

## CD39 Expression Defines Cell Exhaustion in Tumor-Infiltrating CD8<sup>+</sup> T Cells

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### Abstract

The ability of CD8<sup>+</sup> T lymphocytes to eliminate tumors is limited by their ability to engender an immunosuppressive microenvironment. Here we describe a subset of tumor-infiltrating CD8<sup>+</sup> T cells marked by high expression of the immunosuppressive ATP ectonucleotidase CD39. The frequency of CD39<sup>high</sup>CD8<sup>+</sup> T cells increased with tumor growth but was absent in lymphoid organs. Tumor-infiltrating CD8<sup>+</sup> T cells with high CD39 expression exhibited features of exhaustion, such as reduced production of TNF and IL2 and expression of coinhibitory receptors. Exhausted CD39<sup>+</sup>CD8<sup>+</sup> T cells from mice hydrolyzed extracellular ATP, confirming that CD39 is enzymatically active. Furthermore, exhausted CD39<sup>+</sup>CD8<sup>+</sup> T cells inhibited IFN $\gamma$  production by responder CD8<sup>+</sup> T cells. In specimens from breast cancer and melanoma patients, CD39<sup>+</sup>CD8<sup>+</sup> T cells were present within tumors and invaded or metastatic lymph nodes, but were barely detectable within non-

invaded lymph nodes and absent in peripheral blood. These cells exhibited an exhausted phenotype with impaired production of IFN $\gamma$ , TNF, IL2, and high expression of coinhibitory receptors. Although T-cell receptor engagement was sufficient to induce CD39 on human CD8<sup>+</sup> T cells, exposure to IL6 and IL27 promoted CD39 expression on stimulated CD8<sup>+</sup> T cells from human or murine sources. Our findings show how the tumor microenvironment drives the acquisition of CD39 as an immune regulatory molecule on CD8<sup>+</sup> T cells, with implications for defining a biomarker of T-cell dysfunction and a target for immunotherapeutic intervention.

**Significance:** The tumor microenvironment elicits a subset of functionally exhausted CD8<sup>+</sup> T cells by creating conditions that induce cell surface expression of CD39, an immunosuppressive molecule that can be therapeutically targeted to restore effector T-cell function. *Cancer Res*; 78(1); 115–28. ©2017 AACR.

### Introduction

Tumors employ diverse strategies to create a tolerogenic microenvironment, such as production of immunosuppressive cytokines, competition for nutrients and expression of inhibitory ligands, among others. These mechanisms may act in concert to dampen immune cells' activity, particularly CD8<sup>+</sup> T cells, which are essential for the elimination of tumors (1–3).

Regulatory immune cell populations, including Foxp3<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Tregs), myeloid-derived suppressor cells, and tolerogenic dendritic cells (DC) contribute to the establishment of an immunosuppressive tumor microenvironment (4). Among other features, these cells express the immunomodulatory ecto-5'-nucleotidases CD39 and CD73 (5, 6). CD39 is a surface-expressed enzyme that hydrolyzes extracellular ATP (eATP), a proinflammatory metabolite present at high concentrations in the tumor interstice. eATP binds to P2X receptors on T cells inducing cytokine production and proliferation, while its hydrolysis compromises of effector T cells' functionality and favors Tregs expansion (5, 7). CD39 enzymatic activity results in the generation of AMP, which can be further hydrolyzed into adenosine by CD73. Adenosine is a potent immunoregulator that binds to A2A receptors on lymphocytes preventing effector responses (8, 9).

In the tumor microenvironment, inadequate T-cell activation results in dysfunctional states of CD8<sup>+</sup> T cells including anergy, exhaustion, and senescence (10, 11). CD8<sup>+</sup> T cells with features of exhaustion have been observed in a wide variety of animal models and in humans with cancer or chronic viral infections (12–14). Two cardinal features of exhausted CD8<sup>+</sup> T cells are the gradual loss of effector cytokine production and sustained expression of multiple inhibitory receptors (iR) such as PD-1, LAG-3, and Tim-3 (10). Exhausted CD8<sup>+</sup> T cells also show cell-cycle arrest and display a particular pattern of key transcription factors (TF), including T-bet, Eomesodermin (Eomes), Blimp-1, and others (15, 16). Importantly, exhaustion of CD8<sup>+</sup> T cells contributes to poor immune control during chronic infections and cancer.

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**doi:** 10.1158/0008-5472.CAN-16-2684

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Accordingly, current immunotherapies involve T cells' reinvigoration through the blockade of iRs (10, 17).

It has been reported that human tumor-infiltrating CD8<sup>+</sup> T cells can express CD39 (18–20); nevertheless, the specific phenotype and functionality of these cells have not been addressed. Moreover, no studies have delved into the expression of the ectonucleotidases CD39 and CD73 in CD8<sup>+</sup> T cells in cancer models to help understand their biological role.

By using mouse cancer models, we demonstrate that tumor-infiltrating CD8<sup>+</sup> T cells exhibit high expression of CD39. These CD39<sup>high</sup> cells display an exhausted phenotype and high capacity to hydrolyze eATP. Interestingly, we detected CD39-expressing CD8<sup>+</sup> T cells with features of exhaustion within tumors and invaded/metastatic lymph nodes of patients with breast cancer or melanoma. We postulate that the presence of tumor-infiltrating exhausted CD39<sup>+</sup>CD8<sup>+</sup> T cells could be detrimental to tumor control in two different ways; they show poor effector capabilities and, also, may gain regulatory functions. Thus, in the context of the new immunotherapeutic strategies, our results suggest that CD39 should be considered as an immune checkpoint to be targeted to restore the immune response against tumors.

## Materials and Methods

### Mice and human samples

C57BL/6 wild-type, CD73KO (CD73KO-B6.129S1-Nt5et<sup>m1Lj1</sup>), C57BL/6 CD45.1 (B6.SJL-Ptprca Pepcb/Boy), and BALB/c wild-type mice (6–10 weeks) were housed at the animal facility of the CIBICI-CONICET. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the CIBICI-CONICET. CD73KO and CD45.1 mice were purchased from the Jackson Laboratory.

Human tumors and/or tumor-draining lymph nodes (dLN) were collected from 33 untreated breast cancer and 4 melanoma patients undergoing standard-of-care surgery at Institut Curie Hospital (France) and Hospital Rawson (Argentina). All protocols were approved by the Institutional Review Boards and all patients signed an informed consent form. dLNs were classified into invaded/metastatic or noninvaded according to the presence of tumor cells by histology and confirmed by Epcam/CD45 staining by flow cytometry. Blood samples were collected from patients from Hospital Rawson. All studies in patients were conducted in accordance to the ethical guidelines stated in the Declaration of Helsinki.

Tumor and dLNs were disaggregated mechanically and enzymatically with liberase and DNase I (Roche). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque gradients (GE Healthcare).

### Cell lines

B16F10-OVA melanoma cell line was kindly provided by Dr. K. Rock (University of Massachusetts Medical School, Boston, MA), and MCA-OVA (MCA101 fibrosarcoma cell line) was kindly provided by Dr. Clothilde Théry (Institut Curie, PSL Research University INSERM U932, Paris, France). B16F10-OVA and B16F10 cell lines were maintained in DMEM (Gibco), 4T1 and MCA-OVA in RPMI1640. 4T1 and B16F10 cell lines were purchased from ATCC. Media were supplemented with 10% FBS (Gibco), 1 mmol/L L-glutamine (Gibco), 25 mmol/L HEPES (Cellgro) and 40 µg/mL gentamicin sulfate (Richtel).

Hygromycin or geneticin were added to media of MCA-OVA or B16F10-OVA, respectively.

### *In vivo* tumor models

Male C57BL/6 mice were inoculated subcutaneously with  $1 \times 10^6$  B16F10-OVA or B16F10 cells or  $0.5 \times 10^6$  MCA-OVA cells. Female BALB/c mice were inoculated subcutaneously in the mammary gland with  $2 \times 10^4$  4T1 cells. B16F10-OVA and MCA-OVA tumors were excised in the majority of experiments at day 17 postinjection and 4T1 tumors at day 28 postinjection. Tumors were disaggregated mechanically and enzymatically with 2 mg/mL collagenase IV and 50 U/mL DNase I (Roche). In some cases, tumor-infiltrating mononuclear cells were enriched by centrifugation over Ficoll-Hypaque gradients (GE Healthcare).

### Flow cytometry

Single-cell suspensions were stained with mAbs against mouse and human antigens. For fluorochromes and clones, refer Supplementary Table S1. Dead cells were excluded using Fixable Viability Stain 700 (BD Horizon).

For intracellular stainings, cells were fixed/permeabilized with Foxp3 Staining Buffer Set (eBioscience) or Cytofix/Cytoperm and PermWash Buffer (BD Biosciences) according to the manufacturer's instructions.

