were able to recognize neoantigens and react to tumor cells in various types of human cancers [14-16]. Nevertheless, these $CD8^+CD39^+$ tumor-specific T cells exhibited exhausted phenotype, characterized by impaired effector cytokines secretion and increased expression of inhibitory receptors including PD-1, TIM-3, LAG-3, TIGHT, and 2B4 [12]. In chronic virus infection, virus-specific $CD8^+$ T cells also express high levels of CD39 and display terminally exhausted phenotype [17]. These results suggested that CD39 may be up-regulated in $CD8^+$ T cells during long-term exposure to tumoral or viral antigens, thus acquiring antigen specificity and exhausted phenotypes.

In terms of $CD4^+$ T helper cells, CD39 was initially identified as the surface marker of regulatory T cells (Tregs), which cooperated with CD73 and generated immunosuppressive eADO [18]. The recent study revealed that CD39 was also highly expressed in tumor-specific $CD4^+$ TILs, which shared a similar transcriptional profile, tissue residency, and activation state to tumor-specific $CD8^+$ T cells [19]. Therefore, it is likely that both $CD4^+$ and $CD8^+$ tumor-specific T cells express CD39, indicating CD39 as a potential surface marker that determines tumor specificity. However, the divergent function of $CD39^+$ T cells is paradoxical and remains to be revealed. In the present study, T cells from colorectal cancer (CRC) patients and CRC tumor-bearing mice were used to discuss the effects of CD39 expression on T cells.

Methods

Human subjects and mice

Fresh tumor specimens and blood were collected from CRC patients who received colectomy at The First Affiliated Hospital of Soochow University, Suzhou, upon approval by the hospital ethics committee. Case information for CRC patients was listed in Table S1. Informed consent was signed by enrolled patients. Female 6-8 week-old wild-type C57BL/6N mice were purchased from the Vital River Laboratory Animal Technology Co., Ltd., Beijing. CD39-deficient mice (C57BL/6N) were generated by Cyagen Biosciences, Jiangsu.

Cell lines and chemicals

Jurkat cells were cultured in RPMI complete medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. MC38 cell line and its derived CD39-expressing stable cell line were cultured in DMEM complete medium. ATP, adenosine, CD39 inhibitors sodium polyoxotungstate (POM-1) and ARL67156 trisodium salt, and A2A receptor antagonist-2 (Compound 57) were purchased from MCE, Shanghai, China. Concanavalin A (ConA) was purchased from Sigma Aldrich, Missouri, USA. P2X7 antagonists Brilliant Blue G (BBG) and A-740003 were purchased from Selleck Chemicals, Shanghai, China.

Plasmid construction and electroporation

Human ENTPD1 (CD39) and murine Entpd1 (CD39) were cloned into an expression plasmid PCDH containing sequences encoded for GFP and puromycin-resistant genes. Plasmids were electroporated into Jurkat cells. Cells were cultured for 48 hours to allow the expression of exogenous proteins. GFP-expressing cells were sorted using FACS and cultured with 1 μg/ml puromycin.

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was employed to evaluate the mRNA levels of CD39. In brief, total RNA was extracted by RNAiso Plus (Takara, Japan), and reversely transcribed into cDNA using PrimeScript™ RT

Reagent Kit (Takara, Japan). RT-qPCR was performed using SYBR™ Green Master Mix Reagent Kit (Thermo Fisher Scientific, USA). The primers used were as follows: human CD39, forward, 5'-CTGATTCCTGGGAGCACAT-3', reverse, 5'-GACATAGGTGGAGTGGGAGAG-3'; murine CD39, forward, 5'-TGCCAAGTGAAAGGTCCTGG-3', reverse, 5'-CTGCCGATTGTTCGCTTTCC-3'; human β-actin, forward, 5'-GCATGGGTCAGAAGGATTCCT-3', reverse, 5'-TCGTCCCAGTTGGTGACGAT-3'; murine β-actin, forward, 5'-ATTGCTGACAGGATGCAGAA-3', reverse, 5'-GCTGATCCACATCTGCTGGA-3'.

Western blotting (WB)

WB was performed to evaluate the expression of proteins as described previously [20]. Briefly, total cell lysates were prepared, and 30~40 μg proteins were separated by 8%~12% of SDS-PAGE gels. Blots were then transferred to polyvinylidene fluoride membrane, and blocked at 5% non-fat milk. Primary antibodies were incubated with the membrane at 4°C overnight. IgG-HRP-conjugated secondary antibodies were incubated at room temperature for 2 hours. Blots were detected at ChemiDoC MP system (Bio-Rad Laboratories Inc., USA) using ECL peroxide reagents (Tanon, Shanghai).

ATP hydrolysis assay

ATP Assay Kit (Abcam, UK) was employed to evaluate the remaining ATP in the supernatant of cell culture. Briefly, cells were serially diluted and seeded in a 96-well plate. Cells were treated with 10 μM of ATP in the presence or absence of drugs for 1 hour. Cell-free supernatant was collected and detected using a luminometer.

Generation of TILs and peripheral blood lymphocytes (PBLs)

TILs were aseptically extracted from fresh CRC specimens optimizing from the published protocol [21, 22]. Briefly, tissue specimens were washed, and cleaned samples were minced and enzymatically digested in RPMI-1640 containing 1 mg/ml Collagenase IV, 10 μg/ml DNase I, and 25 μg/ml hyaluronidase. Single cell suspensions were filtered and enriched through Ficoll. Rapid expansion protocol (REP) was applied to selected TIL cultures, which were incubated with 30 ng/ml of anti-CD3 antibody (clone OKT3, Biolegend), 3000 IU of rhIL-2, and irradiated PBMC feeders at a ratio of 1:10 in TIL-REP medium consisting of 50% of RPMI and 50% of AIM-V culture medium supplemented with 5% human AB serum. PBMCs were isolated from fresh blood using Lymphocyte®-H (Cedarlane, Canada). PBLs were lymphocytes within the PBMCs, which were cultured in the "human T-cell medium" plus 200 IU/ml rhIL-2.

Isolation of immune cells from spleens and MC38 syngeneic tumors

Spleens and tumors were aseptically harvested from mice and placed in precooling PBS supplemented with 2% FBS before mechanical dissociation and filtration. Splenocytes were extracted after removing red blood cells through Red Blood Cell Lysis Buffer (Beyotime, Beijing) and re-suspended in RPMI medium as described. TILs from MC38 tumors were enriched through Ficoll gradient centrifugation and re-suspended in RPMI medium for further study.

Generation of murine bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells were isolated from the femurs and tibiae of 8-week-old WT C57BL/6N female mice under sterile conditions. To generate BMDCs, bone marrow cells were cultured in RPMI medium plus 20 ng/ml recombinant murine GM-CSF (rmGM-CSF) (PeproTech, USA) and 10 ng/ml rmIL-4 (PeproTech, USA). Supernatant including non-adherent cells was discarded and replaced by fresh complete medium including cytokines at day 3 incubation. On day 5, the fresh medium including cytokines and 100 ng/ml LPS (Sigma, USA) was added in cell culture for DC maturation.

T cells sorting and activation

Magnetic-activated cell sorting (MACS) kits (Miltenyi Biotec, Germany) were employed to isolate primary $CD3^+$ T cells. To obtain $CD3^+CD39^-$ and $CD3^+CD39^+$ T cells, cell pellets were re-suspended in FACS buffer and stained with Live/Dead Dye eFlour™ 506 (Invitrogen) and antibodies against human CD3 (PE-conjugated, Biolegend) and CD39 (Brilliant Violet 421™-conjugated, Biolegend), mouse CD3 (Pacific Blue™-conjugated, Biolegend) and CD39 (PE/cyanine7-conjugated, Biolegend),

respectively. Cell sorting was performed on fluorescence-activated cell sorting (FACS) Aria III device (BD Biosciences). T cells were activated in RPMI medium containing 1 μg/ml anti-CD28 (Biolegend) and 200 IU/ml IL-2 at 24-well plates pre-coated with 1 μg/ml anti-CD3 (Biolegend) for 48 hours.