For *ex vivo* intracellular cytokines' detection, cells were stimulated with 50 ng/mL PMA (Sigma), 1 µg/mL ionomycin (Sigma), Brefeldin A and Monensin (eBioscience) for 5 hours at 37°C. OVA-specific CD8<sup>+</sup> T cells were detected with a PE-conjugated OVA<sup>257-264</sup> H-2Kb dextramer according to the manufacturer's instructions. A PE-conjugated SIY H-2Kb dextramer (Immudex) was used as control for specific staining. Antigen-specific response was addressed stimulating with 5 µmol/L OVA<sup>257-264</sup> peptide (SIINFELK, Invivogen), or 5 µmol/L SIY peptide (SIYRYYGL, Immudex) as control, in the presence of Brefeldin A and Monensin during 5 hours at 37°C.

Samples were acquired in BD FACSCanto II and BD LSR Fortessa flow cytometers (BD Biosciences) and data analyzed with FlowJO software (MFI, geometric mean fluorescence intensity).

### CD8<sup>+</sup> T-cell purification, coculture, and *in vitro* stimulation

Tumor-infiltrating CD8<sup>+</sup> T cells were purified from B16F10-OVA tumor cell suspensions using EasySep Mouse CD8 Positive Selection Kit II (StemCell). Cells were then sorted into PD-1<sup>-</sup>Tim-3<sup>-</sup>, PD-1<sup>+</sup>Tim-3<sup>-</sup> and PD-1<sup>+</sup>Tim-3<sup>+</sup> subsets using a FACS Aria IIb Cell Sorter (BD Biosciences). *In vitro* stimulation was performed for 72 hours with plate-bound anti-CD3 (1 µg/mL, eBioscience), anti-CD28 (0.25 µg/mL, BD Biosciences), and sometimes with 200 U/mL of mouse rIL2 (eBioscience). Cocultures:  $5 \times 10^4$  sorted tumor-infiltrating CD8<sup>+</sup> T cells were incubated in a 1:1 ratio with responder CD8<sup>+</sup> T cells obtained from spleens from CD45.1 or CD73KO mice and stained with CFSE (Invitrogen). Cells were stimulated *in vitro* in the presence or absence of the ecto-ATPase inhibitor ARL67156 (Tocris). For CD39 induction, CD8<sup>+</sup> T cells magnetically purified from mice dLNs nodes were stimulated *in vitro* in the presence of mouse recombinant cytokines: IL6 (20 ng/mL; eBioscience), IL27 (10 ng/mL; eBioscience), or maintained the last 24 hours in a hypoxic atmosphere (1.5% O<sub>2</sub>). Human PBMCs were stimulated with T Cell Activation-Expansion Kit (Miltenyi Biotec) according to the manufacturer's indications. Human CD8<sup>+</sup> T cells were purified using the CD8 Microbeads kit (Miltenyi

Biotec). Then  $2 \times 10^5$  cells were stimulated for 72 hours in the presence or absence of human recombinant IL6 (20 ng/mL; ImmunoTools) and/or IL27 (10 ng/mL; eBioscience).

#### ATP hydrolysis assay

For CD39 activity evaluation,  $5 \times 10^4$  sorted cells were cultured for 10 minutes in phenol red-free RPMI1640 with 20  $\mu$ mol/L of exogenous ATP (Sigma). Cell-free medium with ATP alone was used as control. For ATP hydrolysis inhibition, cells were incubated for 16 hours in presence or absence of 250  $\mu$ mol/L ARL67156 and ATP was added in the last 10 minutes. Cell-free medium was then analyzed for remaining eATP with ENLITEN rLuciferase/Luciferin reagent (Promega). Bioluminescent activity was measured with a Synergy HTX Multi-Mode Reader (BioTek). The percentage of ATP hydrolysis was calculated as described previously (18). Spleen CD8<sup>+</sup> T cells were magnetically purified from tumor-free mice.

#### Immunohistochemistry

Tumors were fixed with 4% paraformaldehyde and embedded in OCT. Tumor sections were stained (fluorochromes and mAbs clones in Supplementary Table S1) and analyzed in a Leica DMI8 fluorescence microscope (Leica). DAPI was used to stain cell nuclei (Life Technologies). Hypoxia was detected using Hypoxyprobe-1 kit (Natural Pharmacia) according to the manufacturer's indications.

#### Statistical analysis

GraphPad Prism software was used for statistical analysis. In experiments with mice, unpaired Student *t* test was used to compare two groups. For human samples, paired Student *t* test was used. One-way ANOVA with Tukey post test was used for multiple comparisons. *P* values <0.05 were considered statistically significant.

## Results

### CD39 is highly expressed by CD8<sup>+</sup> tumor-infiltrating T lymphocytes

To explore the potential contribution of CD8<sup>+</sup> T cells on the purinergic pathways in tumor microenvironment, we examined the expression of the ecto-nucleotidases CD39 and CD73 on CD8<sup>+</sup> T cells from tumors, spleens, and tumor-draining lymph nodes (dLN) from mice bearing the solid tumor B16F10-OVA. At day 17 postinjection, we observed that among CD8<sup>+</sup> tumor-infiltrating T lymphocytes (TIL), cells expressing CD39 comprised the major population, while cells expressing CD73 or coexpressing both enzymes represented smaller subsets. In contrast to tumor, in dLNs and spleens, CD39<sup>+</sup>CD73<sup>-</sup>CD8<sup>+</sup> T cells were nearly absent, whereas CD39<sup>-</sup>CD73<sup>+</sup> cells were the largest population of CD8<sup>+</sup> T cells (Fig. 1A). Comparable frequencies of these cell populations were observed in lymph nodes and spleen from tumor-free mice (Supplementary Fig. S1A). These results indicate that CD39 expression on CD8<sup>+</sup> T cells is associated with the tumor microenvironment.

Similar to the B16F10-OVA model, CD8<sup>+</sup> T cells from mice bearing 4T1 and MCA-OVA tumors also showed a high frequency of CD39<sup>+</sup> cells (Supplementary Fig. S1B), while in lymphoid organs, CD39<sup>+</sup>CD8<sup>+</sup> T cells were present at low frequencies. In these models, a large proportion of CD39<sup>+</sup>CD8<sup>+</sup> TILs also coexpressed CD73. These results allow us to conclude that CD39

expression on CD8<sup>+</sup> TILs is a common feature across tumors of different histologic origin, while CD73 expression is variable among different tumors and mice strains.

Considering that CD39 is expressed by Tregs as part of their immunosuppressive arsenal, we compared CD39 expression on CD8<sup>+</sup> T cells, conventional Foxp3<sup>-</sup>CD4<sup>+</sup> T cells (Tconv), and Tregs from B16F10-OVA tumor-bearing mice. Surprisingly, the levels of CD39 on CD8<sup>+</sup> TILs were comparable with those observed on tumor-infiltrating Tregs, while in spleen and dLNs, Tregs had the highest CD39 expression (Fig. 1B). According to the level of CD39 expression, CD39<sup>+</sup>CD8<sup>+</sup> TILs could be further dissected into two distinct subsets, one with intermediate expression (CD39<sup>int</sup>) and the other with high expression (CD39<sup>high</sup>; Fig. 1C and D). Interestingly, around 50% of CD39-expressing CD8<sup>+</sup> T cells in tumors were CD39<sup>high</sup>, while in dLNs and spleens this population was absent. Consistent with our observations in B16F10-OVA tumors, CD39<sup>high</sup>CD8<sup>+</sup> T cells were also exclusively present in 4T1 and MCA-OVA tumors, but not in lymphoid organs (Supplementary Fig. S1C). Interestingly, CD8<sup>+</sup> TILs from mice bearing B16F10-OVA tumors showed higher frequency of CD39<sup>high</sup> cells than counterparts from mice with parental B16F10 tumors, suggesting that higher immunogenicity favors CD39 expression (Supplementary Fig. S2A).