Flow cytometry

Cell pellets were re-suspended in FACS buffer and stained with Live/Dead Dye eFlour™ 506 and various of antibodies against cell surface markers as indicated. For detection of cytokines release, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) and 500 ng/ml Ionomycin (Fcmacs Biotech, Nanjing) in the persistence of GolgiPlug Brefeldin A (5 μg/ml, Biolegend) and GolgiStop Monensin (2 μM, Biolegend). Cells were harvested and stained with indicated cell markers before fixation and permeabilization. Fixed and permeabilized cells were thereafter stained for anti-human IFN-γ (PE-Cy7, BD Biosciences), IL-2 (APC, BD Biosciences), TNF-α (FITC, BD Biosciences). For detection of transcription factors Foxp3, cells were firstly stained with indicated cell surface markers before staining of anti-mouse Foxp3 (PE, eBiosciences) using the eBiosciences™ Foxp3 staining kit (Invitrogen).

Enzyme-linked immunosorbent assay (ELISA) & enzyme-linked ImmunoSpot (ELISpot)

The secretion of cytokines IL-2 and IFN-γ were evaluated by ELISA according to manufacturer's instructions (Invitrogen). ELISpot assay was performed using BD™ ELISPOT Human IFN-γ ELISPOT Pair (No. 551873) or Mouse IFN-γ ELISPOT set (No. 551083) according to manufacturer's protocols. For the identification of neoantigens, tumor-reactive T cells were stimulated with syngeneic BMDC-loaded peptides or TCLs. For re-stimulation of splenocytes with peptides, splenocytes isolated from vaccinated mice were activated with peptides (2 μg/ml) or PMA (50 ng/ml) & ionomycin (500 ng/ml) as the positive control at ELISpot plates. For analysis of T-cell response, sorted $CD39^-$ or $CD39^+$ T cells were co-cultured with syngeneic BMDC-loaded peptides or TCLs.

In vitro cytotoxicity assay

To measure the cytolytic activity of T cells to targeted tumor cells, the calcein AM release-based cytotoxicity assay was performed as described previously [23]. Briefly, MC38 target cells were stained in the cell culture medium containing 5 μg/ml of calcein AM (Invitrogen, USA) at 37°C for 1 hour. T cells were co-cultured with stained MC38 cells for 4 hours. Cell-free supernatant was harvested and analyzed in a fluorescence reader.

Statistical analysis

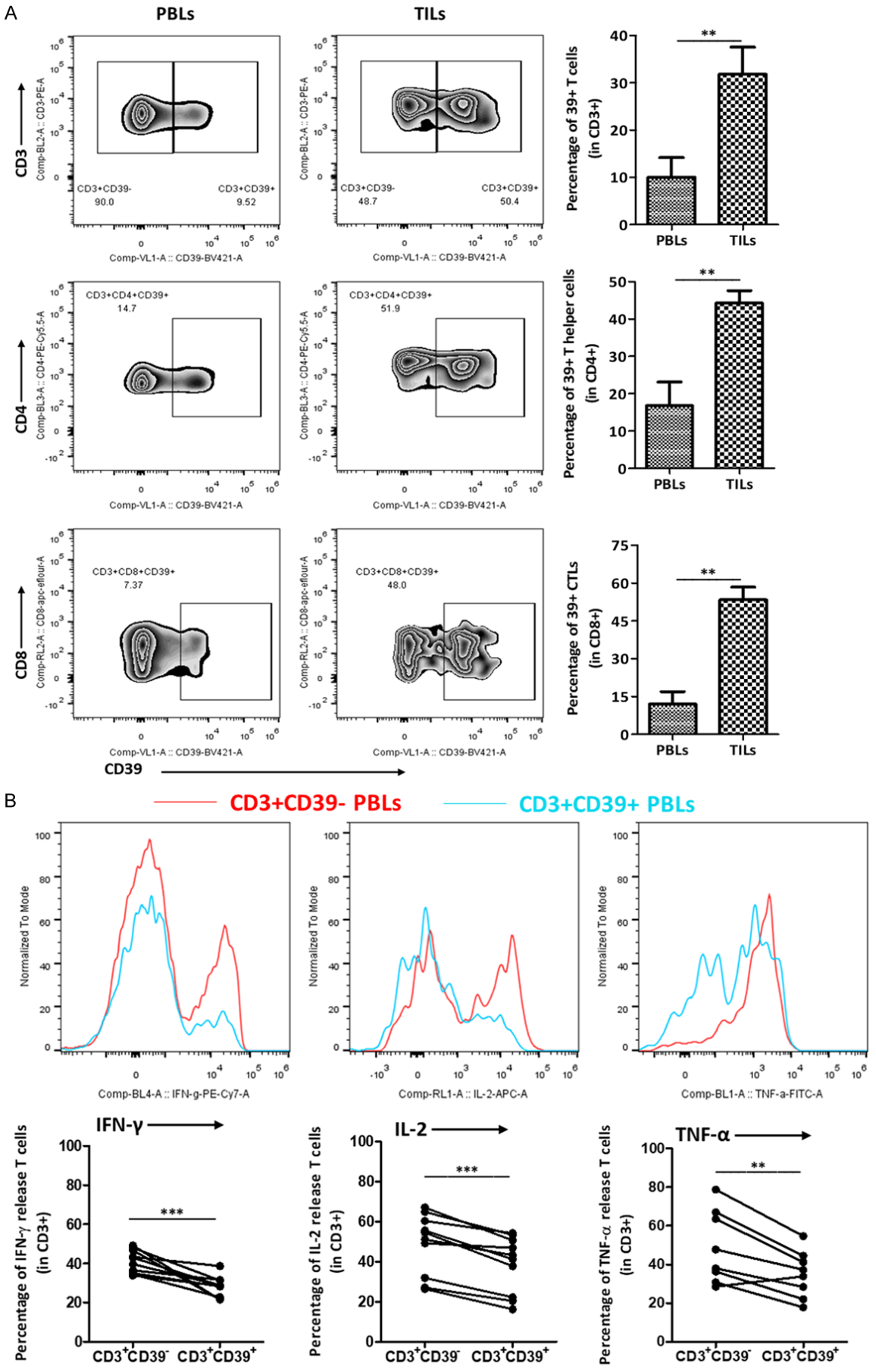
All experiments were performed in triplicates and data were shown as means ± SEM, Correlation analysis was performed by GraphPad Prism 5.0. Statistical significance was determined by Student's t-test ($P < 0.05$).

Results

CD39 is elevated in intratumoral T cells from CRC patients, and T cell activation induces CD39 expression

We firstly evaluated the expression of CD39 in T cells derived from peripheral blood and tumor surgical specimens of CRC patients. In contrast with PBLs, the percentages of $CD3^+CD39^+$ T cells, $CD3^+CD4^+CD39^+$ T helper cells, and $CD3^+CD8^+CD39^+$ cytotoxicity T lymphocytes (CTLs) were significantly increased in TILs as indicated in **Figure 1A**. To better understand the correlation between CD39 expression and T-cell functionality, we performed a cytokine secretion assay of T cells in response to PMA and ionomycin. As a result, the percentages of cytokines-releasing T cells including $IFN\text{-}\gamma^+$, $IL\text{-}2^+$ and $TNF\text{-}\alpha^+$ T cells were significantly enriched in the subset of $CD39^-$ T cells in both PBLs and TILs, which indicated that $CD39^+$ T cells from CRC patients were less sensitive to PMA and ionomycin stimulation (**Figure 1B** and **1C**).