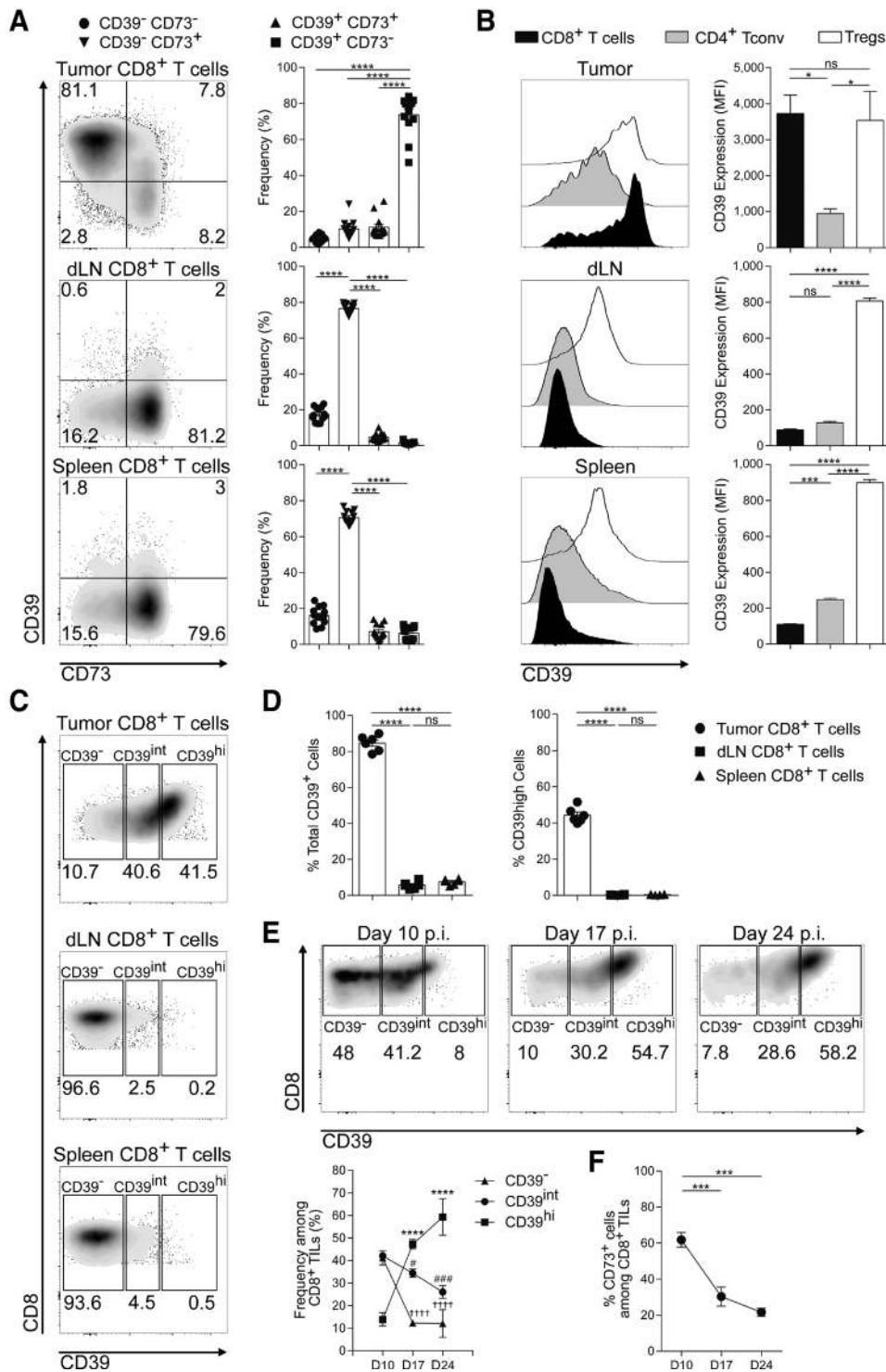
A kinetic study in B16F10-OVA tumors revealed that the frequency of CD39<sup>high</sup> cells within CD8<sup>+</sup> TILs increased by days 17 and 24 postinjection, compared with day 10 postinjection, while CD39<sup>-</sup> and CD39<sup>int</sup> CD8<sup>+</sup> TILs decreased (Fig. 1E). Notably, the frequency of CD73<sup>+</sup>CD8<sup>+</sup> T cells decreased with tumor progression (Fig. 1F). These results reveal that the upregulation of CD39 in CD8<sup>+</sup> TILs is associated with tumor burden.

Immunohistochemistry on tumor sections revealed infiltration of CD39-expressing CD45<sup>+</sup> cells and particularly CD39<sup>+</sup>CD8<sup>+</sup> T cells in tumors from B16F10-OVA tumor-bearing mice (Supplementary Fig. S2B). The analysis performed by flow cytometry showed that CD8<sup>+</sup> T cells and NK cells were the most abundant populations within CD45<sup>+</sup> leukocytes (Supplementary Fig. S2C). Moreover, other leukocyte subsets besides CD8<sup>+</sup> T cells and NK cells, such as DCs and granulocytic myeloid-derived suppressor cells (G-MDSC), exhibited important frequencies of CD39<sup>+</sup> cells. Tregs, G-MDSCs and DCs showed the highest frequency of CD39<sup>+</sup> cells coexpressing CD73 (Supplementary Fig. S2C and S2D). In addition, the frequency of CD39<sup>high</sup> cells within Tregs and G-MDSCs was maintained during tumor progression, while CD39<sup>high</sup> expressing DCs, NK cells, and monocytic-MDSCs (M-MDSC) cells increased by day 17 postinjection (Supplementary Fig. S2E).

### CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibit an effector memory phenotype and impaired IL2 and TNF production

We further examined the activation/differentiation phenotype of CD8<sup>+</sup> TILs from B16F10-OVA tumor-bearing mice. Most of CD39<sup>high</sup> and CD39<sup>int</sup> CD8<sup>+</sup> T cells exhibited an effector memory phenotype (CD62L<sup>-</sup>CD44<sup>+</sup>). However, CD39<sup>-</sup>CD8<sup>+</sup> TILs seemed to be more heterogeneous comprising also naive and central memory cells (Fig. 2A).

We next evaluated cytokine production and found that CD39<sup>+</sup> as well as CD39<sup>-</sup>CD8<sup>+</sup> TILs were able to produce IFN $\gamma$  when stimulated *ex vivo* with PMA/ionomycin. Despite the fact that CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibited the highest percentage of IFN $\gamma$ <sup>+</sup> cells, they showed a profound impairment in TNF and IL2

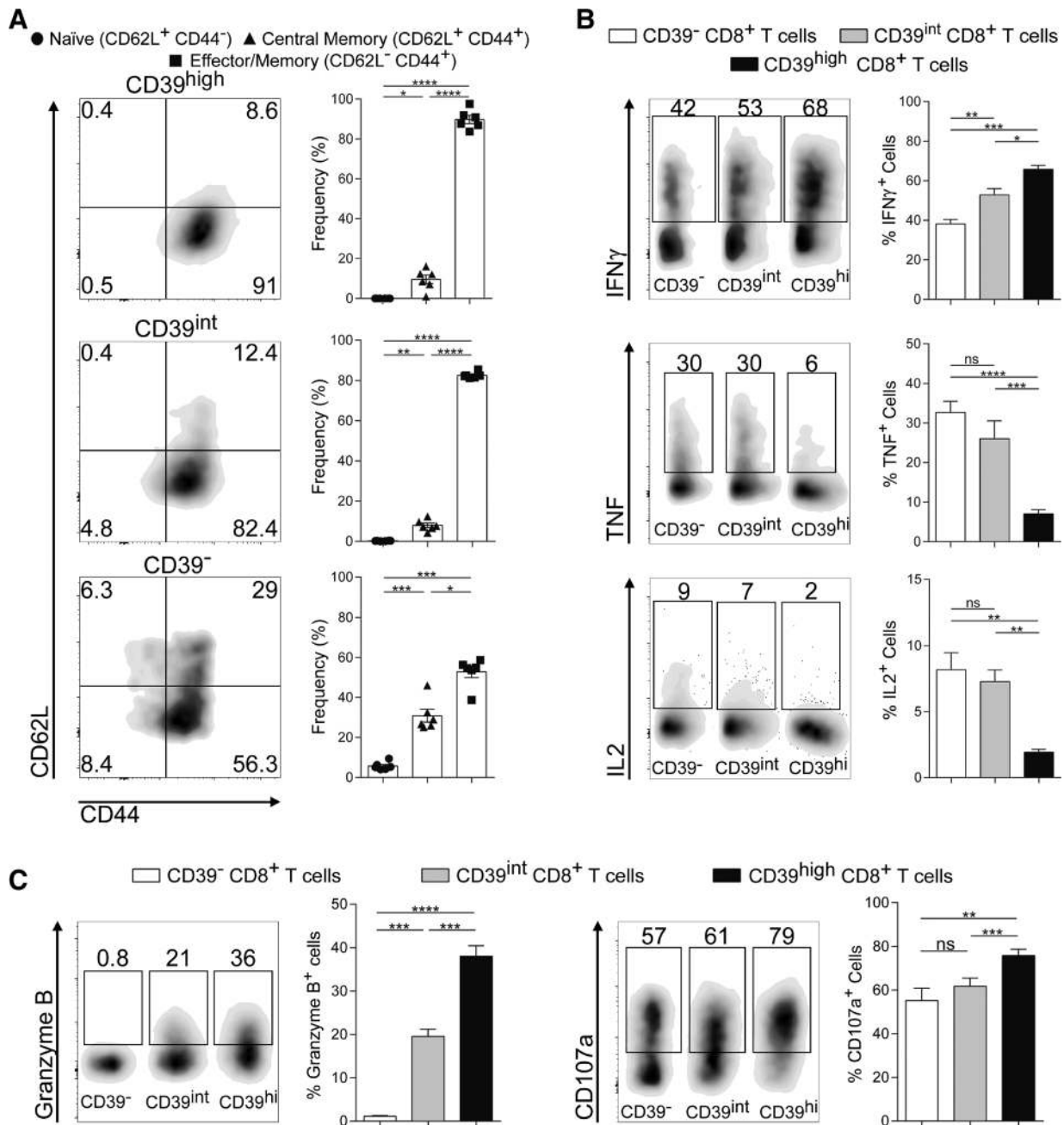


**Figure 1.** B16F10-OVA tumor-infiltrating CD8<sup>+</sup> T cells exhibit high expression of CD39. **A–D**, Cells obtained at day 17 postinjection (D17) from tumors, dLNs, and spleens. **A**, Representative density plots and graphs show frequency of CD39<sup>+</sup> and CD73<sup>+</sup> cells in CD3<sup>+</sup>CD8<sup>+</sup> T cells. **B**, Histograms and graphs show CD39 expression on Tregs, CD4<sup>+</sup> Tconv, and CD8<sup>+</sup> T cells. **C**, Frequency of CD39<sup>-</sup>, CD39<sup>int</sup>, and CD39<sup>high</sup> (CD39<sup>hi</sup>) CD8<sup>+</sup> T cells. **D**, Frequency of total CD39<sup>+</sup> (CD39<sup>int</sup> + CD39<sup>high</sup>) and CD39<sup>high</sup> cells in CD3<sup>+</sup>CD8<sup>+</sup> T cells. **E**, Frequency of CD39<sup>-</sup>, CD39<sup>int</sup>, and CD39<sup>high</sup> CD3<sup>+</sup>CD8<sup>+</sup> TILs during tumor progression; mean values and statistical analyses (D10 vs. D17 and D24) are shown. **F**, Frequency of CD73<sup>+</sup> cells in CD3<sup>+</sup>CD8<sup>+</sup> TILs at different days postinjection. All results are representative of 3–5 independent experiments (*n* = 6–15 mice per experiment). **A, B, D–F**, data presented as mean ± SEM. ns, nonsignificant; \* or #, *P* ≤ 0.05; \*\*, *P* ≤ 0.01; \*\*\* or ###, *P* ≤ 0.001; \*\*\*\* or tttt, *P* ≤ 0.0001.

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production when compared with CD39<sup>-</sup> and CD39<sup>int</sup>CD8<sup>+</sup> TILs (Fig. 2B). Furthermore, in accordance with their IFN $\gamma$ -producer phenotype, CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibited the highest cytotoxic potential, evaluated by expression of granzyme B, and CD107a mobilization (Fig. 2C). CD39<sup>high</sup>CD8<sup>+</sup> TILs from B16F10-OVA tumor-bearing mice showed higher frequency of IFN $\gamma$ <sup>+</sup> and

granzyme B<sup>+</sup> cells as well as higher proliferative potential than CD39<sup>high</sup>CD8<sup>+</sup> TILs from B16F10 tumor-bearing mice, which may be attributed to the higher immunogenicity of the B16F10-OVA tumor. However, the frequency of TNF<sup>+</sup> and IL2<sup>+</sup> cells within B16F10 CD39<sup>high</sup>CD8<sup>+</sup> TILs was as low as that detected in the OVA-expressing melanoma (Supplementary Fig. S3A).



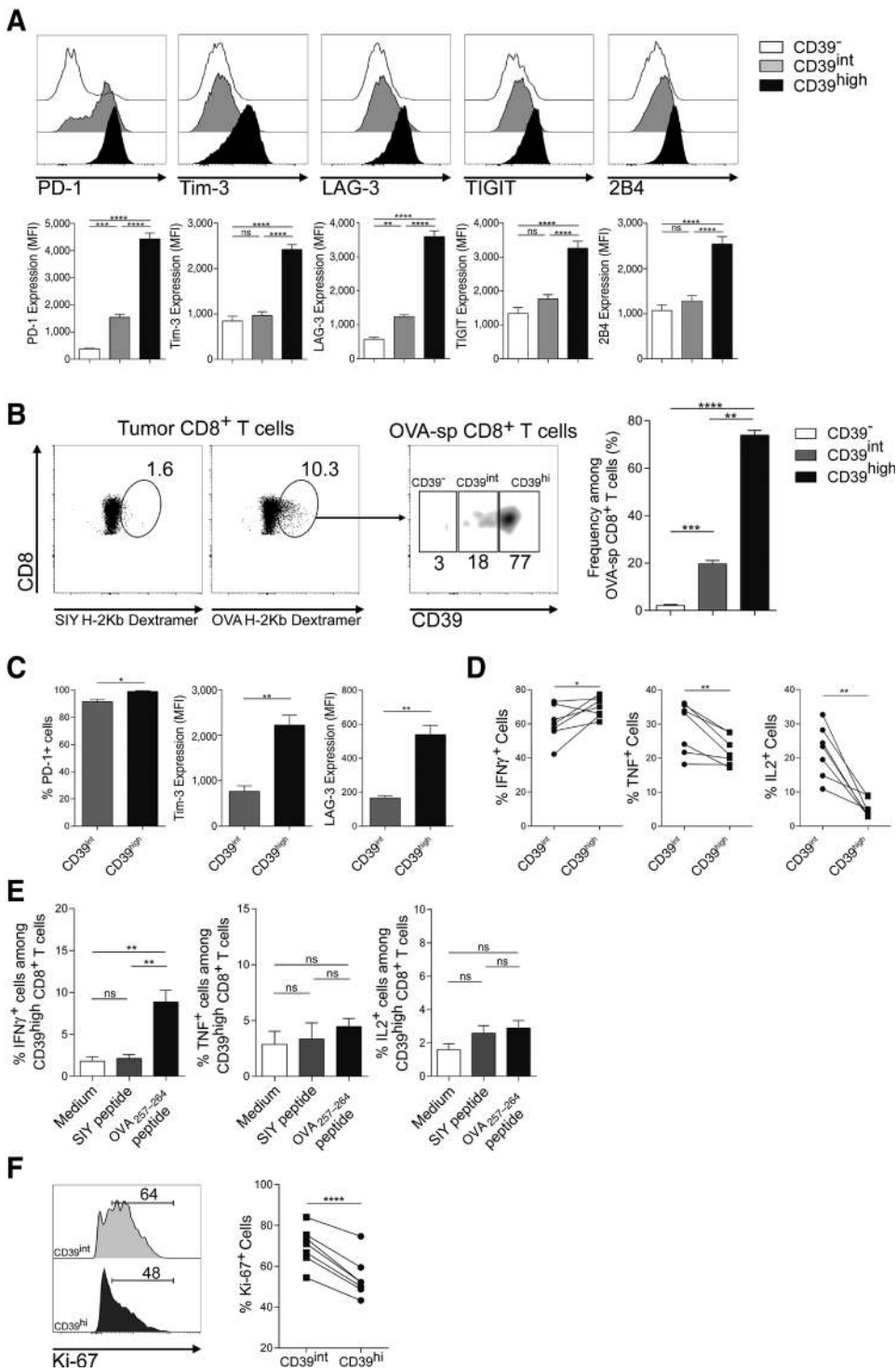
**Figure 2.** B16F10-OVA CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibit an effector/memory phenotype and a particular profile of effector functions. TILs obtained at day 17 postinjection. **A**, Frequency of cells expressing CD44 and CD62L within CD3<sup>+</sup>CD8<sup>+</sup> TILs. **B** and **C**, Frequency of CD3<sup>+</sup>CD8<sup>+</sup> TILs showing different expression of CD39 that produce IFN $\gamma$ , TNF, and IL2 (**B**) and express granzyme B and CD107a (**C**) after PMA/ionomycin stimulation. All results are representative of 3–5 independent experiments ( $n = 6$ –15 mice per experiment). Data presented as mean  $\pm$  SEM. ns, nonsignificant; \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

**CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibit a phenotype associated with exhaustion**

Exhausted CD8<sup>+</sup> T cells are characterized by early impairment in IL2 and TNF secretion and expression of multiple iRs (10). Considering that CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibit significantly lower IL2 and TNF production, we further evaluated iRs and TFs associated with exhaustion. Among CD8<sup>+</sup> TILs, CD39<sup>high</sup>CD8<sup>+</sup> T cells

exhibited the highest expression of all iRs tested (PD-1, Tim-3, LAG-3, TIGIT, and 2B4; Fig. 3A). Similar profiles of iR expression were also observed on CD39<sup>high</sup>CD8<sup>+</sup> TILs in 4T1 and MCA-OVA tumors (Supplementary Fig. S3B). Consistent with the absence of CD39<sup>high</sup>CD8<sup>+</sup> T cells in dLNs and spleens, no significant expression of iRs was observed on CD39<sup>-</sup> and CD39<sup>int</sup>CD8<sup>+</sup> T cells from these organs (Supplementary Fig. S3C).