The percentage of $CD3^+CD39^+$ T cells from primary PBLs was also significantly increased after cultivation with the antibodies against CD3 and CD28 (cultured PBLs) (**Figure 2A** and **2B**). However, primary or cultured PBLs and TILs were cultured with autologous tumor cells ($CD3^-$ sorted tumor single-cell suspension) or PMA/ionomycin cocktail for 24 hours, and ELISA assay showed that the secretion of IFN-γ was enhanced in cultured TILs, but not the PBLs (**Figure 2C**). Moreover, activation of T cell receptor (TCR) signaling via PMA/ionomycin, ConA as well as antibodies against CD3 and


A
PBLs
TILs
CD3
CD4
CD8
CD39
CD3+CD39- 90.0
CD3+CD39+ 9.52
CD3+CD39- 48.7
CD3+CD39+ 50.4
CD3+CD4+CD39+ 14.7
CD3+CD4+CD39+ 51.9
CD3+CD8+CD39+ 7.37
CD3+CD8+CD39+ 48.0
Comp-VL1-A :: CD39-BV421-A
Percentage of 39+ T cells (in CD3+)
Percentage of 39+ T helper cells (in CD4+)
Percentage of 39+ CTLs (in CD8+)
**
B
CD3+CD39- PBLs
CD3+CD39+ PBLs
Normalized To Mode
Comp-BL4-A :: IFN-g-PE-Cy7-A
Comp-RL1-A :: IL-2-APC-A
Comp-BL1-A :: TNF-a-FITC-A
IFN-γ
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Percentage of IFN-γ release T cells (in CD3+)
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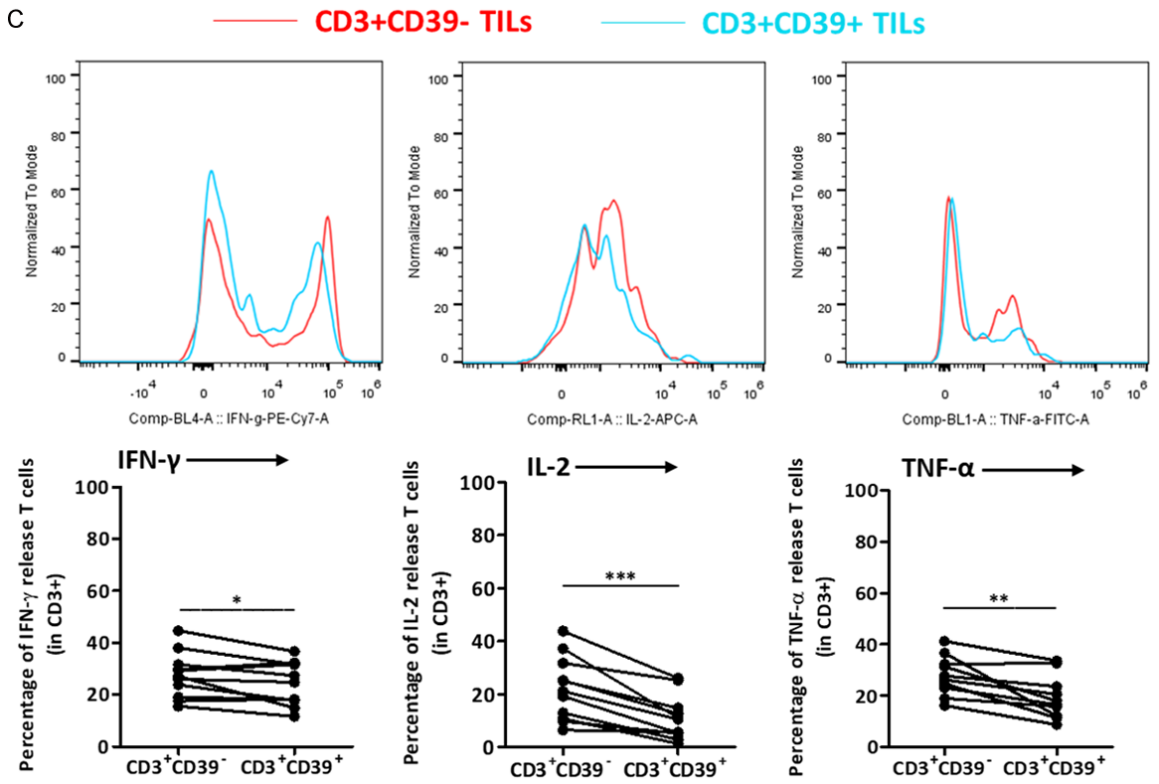


Figure 1. CD39 is highly expressed in intratumoral T cells and negatively correlated with cytokine secretion. (A) Tumor-infiltrating lymphocytes (TILs) and peripheral blood lymphocytes (PBLs) were isolated from colorectal cancer (CRC) patients (n = 11) and stained with antibodies against FDV506, CD3, CD4, CD8 and CD39. Representative flow cytometry plots and statistical analysis in TILs and PBLs. (B and C) TILs and PBLs from CRC patients (n = 8) were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 hours before blocking cellular protein transportation via Brefeldin A and Monensin. Cells were harvested and stained with antibodies against FDV506, CD3 and CD39 before fixation and permeabilization. Permeabilized cells were intracellularly stained with antibodies against IFN-γ, IL-2 and TNF-α. Cytokines-releasing T cells (PBLs shown in B; TILs shown in C) were recorded based on the expression of CD39. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

CD28 resulted in the up-regulation of CD39 on T cells (**Figure 2D** and **2E**).

CD39⁺ T cells from CRC tumor-bearing mice are more reactive to tumor antigens

To further explore the tumor reactivity of CD39⁺ T cells, a murine CRC cell line (MC38)-derived tumor engraftment model was established. We extracted T cells from the spleens and tumors of tumor-bearing mice. $CD3^+CD39^+$ T cells and $CD3^+CD39^-$ T cells were fluorescently sorted, and were then co-cultured with MC38 cells or BMDCs loaded with a pool of neo-peptides for 24 hours. The secretion of IFN-γ was employed as the indicator of specific T-cell recognition when polyclonal T cells were stimulated with corresponding antigens. As shown in **Figure 3A-D**, $CD3^+CD39^+$ splenocytes and $CD3^+CD39^+$ TILs produced more IFN-γ in response to autologous MC38 cells or neo-peptide-loaded BMDCs compared with $CD3^+CD39^-$ splenocytes and $CD3^+CD39^-$ TILs, respectively. These results indicated that tumor-reactive T cells were accumulated in the subset of CD39⁺ T cells. Moreover, CD39⁺ T cells in the spleens of tumor-bearing mice exhibited the stronger anti-tumor activity *in vitro* than $CD39^-$ T cells (**Figure 3E**). However, there was no significant difference in the anti-tumor activities between $CD39^-$ TILs and CD39⁺ TILs, probably due to the defective function of T cells in the tumors (**Figure 3F**). Indeed, TILs were exhausted and less sensitive to PMA & ionomycin stimulation compared with T cells in the spleens (**Figure 3A** and **3C**). Hence, the results further suggested that CD39 was highly expressed on tumor-reactive T cells or neoantigen-specific T cells.

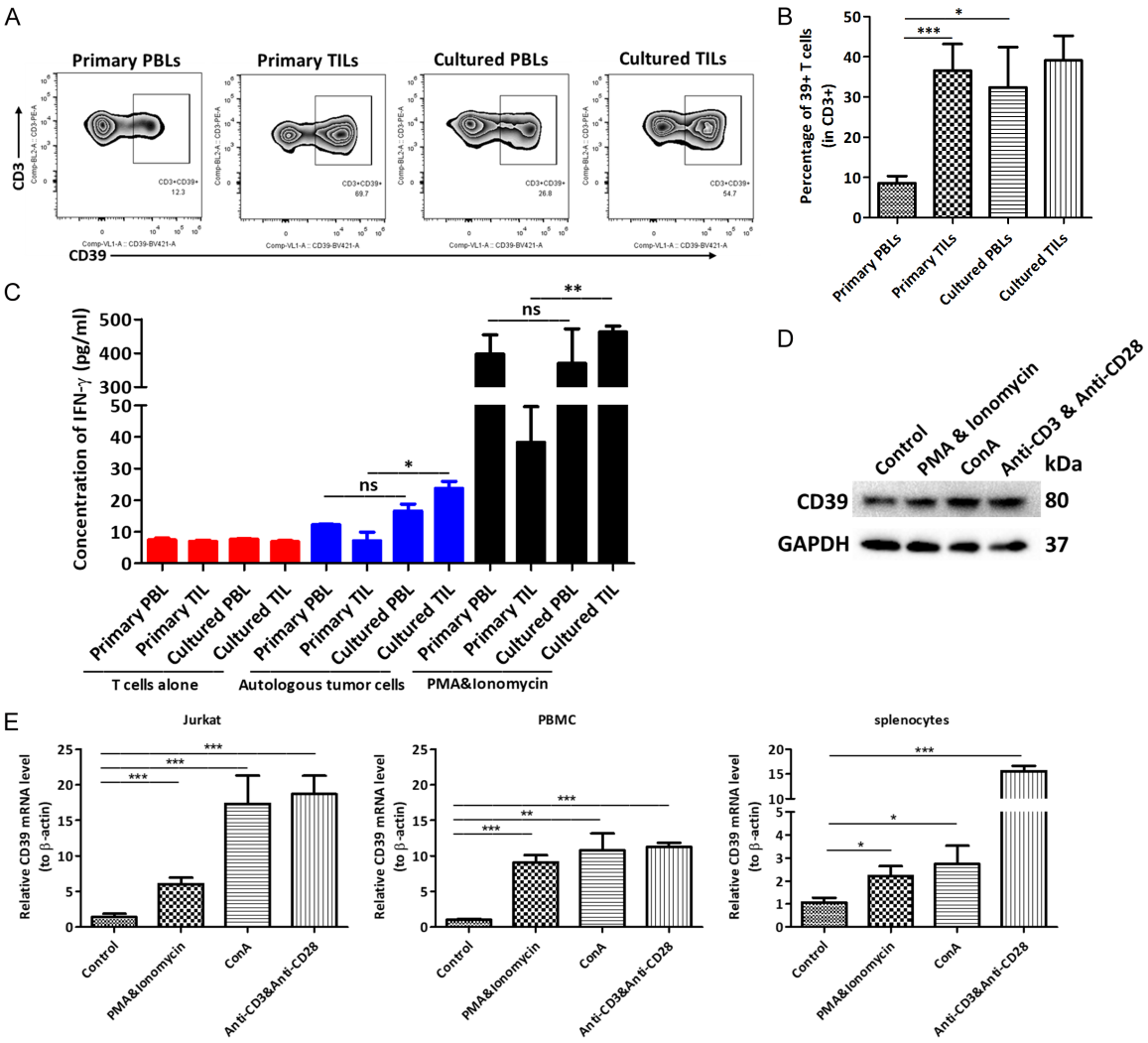


Figure 2. T cell activation induces CD39 expression. (A and B) Primary TILs and PBLs isolated from CRC patients were sorted by MACS. Sorted primary CD3⁺ T cells were aliquoted for cyto-preservation or cultivation for 10~14 days. Paired primary or cultured PBLs and TILs were thawed and stained with antibodies against FDV506, CD3 and CD39. Representative FACS plots (A) and statistical analysis (B) were shown. (C) Primary or cultured PBLs and TILs were cultured with autologous tumor cells (CD3⁻ sorted tumor single-cell suspension) at the ratio of 5:1 or PMA/Ionomycin cocktail for 24 hours. Cell culture supernatant was harvested for detecting IFN-γ secretion by ELISA. (D and E) Mouse splenocytes, Jurkat and PBMC from healthy donors were harvested and stimulated with 1 μg/ml Concanavalin A (ConA), 1 μg/ml anti-CD3 & 1 μg/ml anti-CD28 antibodies and a cocktail of 20 ng/ml PMA & 1 μg/ml ionomycin, respectively for 24 hours. Cell lysates from Jurkat were prepared for WB (D). CD39 mRNA levels were examined by RT-qPCR (E). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

CD39⁺ T cells are antigen-experienced T cells and more immunosuppressive than CD39⁻ T cells

T cells isolated from the spleens and tumors of tumor-bearing mice were collected and analyzed using multi-colour flow cytometry (**Figure 4A** and **4B**). CD44⁻CD62L⁺ T cells were characterized as Naïve T cells, CD44⁺CD62L⁻ T cells were characterized as effect tor T cells, and CD44⁺CD62L⁺ T cells were characterized as central memory T cells [24]. Approximately 40% of T cells in the spleens were found characterized as naïve T cells, whereas naïve T cells were rarely observed in the tumors (**Figure 4C**). According to the expression of CD39, naïve T cells mainly existed in the CD39⁻ T cell subset, whereas CD39⁺ T cells were either memory or

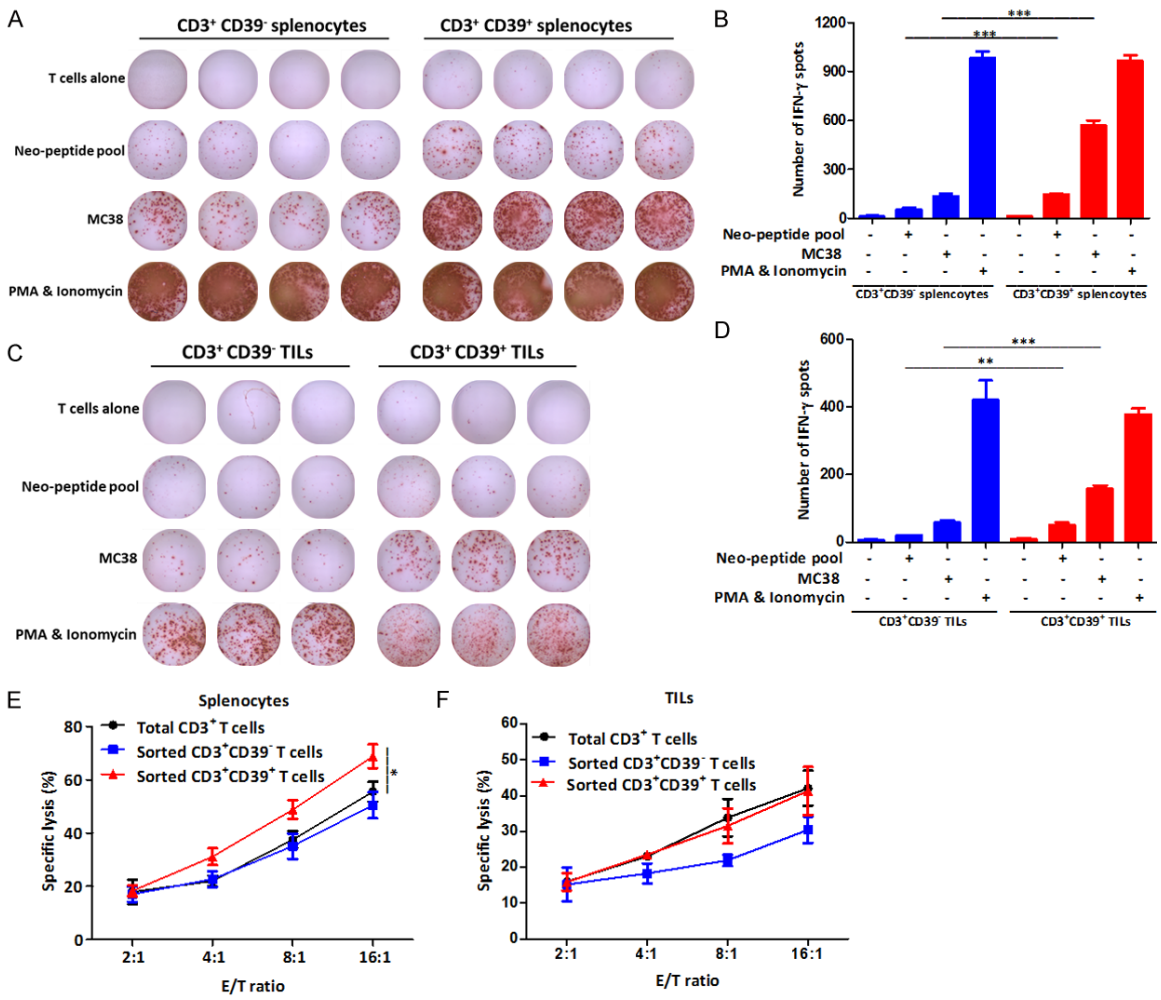
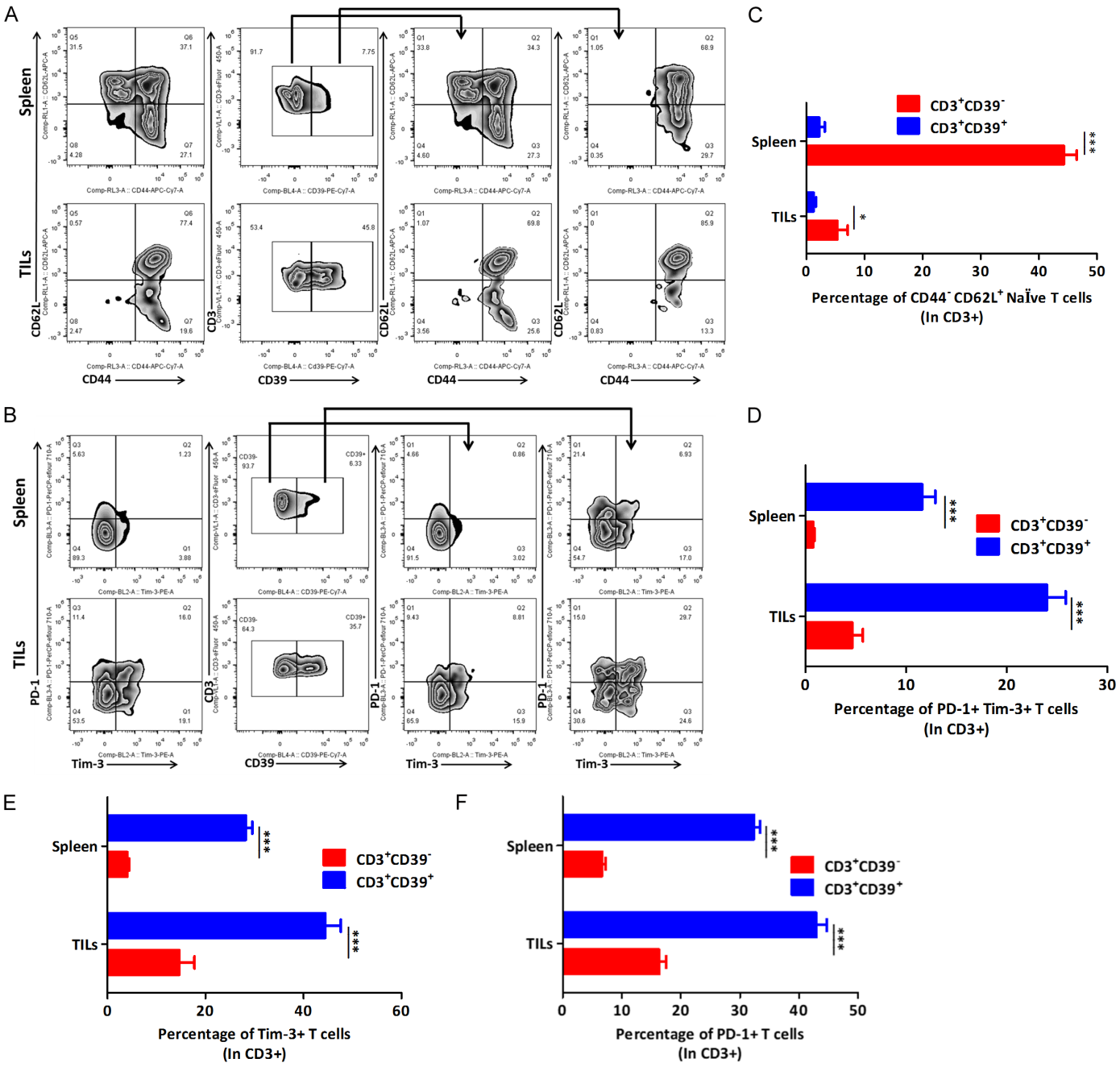