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**Figure 3.** High expression of CD39 identifies CD8<sup>+</sup> T cells that express iRs associated with exhaustion. TILs obtained at day 17 postinjection. **A**, Expression of iRs in total CD39<sup>-</sup>, CD39<sup>int</sup> and CD39<sup>high</sup> CD8<sup>+</sup> TILs. **B**, Representative plots show frequency of OVA-specific CD8<sup>+</sup> TILs and plot and graph shows frequency of CD39<sup>+</sup> cells within OVA-specific CD8<sup>+</sup> TILs. SIY H-2Kb dextramer was used as control for specific staining. **C**, Frequency or MFI of PD-1, Tim-3, and LAG-3 among CD39<sup>int</sup> and CD39<sup>high</sup> OVA-specific CD8<sup>+</sup> TILs. **D**, Frequency of IFN $\gamma$ <sup>+</sup>, TNF<sup>+</sup>, and IL2<sup>+</sup> cells within OVA-specific CD39<sup>high</sup>CD8<sup>+</sup> TILs versus CD39<sup>int</sup>CD8<sup>+</sup> TILs after stimulation with PMA/ionomycin. **E**, Frequency of cytokine-producing CD39<sup>high</sup>CD8<sup>+</sup> TILs after stimulation with OVA<sup>257-264</sup> peptide or SIY peptide (control). **F**, Frequency of Ki-67<sup>+</sup> cells within CD39<sup>int</sup> and CD39<sup>high</sup> OVA-specific CD8<sup>+</sup> TILs after anti-CD3/anti-CD28 stimulation. All results are representative of 2-5 independent experiments ( $n = 10-15$  mice per experiment). Data presented as mean  $\pm$  SEM. ns, nonsignificant; \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

CD39<sup>high</sup>CD8<sup>+</sup> TILs also exhibited higher expression of effector and exhaustion-associated TFs such as T-bet, Eomes, and Blimp-1 when compared with CD39<sup>-</sup> and CD39<sup>int</sup>CD8<sup>+</sup> TILs (Supplementary Fig. S3D). These results sustain that CD39<sup>high</sup>CD8<sup>+</sup> TILs constitute a defined subset that shows particular features different from other CD8<sup>+</sup> TILs.

Analyzing antigen-specific CD8<sup>+</sup> TILs, we found that around 75% of OVA-specific CD8<sup>+</sup> TILs were CD39<sup>high</sup> and these cells

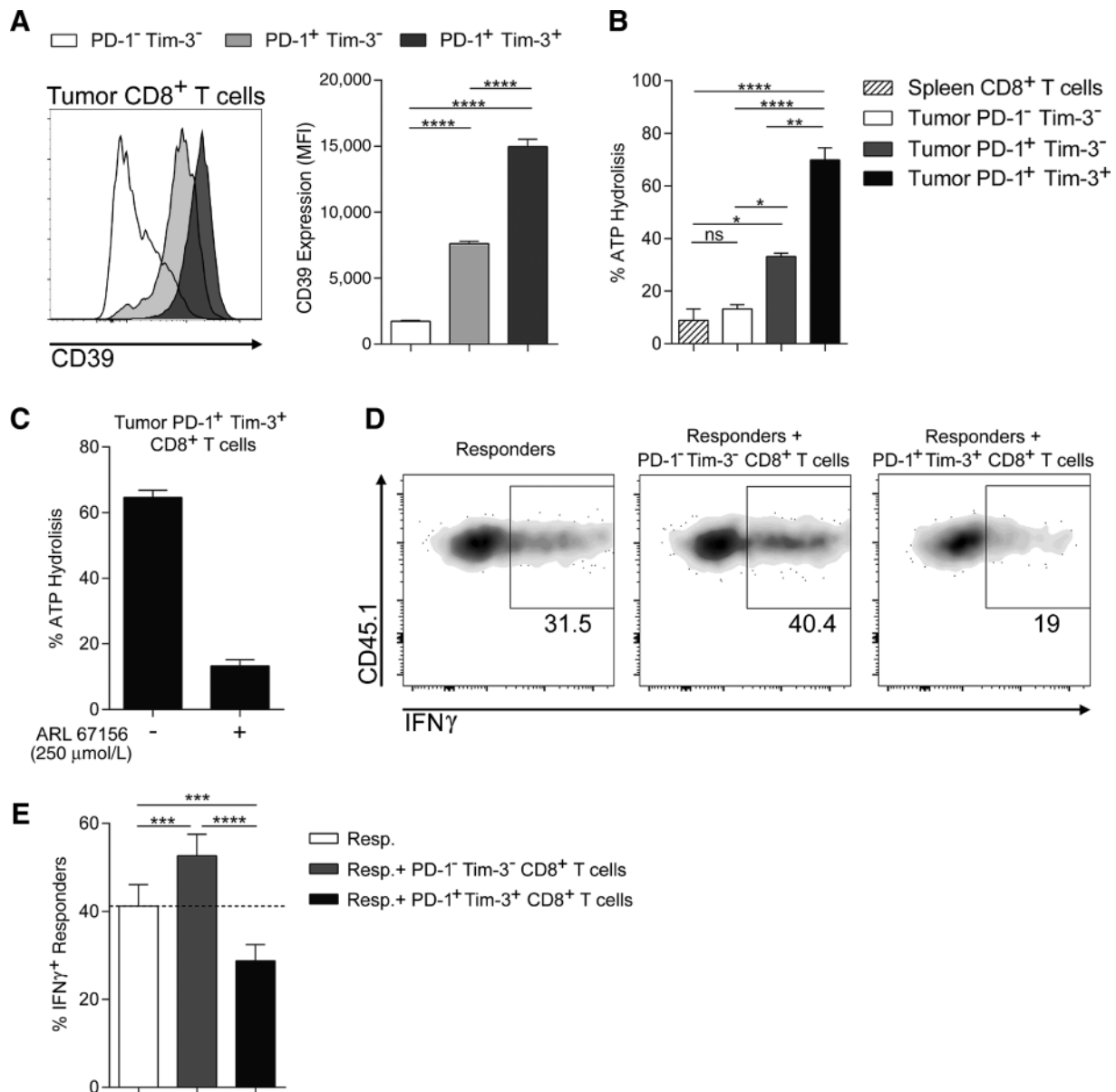
exhibited higher expression of iRs than OVA-specific CD39<sup>int</sup>CD8<sup>+</sup> TILs (Fig. 3B and C). After PMA/ionomycin stimulation, OVA-specific CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibited higher frequency of IFN $\gamma$ <sup>+</sup> cells but reduced percentage of TNF<sup>+</sup> and IL2<sup>+</sup> cells than OVA-specific CD39<sup>int</sup>CD8<sup>+</sup> TILs (Fig. 3D). Upon OVA<sup>257-264</sup>-specific peptide stimulation, CD39<sup>high</sup> CD8<sup>+</sup> TILs showed increased frequency of IFN $\gamma$ -producing cells, but no significant induction of TNF and IL2. When analyzing the

proliferative potential of OVA-specific CD8<sup>+</sup> TILs, OVA-specific CD39<sup>high</sup> CD8<sup>+</sup> TILs showed lower frequency of Ki-67<sup>+</sup> cells than CD39<sup>int</sup> CD8<sup>+</sup> TILs after *in vitro* stimulation (Fig. 3E and F).

**Intratumoral exhausted CD8<sup>+</sup> T cells show high expression of CD39 and ability to hydrolyze extracellular ATP**

The coexpression of PD-1 and Tim-3 has been described as hallmark of CD8<sup>+</sup> T-cell exhaustion in cancer and chronic infec-

tions (10, 12). We identified PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs in mice bearing B16F10-OVA tumors and determined that PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs show higher expression of CD39 than PD-1<sup>+</sup>Tim-3<sup>-</sup> and PD-1<sup>-</sup>Tim-3<sup>-</sup> CD8<sup>+</sup> TILs, which exhibited intermediate and low expression of the ecto-enzyme, respectively (Fig. 4A). We further confirmed that PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> T cells (CD39<sup>high</sup>) are functionally exhausted as, in contrast to the other CD8<sup>+</sup> T-cell subsets, they showed cell-cycle arrest (indicated by low frequency



**Figure 4.** Exhausted CD8<sup>+</sup> TILs exhibit high expression of enzymatically active CD39 and modulate IFN $\gamma$  production by spleen CD8<sup>+</sup> T cells. TILs obtained at day 17 postinjection. **A**, CD39 expression on PD-1<sup>-</sup>Tim-3<sup>-</sup>, PD-1<sup>+</sup>Tim-3<sup>-</sup>, and PD-1<sup>+</sup>Tim-3<sup>+</sup> CD8<sup>+</sup> TILs. **B**, Percentage of ATP hydrolysis after culture of sorted CD8<sup>+</sup> TIL subsets incubated with exogenous ATP ( $n = 3$ , representative from two independent experiments). **C**, Percentage of eATP hydrolysis by PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs preincubated or not with ARL67156 ( $n = 2$ , data from two independent experiments performed with pools of cells from 6 mice per pool). **D**, Frequency of IFN $\gamma$ <sup>+</sup> responder cells (CD8<sup>+</sup> T cells from CD45.1 tumor-free mice), stimulated alone or in coculture with CD45.2<sup>+</sup> PD-1<sup>-</sup>Tim-3<sup>-</sup> or PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs (1:1 ratio). **E**, Frequency of IFN $\gamma$ <sup>+</sup> responder cells described in **D** ( $n = 11$ , data from 6 independent experiments). **A**, Results are representative of five independent experiments ( $n = 6$ -15 mice per experiment). **A-C** and **E**, Data presented as mean  $\pm$  SEM. ns, nonsignificant; \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

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of Ki-67<sup>+</sup> cells), lower T-bet expression and decreased IFN $\gamma$  production after *in vitro* restimulation with anti-CD3/anti-CD28 and rIL2 for 72 hours (Supplementary Fig. S4A and S4B). Collectively, our data support the concept that coexpression of PD-1 and Tim-3 along with high expression of CD39 mark the most exhausted population of CD8<sup>+</sup> TILs.