Figure 3. CD39⁺ T cells are more reactive to tumor antigens. (A-D) Age-matched female C57BL/6N mice (n = 6) were subcutaneously inoculated with 1×10^6 MC38 cells and bred until sacrifice at Day 28 after tumor engraftment. Spleens and tumors were extracted and squeezed into single cell suspension. T cells were stained with antibodies against FDV506, CD3 and CD39 before fluorescently sorting. Live CD3⁺CD39⁻ T cells and CD3⁺CD39⁺ T cells were harvested from splenocytes and TILs, respectively. Sorted splenocytes (A) and TILs (C) were co-cultured with 1 μg/ml neo-peptides-loaded bone-marrow derived dendritic cells (BMDCs), MC38 cells at the ratio of 5:1 or a cocktail of PMA and ionomycin for 24 hours. The secretion of IFN-γ was detected through ELISpot assay and the number of spots was analyzed (B and D). (E and F) MC38 in vitro killing assay was performed by co-culture of CD3-sorted splenocytes (E) or TILs (F) with Calcein AM-labelled MC38 cells for 4 hours. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

effector T cells, which indicated that CD39⁺ T cells had experienced antigen stimulation (**Figure 4A**).

The expression of negative immune regulatory molecules such as PD-1 and Tim-3 was significantly increased in TILs compared with the spleen (**Figure 4B**). Moreover, the enrichment of PD-1⁺, Tim-3⁺, and PD-1⁺Tim-3⁺ double-positive T cells was also observed in the subset of CD39⁺ T cells (**Figure 4D-F**). Of note, there were only around 5% of PD-1⁺ T cells, 3% of Tim-3⁺ T cells, and less than 1% of PD-1⁺Tim-3⁺ double-positive T cells in CD39⁻ T-cell subset, which were accounted for more than 90% of total T cells within the spleen of tumor-bearing mice (**Figure 4D-F**). In contrast, 21% of PD-1⁺ T cells, 17% of Tim-3⁺ T cells and 7% of PD-1⁺Tim-3⁺ double-positive T cells were found in CD39⁺ T-cell subset in spleen (approximately 7% of entire T cells in spleen) (**Figure 4D-F**). Similar results also appeared in T cells within TILs. Especially, PD-1 was expressed in 18.24% of CD39⁻ T cells and 44.7% of CD39⁺ T cells; Tim-3 was expressed in 24.71% of CD39⁻ T cells and 54.3% of CD39⁺ T cells; The percentage of


A
Spleen
TILs
CD62L
CD44
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B
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Tim-3
C
CD3⁺CD39⁻
CD3⁺CD39⁺
Spleen
TILs
Percentage of CD44⁻ CD62L⁺ Naïve T cells (In CD3+)
D
Percentage of PD-1+ Tim-3+ T cells (In CD3+)
E
Percentage of Tim-3+ T cells (In CD3+)
F
Percentage of PD-1+ T cells (In CD3+)

Figure 4. CD39+ T cells are antigen-experienced T cells and more immunosuppressive than CD39- T cells. (A and B) Splenocytes and TILs generated from tumor-bearing mice were stained with antibodies against FDV506, CD3, CD44, CD62L, PD-1, TIM-3 and CD39. The representative FACS results were as shown in (A and B). (C-F) Data from the FACS results were analyzed as bar chart. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

PD-1+Tim-3+ double-positive T cells was increased from 8.81% in CD39- T cells to 29.7% in CD39+ T cells (**Figure 4D-F**).

In tumor-bearing mice, the percentage of Treg cells was elevated in tumors compared with the spleens (**Figure 5A** and **5B**). Remarkably, 3.89% of Treg cells were observed in CD39- splenocytes, whereas the proportion of Treg cells increased to 13.3% in CD39+ splenocytes (**Figure 5C**). Similar results indicating that Treg cells were significantly enriched in the CD3+CD4+CD39+ T cells subset were observed in TILs (**Figure 5C**).

Therefore, CD39+ T cells from tumor-bearing mice expressed higher checkpoint molecules PD-1 and Tim-3, and containing a higher proportion of Treg cells than CD39- T cells, suggesting that CD39+ T cells may be correlated with an immunosuppressive phenotype.

CD39 expression on T cells aggravated eATP-induced IL-2 suppression via generating adenosine

It has been reported that CD39 was an extracellular ATPase, which facilitated the hydrolysis of ATP to adenosine, which indicated CD39+ T cells might be involved in regulating eATP and adenosine conversion [25]. Notably, a dramatic decrease of IL-2 production was observed when eATP-treated Jurkat T cells were activated via diverse stimulus including ConA, PMA & ionomycin as well as the antibodies against CD3 and CD28 (**Figure 6A**). Furthermore, pharmacological blockade A2AR could rescue eATP-induced IL-2 suppression, which indicated A2A-mediated signaling was involved in eATP-induced IL-2 suppression (**Figure 6B**). Moreover, the generation of adenosine could be significantly prevented by ENTPDase inhibitors POM-1 and ARL67156, resulting in the impediment of eATP-induced IL-2 reduction (**Figure 6C** and **6D**). Similar results were also seen in PBMC cells from healthy donors and mice splenocytes (Figure S1). Enhanced expression of CD39 in Jurkat T cells did not show influence on T cell receptor signaling, as there was no significant difference of CD3ζ, p38, ERK, AKT, and p65 phosphorylation between Jurkat-PCDH-Empty and Jurkat-PCDH-CD39 cells in response to OKT3 stimulation (**Figure 6E**). Taken together, the expression of CD39 induced by TCR activation may not affect TCR signaling directly but contribute to T cell dysfunction via converting pro-inflammatory eATP to immunosuppressive eADO.