We next tested the ability of exhausted CD8<sup>+</sup> TILs to hydrolyze eATP. Consistent with their increased CD39 expression, sorted PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs showed the highest capacity to hydrolyze eATP (Fig. 4B). In addition, PD-1<sup>+</sup>Tim-3<sup>-</sup>CD8<sup>+</sup> TILs, which have intermediate CD39 expression, exhibited higher eATP hydrolysis than PD-1<sup>-</sup>Tim-3<sup>-</sup>CD8<sup>+</sup> TILs and spleen CD8<sup>+</sup> T cells (Fig. 4B). To confirm that CD39 was responsible for ATP hydrolysis, exhausted CD8<sup>+</sup> TILs were preincubated with the ATPase inhibitor, ARL67156, resulting in a marked reduction in the percentage of ATP hydrolysis (Fig. 4C). Considering these results and that ATP hydrolysis by T cells has been reported to be substantially independent of other ecto-ATPases (6, 21), we conclude that eATP hydrolysis by exhausted CD8<sup>+</sup> TILs is due to CD39 expression.

Considering that CD39 has been linked to an immunoregulatory functionality, we evaluated whether CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs were able to modulate cytokine production or proliferative responses by responder CD8<sup>+</sup> T cells obtained from normal spleens. Coculture assays showed that sorted CD39<sup>-</sup>PD-1<sup>-</sup>Tim-3<sup>-</sup>CD8<sup>+</sup> TILs increased the frequency of IFN $\gamma$ -producing responder cells compared with responders cultured alone. On the contrary, CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs significantly diminished IFN $\gamma$ -producing responder cells (Fig. 4D and E). This decrease in IFN $\gamma$  production was not restored when CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs and responders were cocultured in the presence of ARL67156 or when responders were CD73 deficient, indicating that IFN $\gamma$  modulation is not mediated by CD39 at least in our *in vitro* settings (Supplementary Fig. S4C and S4D). In contrast to the reported effects of CD39<sup>+</sup>Tregs (6), CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs did not suppress the proliferation of responder cells (Supplementary Fig. S4E).

#### CD39<sup>+</sup>CD8<sup>+</sup> T cells accumulate within tumors and invaded lymph nodes from cancer patients and exhibit features of exhaustion.

The knowledge about the specific infiltrate of immune cells and their functionality in patients with cancer is becoming important for prognosis and therapy design (22). Thus, we evaluated ectonucleotidases expression on CD8<sup>+</sup> T cells from human primary breast tumors, invaded/metastatic and noninvaded dLNs and peripheral blood. In primary tumors, we noticed defined CD39<sup>+</sup> and CD73<sup>+</sup> CD8<sup>+</sup> T-cell populations, although practically no cells coexpressed both ecto-enzymes. We observed a mean frequency of 18.5%  $\pm$  4.3% CD39<sup>+</sup>CD8<sup>+</sup> TILs (Fig. 5A; Supplementary Table S2), while a mean of 11.2%  $\pm$  1.7% CD8<sup>+</sup> TILs exhibited CD73 expression. Interestingly, invaded/metastatic LNs (I-LN) showed a higher frequency of CD39<sup>+</sup>CD8<sup>+</sup> T cells than noninvaded LNs (NI-LN) but reduced frequency of CD73<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 5B). Furthermore, CD8<sup>+</sup> T cells from peripheral blood from breast cancer patients did not exhibit CD39 expression (Fig. 5C). Our results associate the presence of CD39<sup>+</sup>CD8<sup>+</sup> T cells to locations where tumor cells are also present, suggesting that the microenvironment induced by tumor cells may be critical to elicit CD39 expression on CD8<sup>+</sup> T cells. Of note, we also observed that similar to the B16F10-OVA model, tumors from breast cancer

patients are also infiltrated by CD39<sup>+</sup>CD4<sup>+</sup> Tconv cells (Supplementary Fig. S5A).

Most of CD39<sup>+</sup>CD8<sup>+</sup> T cells from tumors and I-LNs exhibited a phenotype compatible with effector/memory cells, indicating that CD39 expression is predominantly associated to activated CD8<sup>+</sup> T cells (Supplementary Fig. S5B). Conversely, CD39<sup>-</sup>CD8<sup>+</sup> T cells showed higher frequency of naïve cells, particularly in I-LNs.

We further evaluated the expression of iRs on CD8<sup>+</sup> T cells from tumors (Fig. 5D) and I-LNs (Fig. 5E). We first gated out naïve cells as they do not express iRs and may influence the results (gating out CD45RA<sup>+</sup>CD27<sup>+</sup> cells). In comparison with their CD39<sup>-</sup> counterparts, CD39<sup>+</sup>CD8<sup>+</sup> T cells from both sites showed significantly higher expression of PD-1 and TIGIT as well as BTLA, particularly in I-LNs. Other iRs, such as Tim-3 and LAG-3, were not detected either in tumors or in I-LNs from breast cancer patients (data not shown), although it has been described that these receptors are present in CD8<sup>+</sup> TILs from other types of cancer (23–25).

Analysis of coexpression of iRs on CD8<sup>+</sup> T-cell subsets revealed that the frequency of CD8<sup>+</sup> T cells that coexpress two or more iRs was increased in CD39<sup>+</sup> population compared with CD39<sup>-</sup>CD8<sup>+</sup> T cells (Supplementary Fig. S5C). The proportion of CD39<sup>+</sup>CD8<sup>+</sup> T cells expressing 3 iRs was more prominent in I-LNs than in tumors. This may be related to the fact that the type of coexpressed iRs on human CD8<sup>+</sup> T cells depends on the anatomic localization (26). Alternatively, CD73 presence was not associated to iRs expression in LNs, in fact CD39<sup>-</sup>CD73<sup>+</sup>CD8<sup>+</sup> T cells did not express PD-1 (Supplementary Fig. S5D).

#### CD39-expressing CD8<sup>+</sup> T cells from cancer patients exhibit an impairment in effector cytokine production

The effector function of CD39<sup>+</sup>CD8<sup>+</sup> T cells from tumors and I-LNs was assessed analyzing their ability to produce IL2, IFN $\gamma$ , and TNF upon activation. To compare the effector response between CD39<sup>-</sup> and CD39<sup>+</sup>CD8<sup>+</sup> T cells, we focused our analysis on non-naïve cells (gating out CD45RA<sup>+</sup>CD27<sup>+</sup> cells). CD39<sup>+</sup>CD8<sup>+</sup> T cells from tumors (Fig. 6A) and I-LNs (Fig. 6B) exhibited reduced frequency of TNF and IFN $\gamma$ -producing cells compared with CD39<sup>-</sup>CD8<sup>+</sup> T cells. Furthermore, CD39<sup>+</sup>CD8<sup>+</sup> T cells from I-LNs also showed a compromise in IL2 production. In addition, CD39<sup>+</sup>CD8<sup>+</sup> T cells from tumors and I-LNs exhibited higher frequency of CD107a<sup>+</sup> cells than their CD39<sup>-</sup> counterparts (Fig. 6A and B).