Tumor-reactive T cells induced from CD39-deficiency mice were able to recognize targeted tumor cell

To address the question whether CD39 was required in T-cell-mediated neoantigen recognition and tumor eradication, wild-type mice and CD39-/- mice were vaccinated with predicted neo-peptides (nP) and irradiated MC38 tumor cells (iT) respectively (**Figure 7A**). Interestingly, both T cells from the vaccinated-wild-type mice and CD39-/- mice could recognize tumor cells *in vitro* that secreted the same amount of IFN-γ (**Figure 7B**). Next, T cells sorted from vaccinated mice were adoptively transferred into irradiated tumor-bearing mice. Consequently, adoptive transfer of these T cells resulted in tumor growth inhibition in tumor-bearing mice (**Figure 7C**). Together, these results indicated that CD39 was not involved in the reactivity to tumor antigens, and not required for establishing tumor-reactive T cells. The diagram of purposed CD39 function in the tumor microenvironment was shown as in **Figure 7D**.

Discussion

Our data confirmed the increased expression of CD39 on intratumoral T cells (TILs) compared with circulating T cells (PBLs). Transcriptional analysis of CD8+CD39+ and CD8+CD39- TIL populations revealed that CD39+ TILs were associated with T-cell exhaustion signature, which highly expressed negative modulatory genes such as PD-1, Tim-3, and CTLA4 [15]. In addition, the increased expression of CD39 was observed in viral antigen-specific T cells during chronic virus infection and associated with the dysfunctional phenotype [17]. Taken together, these results suggested that CD39+ T cells

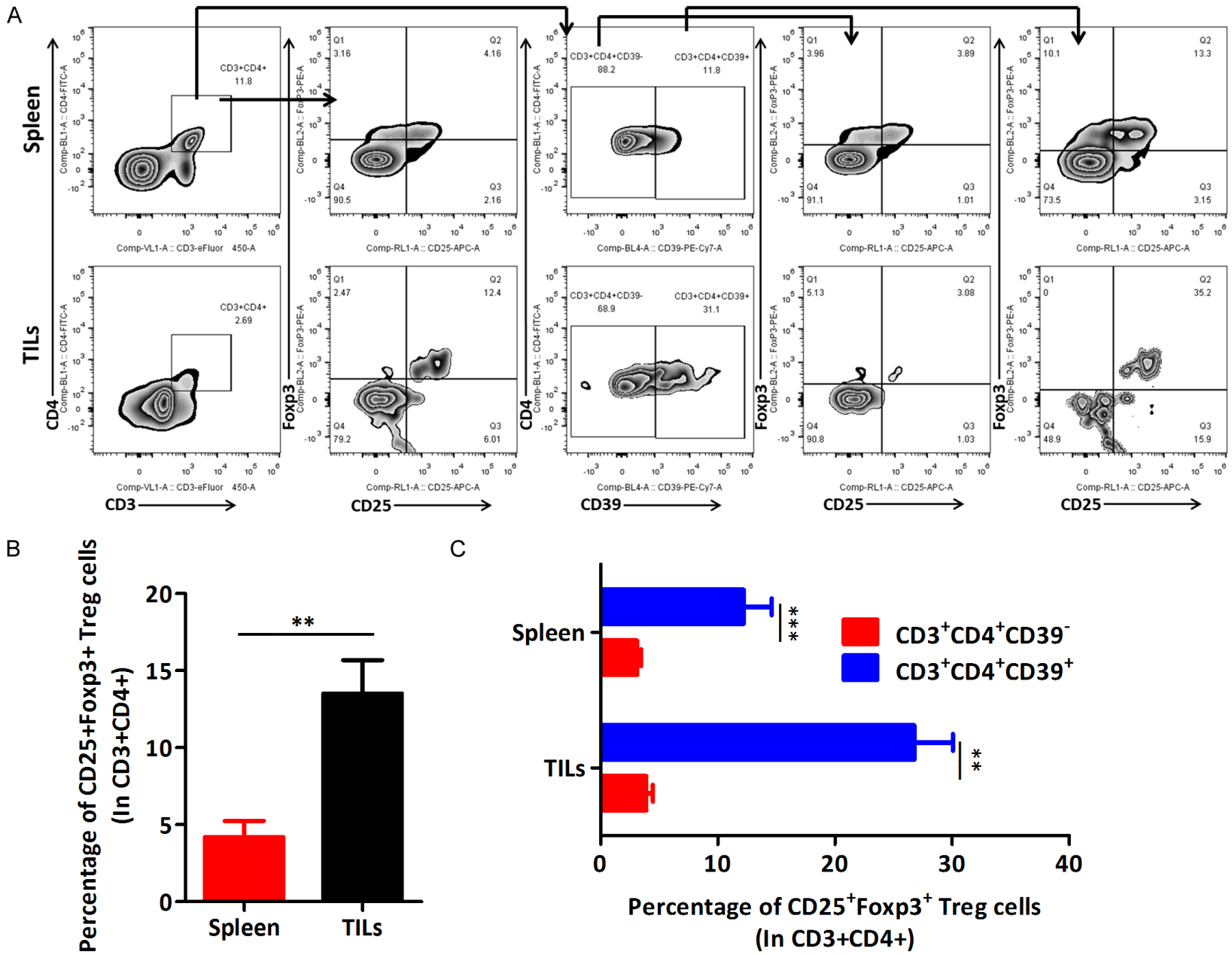
A
Spleen
TILs
CD3+CD4+
11.8
CD3+CD4+
2.69
Q1 3.16
Q2 4.16
Q4 90.5
Q3 2.16
Q1 2.47
Q2 12.4
Q4 79.2
Q3 6.01
CD3+CD4+CD39-
88.2
CD3+CD4+CD39+
11.8
CD3+CD4+CD39-
68.9
CD3+CD4+CD39+
31.1
Q1 3.96
Q2 3.89
Q4 91.1
Q3 1.01
Q1 5.13
Q2 3.08
Q4 90.8
Q3 1.03
Q1 10.1
Q2 13.3
Q4 73.5
Q3 3.15
Q1 0
Q2 35.2
Q4 48.9
Q3 15.9
CD4
Foxp3
CD3
CD25
CD39
CD25
Foxp3
CD25
B
Percentage of CD25+Foxp3+ Treg cells (In CD3+CD4+)
20
15
10
5
0
**
Spleen
TILs
C
Spleen
TILs
***
**
CD3+CD4+CD39-
CD3+CD4+CD39+
0
10
20
30
40
Percentage of CD25+Foxp3+ Treg cells (In CD3+CD4+)

Figure 5. CD4⁺CD39⁺ T cells are significantly enriched in Treg cells expressing CD25 and Foxp3. A. Splenocytes and TILs harvested from tumor-bearing mice were stained with antibodies against FDV506, CD3, CD4, CD39, CD25 and Foxp3. The representative FACS results were shown. B and C. Data from the FACS results were analyzed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

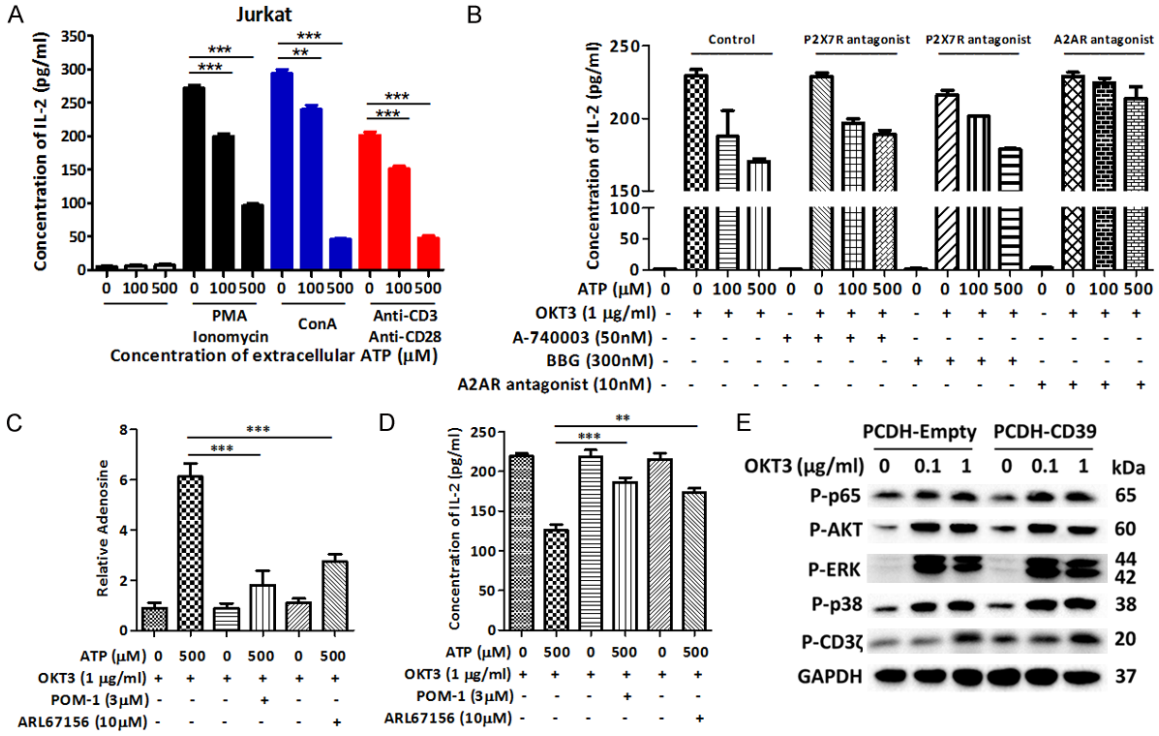

Figure 6. CD39 expression aggravates eATP-induced IL-2 suppression via generating adenosine. (A) Jurkat T cells were treated with indicated dose eATP with or without indicated activation cocktails for 24 hours. (B) Jurkat cells were pre-treated with P2X7 receptor inhibitors or A2A receptor for 1 hour before challenging with eATP and OKT3 for 24 hours. (C and D) Jurkat cells were pre-treated with CD39 inhibitors POM-1 and ARL67156 for 1 hour before adding eATP and OKT3. Cell-free supernatant was harvested for the detection of adenosine (C) and IL-2 (D). (E) Jurkat-PCDH-empty and Jurkat-PCDH-CD39 cells were treated with indicated concentration of OKT3 for 30 minutes. Cell lysates were prepared for WB. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

were likely exhausted and associated with impaired cytokine production capacity.

T cells harvested from tumor-bearing mice were applied to explore the relationship between CD39 expression and T cell functionality. By co-culturing with tumor cells or neo-peptide pool, the majority of tumor-reactive T cells were found enriched in CD39⁺ subset, which produced higher level of IFN-γ in response to rechallenge of antigens. In fact, it has been demonstrated that the adoptive transfer of T cells with a less differentiated phenotype but a higher proliferative capacity resulted in a better prognosis in patients [26]. These results indicated that CD39⁺ T cells from tumor-bearing mice were reactive to tumor cells, which could recognize and kill autologous tumor cells in vitro.

At the mechanistic level, the analysis of phenotypic markers of T cells isolated from tumor-bearing mice suggested that CD39⁺ T cells were mainly antigen-experienced T cells, especially central memory T cells. It has been demonstrated that central memory T cells exhibited higher *in vivo* proliferative and self-renewal capacity [27]. Our results also indicated that approximately 50% of CD39⁺ T cells within the spleen of tumor-bearing mice expressed negative regulators such as PD-1, Tim-3, or both while only less than 10% of CD39⁻ T cells expressed PD-1, Tim-3, or both, which indicated that CD39⁺ T cells displayed the more dys-

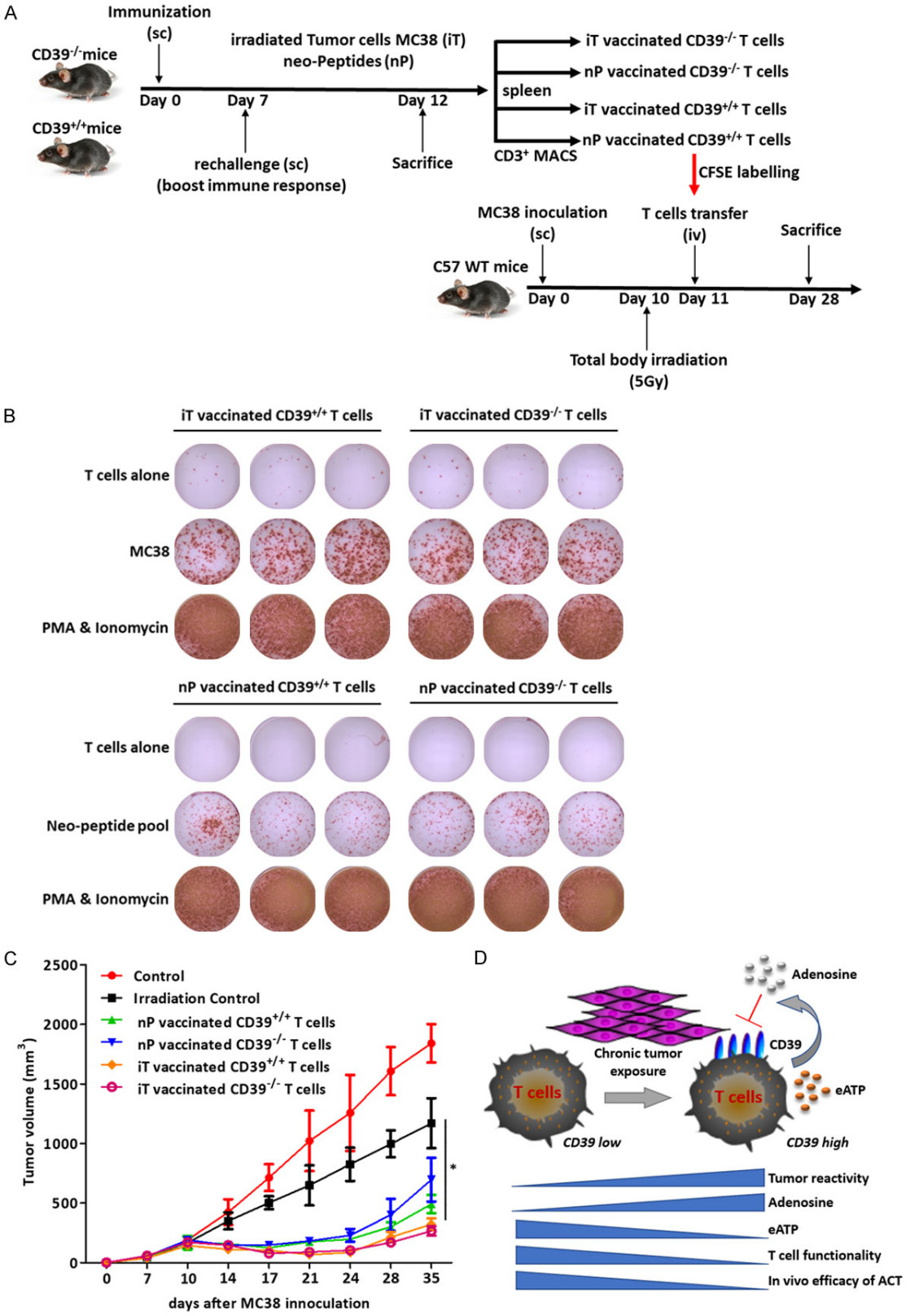

Figure 7. CD39 deficiency mice were able to elicit neoantigen-specific or tumor-reactive T cells that targeted tumor cells. A. Schematic diagram of inducing CD39$^{-/-}$ or CD39$^{+/+}$ T cells that targeting to MC38 tumor through vaccination of CD39 deficiency or wild type mice. nP, neo-peptides; iT, irradiated MC38 tumor cells. B. Specific recognition of MC38 tumor cells was indicated by the secretion of IFN-γ, which was measured by ELISpot assay. C. Tumor-reactive T cells generated from CD39$^{-/-}$ and CD39$^{+/+}$ mice were adoptively transferred into tumor-bearing mice one day after total body irradiation. Tumor volumes were monitored. D. Diagram of purposed CD39 function in the tumor micro-environment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

functional phenotype than CD39- T cells. Moreover, our data showed that CD39 expression was significantly increased in Treg cells. It has been reported that the expression of CD39 was identified in a Treg-enriched subset of autoimmune diseases such as rheumatism [28].