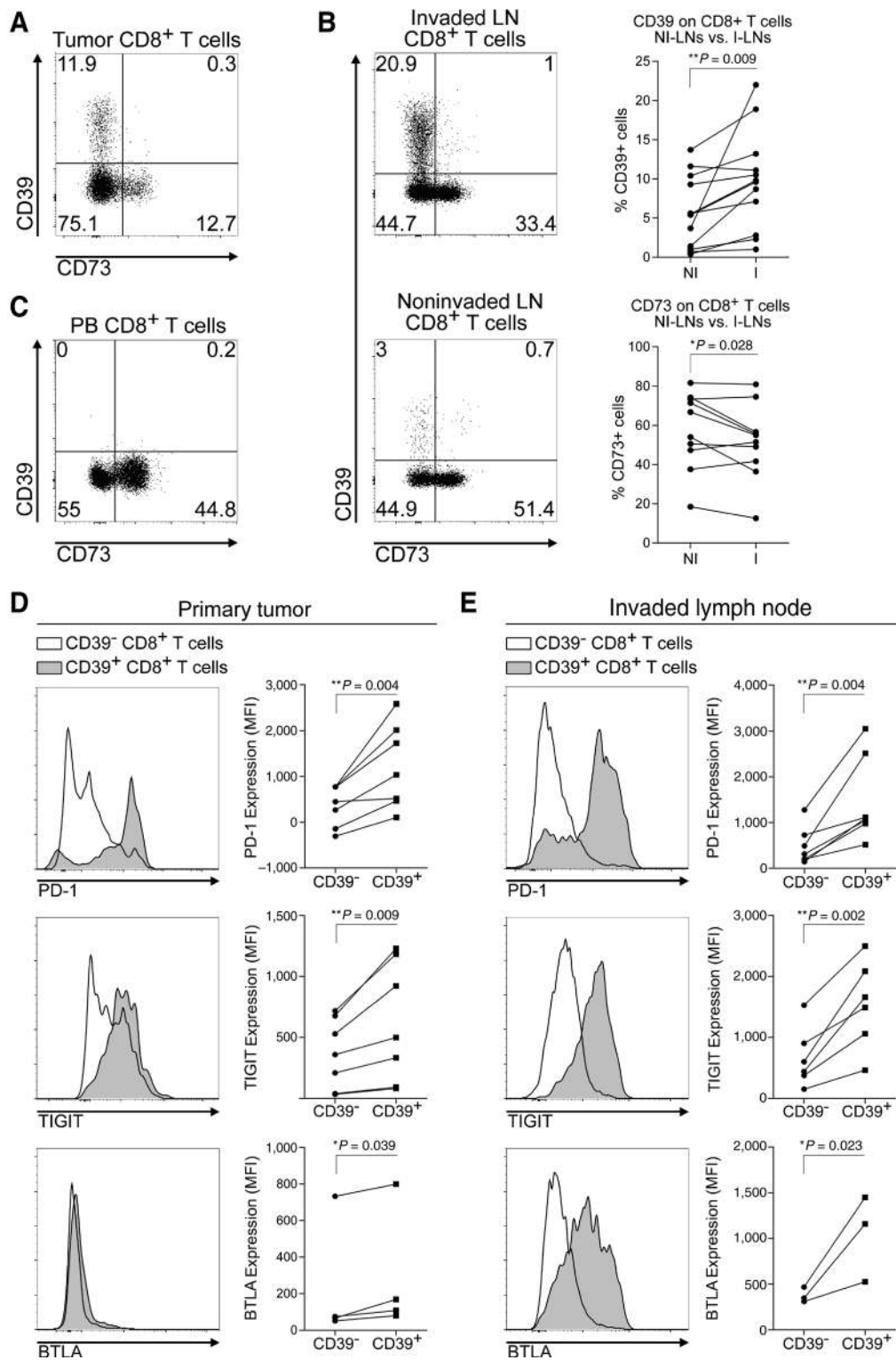
In addition, we observed that melanoma patients also exhibited a higher frequency of CD39<sup>+</sup>CD8<sup>+</sup> T cells in I-LNs, compared with NI-LNs. Moreover, CD39<sup>+</sup>CD8<sup>+</sup> T cells showed high PD-1 and TIGIT expression and impairment in TNF and IL2 production (Supplementary Fig. S6A–S6C).

Altogether, these results demonstrate that CD39<sup>+</sup>CD8<sup>+</sup> T cells with functional features of exhaustion are present in tumors and metastatic LNs from cancer patients.

#### CD8<sup>+</sup> T cells upregulate CD39 expression upon *in vitro* TCR stimulation plus IL6 and IL27

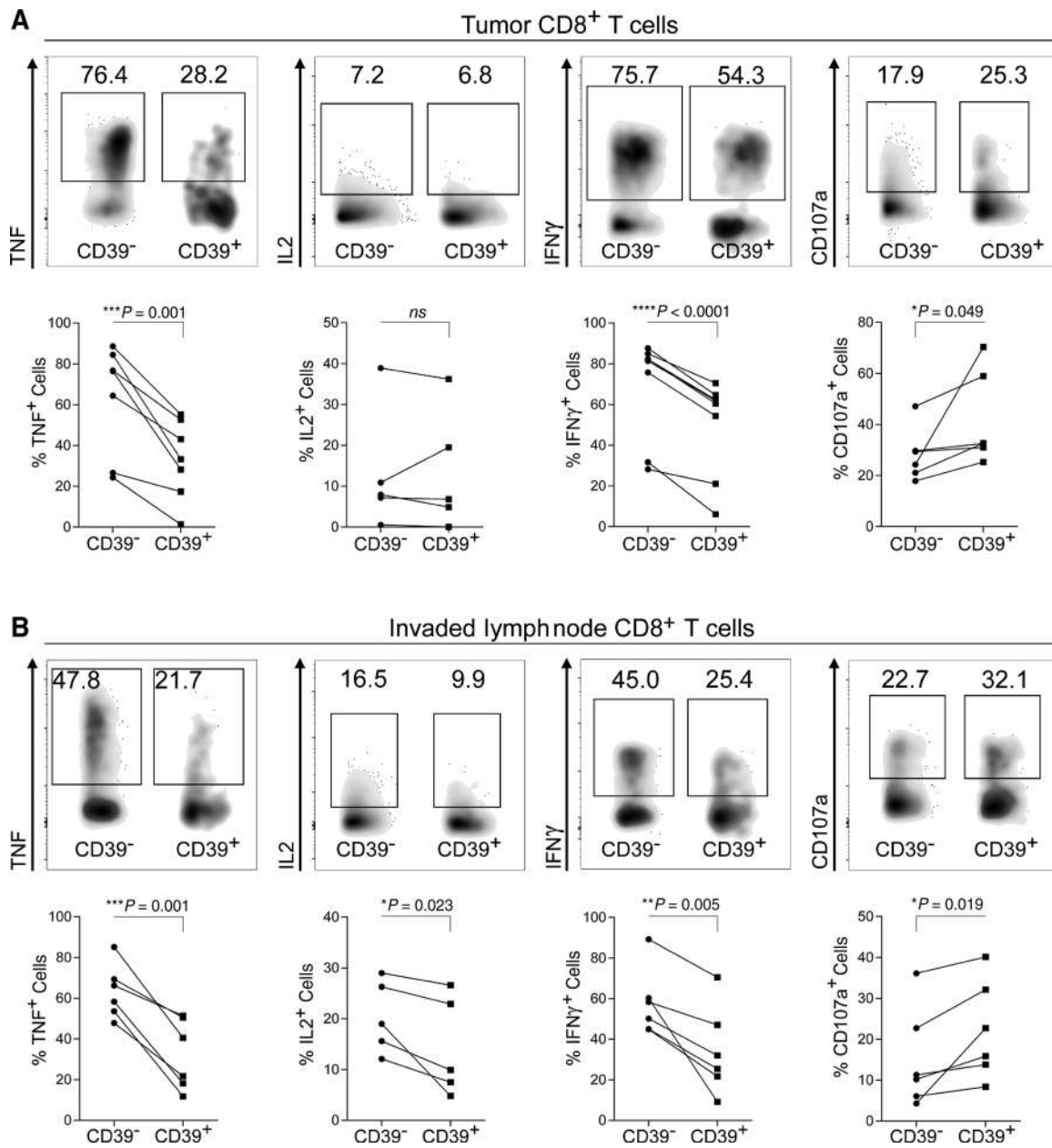
We further evaluated signals that may influence CD39 upregulation. CD8<sup>+</sup> T cells from dLNs from B16F10-OVA tumor-bearing mice were stimulated *in vitro* with anti-CD3/anti-CD28 and cytokines known to be involved in the induction of exhaustion (27, 28). TCR stimulation alone did not modify





**Figure 5.** CD39<sup>+</sup>CD8<sup>+</sup> T cells infiltrate human breast tumors and invaded/metastatic lymph nodes and exhibit high expression of iRs. **A**, Representative dot plot shows frequency of CD39<sup>+</sup> and CD73<sup>+</sup> cells in CD3<sup>+</sup>CD8<sup>+</sup> TILs (one out of nine breast tumor samples). **B**, Frequency of CD39<sup>+</sup> and CD73<sup>+</sup> cells in CD3<sup>+</sup>CD8<sup>+</sup> T cells from NI-LNs and I-LNs from breast cancer patients (*n* = 11). **C**, Representative dot plot shows frequency of CD39<sup>+</sup> and CD73<sup>+</sup> cells in peripheral blood (PB) CD3<sup>+</sup>CD8<sup>+</sup> T cells (1/7 samples). **D** and **E**, Expression of PD-1, TIGIT, and BTLA on CD39<sup>-</sup> and CD39<sup>+</sup> CD8<sup>+</sup> T cells from breast tumors (**D**) and I-LNs from breast cancer patients (**E**; *n* = 3-7). Lines indicate that data are paired.