As the main metabolite of ATP hydrolysis, adenosine could increase the intracellular cAMP and inhibit immune response [29]. Of note, the immunosuppressive role of CD39 and CD73 has been reported in myeloid cells, cancer cells, and even cancer exosomes, which generated extracellular adenosine and suppressed T-cell functions [30, 31]. In contrast, low doses of eATP activated the P2X7 receptor, which facilitated the intracellular Ca^{2+}, thus promoting T cells activation [32]. Therefore, the mechanism of high doses of eATP-induced T-cell suppression was attributed to the generation of immunosuppressive adenosine rather than eATP-P2X7R mediated signaling. Indeed, blocking CD39 or adenosine receptor A2A could significantly attenuate the eATP-induced suppression in T cells, whereas blocking P2X7 receptors cannot. eADO was mainly generated by the dominant CD39- and CD73-expressing tumor cells or infiltrated myeloid cells within the TME. Consumption of eATP and generation of adenosine are major immunosuppressive roles of $CD39^+CD73^+$ Treg cells. Together, CD39- and CD73-expressing cells within the TME exacerbated the conversion of eATP to eADO and worsened the TME, resulting in the stresses within the TME and release of immunosuppressive factors, which may further up-regulate the expression of CD39. Therefore, the positive feedback of CD39 resulted in an immunosuppressive TME, which may be a strategy of immune escape. Therefore, targeting CD39, purinergic, and adenosinergic signaling pathways may be a promising anti-tumor treatment.

Conclusion

In summary, our results emphasize that $CD39^+$ tumor infiltrating T cells from colorectal cancers are reactive to tumor antigens, but exhibit dysfunctional phenotype.

Acknowledgements

We are grateful to GenScript (Nanjing, China) for synthesizing the peptides for us. This study was supported by the National Natural Sciences Foundation of China (NSF81973358).

Written informed consent was signed from all patients.

Disclosure of conflict of interest

None.

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Table S1. Case information for colorectal cancer patients

ID	Gender	Age	Diagnosis	Biopsy morphology
#1	F	33	Rectal carcinoma	Infiltrative
#2	F	46	Sigmoid colon carcinoma	Massive-lump
#3	F	51	Colon carcinoma	Infiltrative
#4	M	65	Colon carcinoma	Infiltrative
#5	F	83	Sigmoid colon carcinoma	Ulcerative
#6	M	62	Rectal carcinoma	Massive-lump
#7	F	57	Rectal carcinoma	Massive-lump
#8	F	68	Rectal carcinoma	Massive-lump
#9	M	60	Rectal carcinoma	Infiltrative
#10	F	63	Colon carcinoma	Massive-lump
#11	F	65	Colon carcinoma	Ulcerative
#12	M	64	Ileocecal carcinoma	Massive-lump
#13	M	62	Colon carcinoma	Ulcerative
#14	F	68	Rectal carcinoma	Infiltrative
#15	M	72	Rectal carcinoma	Massive-lump
#16	F	57	Ascending colon carcinoma	Infiltrative
#17	M	41	Sigmoid colon carcinoma	Infiltrative
#18	F	63	Rectal carcinoma	Massive-lump
#19	F	89	Rectal carcinoma	Massive-lump

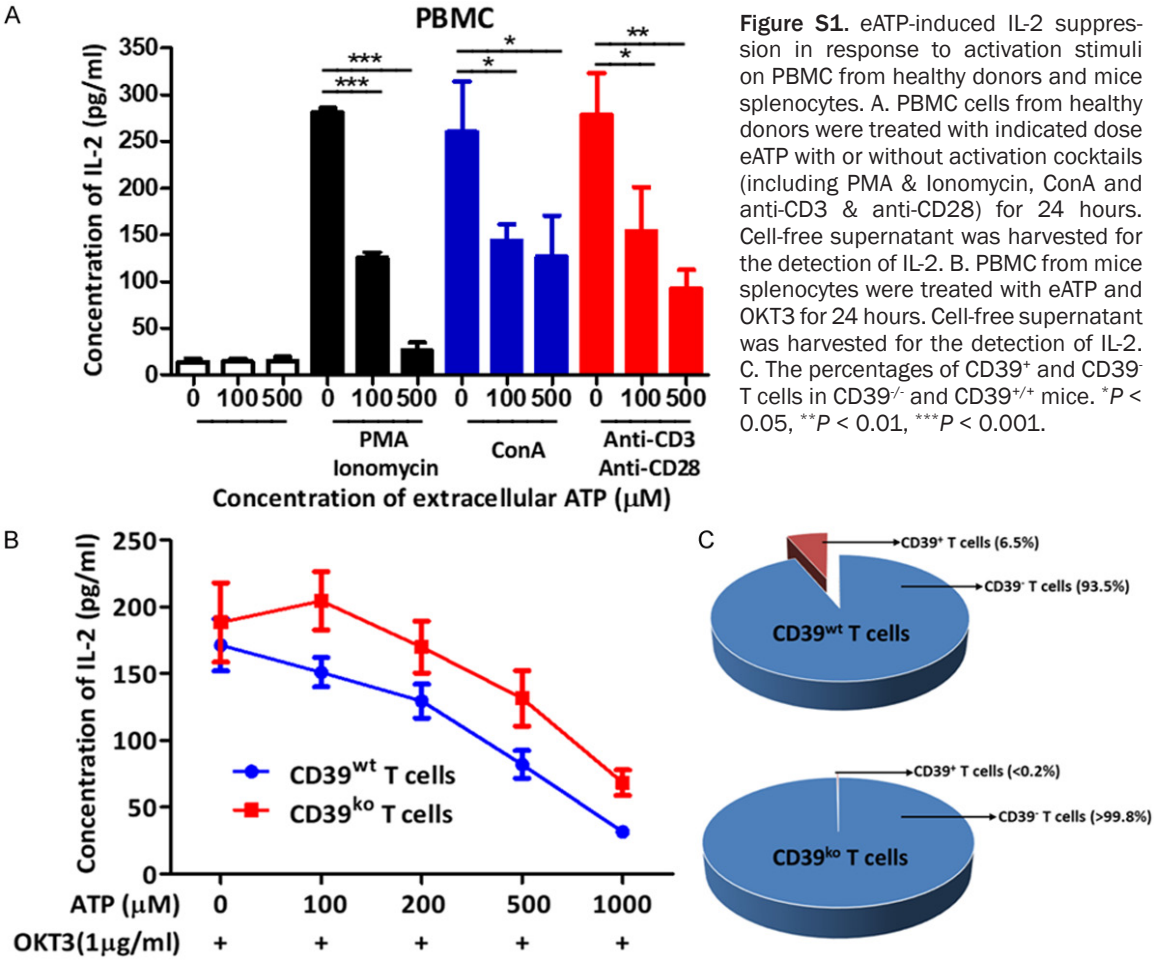

Figure S1. eATP-induced IL-2 suppression in response to activation stimuli on PBMC from healthy donors and mice splenocytes. A. PBMC cells from healthy donors were treated with indicated dose eATP with or without activation cocktails (including PMA & Ionomycin, ConA and anti-CD3 & anti-CD28) for 24 hours. Cell-free supernatant was harvested for the detection of IL-2. B. PBMC from mice splenocytes were treated with eATP and OKT3 for 24 hours. Cell-free supernatant was harvested for the detection of IL-2. C. The percentages of $CD39^+$ and $CD39^-$ T cells in $CD39^{-/-}$ and $CD39^{+/+}$ mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.