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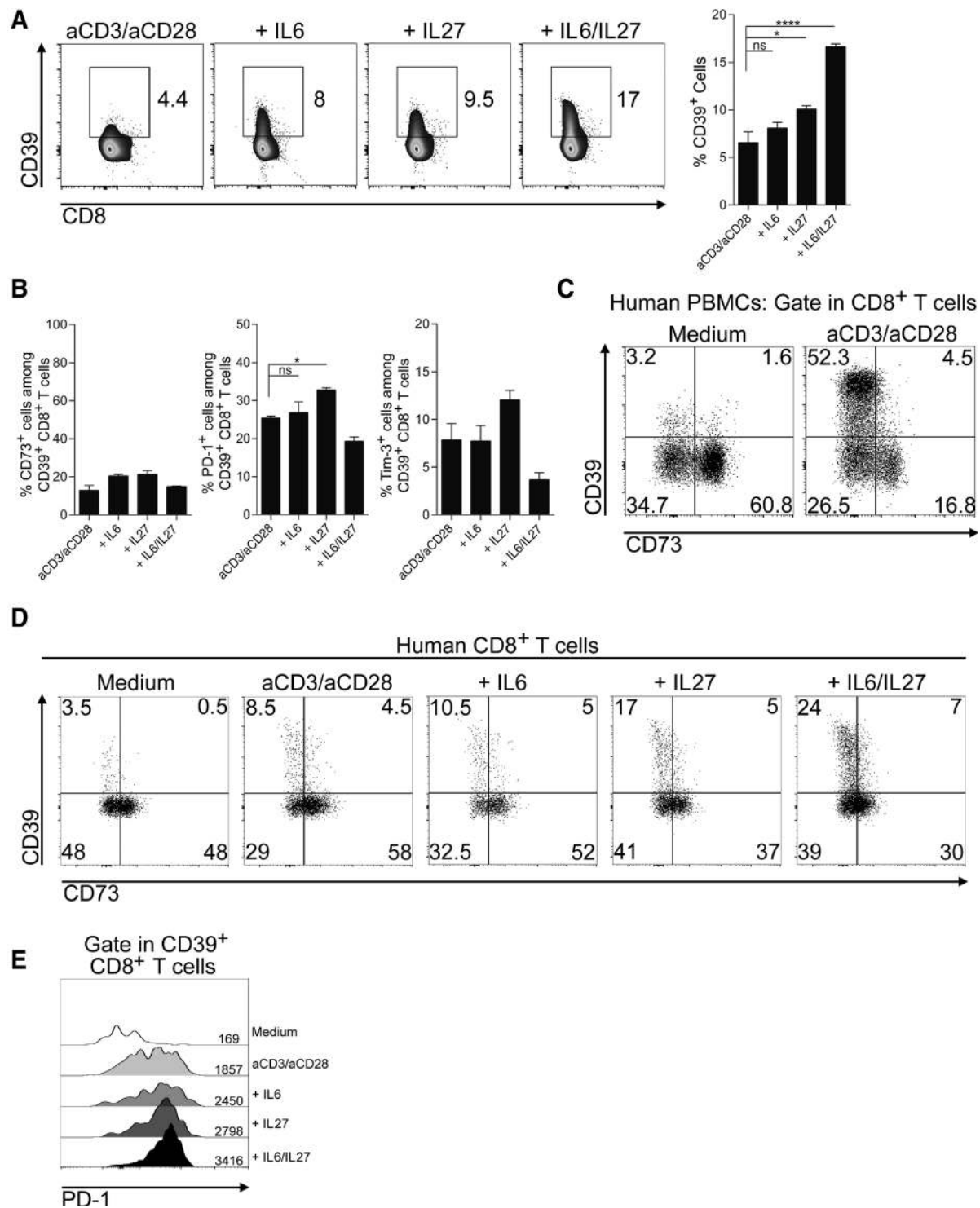


**Figure 6.** Human CD39<sup>+</sup>CD8<sup>+</sup> T cells from tumors and invaded/metastatic lymph nodes exhibit impaired production of effector cytokines. Frequency of cytokine-producing cells (IL2, TNF, and IFN $\gamma$ ) and CD107a<sup>+</sup> cells in CD39<sup>-</sup> and CD39<sup>+</sup> CD8<sup>+</sup> T cells from breast tumors (A) or I-LNs (B) from breast cancer patients after PMA/ionomycin stimulation ( $n = 5-7$ ). Analysis performed within the non-naïve population (gating out CD45RA<sup>+</sup>CD27<sup>+</sup> cells). Lines indicate that data are paired.

the CD39 expression observed *ex vivo*; however, IL27 drove CD39 expression on stimulated cells, especially when combined with IL6 (Fig. 7A). Phenotypic characterization showed that most of these induced CD39<sup>+</sup> cells did not express CD73, but they showed a significant increment of PD-1 and a tendency to increase Tim-3 expression upon culture with IL27 but not with IL6 or IL6/IL27 combination (Fig. 7B).

Stimulation of PBMCs from breast cancer patients with anti-CD3/anti-CD28 was sufficient to induce an impressive CD39 expression on CD8<sup>+</sup> T cells (Fig. 7C). Next, to better identify

signals that induce CD39 on CD8<sup>+</sup> T cells and to rule out the interaction with other mononuclear cells, we stimulated purified CD8<sup>+</sup> T cells with anti-CD3/anti-CD28. We observed a lower increase in CD39 expression in comparison with total PBMCs, but it was further upregulated in the presence of IL27 or the combination of IL6 and IL27. Moreover, TCR-stimulated CD8<sup>+</sup> T cells in presence of IL27 plus IL6 exhibited higher expression of PD-1 than CD8<sup>+</sup> T cells stimulated with anti-CD3/anti-CD28 alone (Fig. 7D and E; Supplementary Table S3).



**Figure 7.** CD39 expression is promoted on CD8<sup>+</sup> T cells by TCR stimulation plus IL6 and IL27. **A**, CD8<sup>+</sup> T cells from dLNs from B16F10-OVA tumor-bearing mice (D17) were stimulated in the presence of recombinant IL6 and/or IL27. Frequency (density plots and graph) of CD39<sup>+</sup> CD8<sup>+</sup> T cells induced in each condition. **B**, Frequency of CD73<sup>+</sup>, PD-1<sup>+</sup>, and Tim-3<sup>+</sup> cells within mouse CD39<sup>+</sup> CD8<sup>+</sup> T cells induced in each condition. **C**, Frequency of CD39<sup>+</sup> and CD73<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> T cells from PBMCs from breast cancer patient stimulated with anti-CD3/anti-CD28 or unstimulated. **D**, Frequency of CD39<sup>+</sup> and CD73<sup>+</sup> cells within CD8<sup>+</sup> T cells purified from PBMCs from breast cancer patient after anti-CD3/anti-CD28 stimulation in presence or absence of IL6 and/or IL27. **E**, Representative histograms show PD-1 expression (MFI indicated) on CD39<sup>+</sup> CD8<sup>+</sup> T cells in each condition. **A** and **B**, Data presented as mean ± SEM. ns, nonsignificant; \*, P < 0.05; \*\*\*\*, P < 0.0001.

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Although murine and human CD8<sup>+</sup> T cells express CD39 upon *in vitro* stimulation, these cells showed high frequency of IFN $\gamma$ <sup>+</sup> and TNF<sup>+</sup> cells when stimulated with PMA/ionomycin (Supplementary Fig. S7A and S7B), indicating that longer stimulation and/or additional signals may be necessary to induce exhaustion.

Hypoxia has also been linked to ecto-nucleotidases induction (29). Accordingly, CD39<sup>+</sup>CD45<sup>+</sup> cells were mostly localized within hypoxic foci in tumor sections. However, CD39 was not induced in CD8<sup>+</sup> T cells stimulated under *in vitro* hypoxic conditions, indicating that probably a longer exposure to hypoxic conditions or additional factors may be necessary (Supplementary Fig. S7C and S7D).

## Discussion

Increasing evidence suggests that cancer and immune cells closely interact to generate an immunosuppressive environment that supports neoplastic growth (30). In this regard, several studies have pointed to the critical task carried out by CD39 and CD73 in generating this immunosuppressive environment (5). Our work demonstrates that an impressive frequency of tumor-infiltrating CD8<sup>+</sup> T cells in different experimental mice models exhibits high CD39 expression. Interestingly, CD39 was associated with a specific subset of CD8<sup>+</sup> T cells with exhaustion features. Moreover, CD39<sup>+</sup>CD8<sup>+</sup> T cells with exhausted phenotype were also present in tumors and invaded/metastatic lymph nodes from breast cancer and melanoma patients. While CD39 was expressed on CD8<sup>+</sup> TILs from all tested experimental models as well as in CD8<sup>+</sup> T cells from cancer patients, CD73 was only coexpressed by CD8<sup>+</sup> TILs from 4T1 and MCA-OVA tumor-bearing mice. These variances in CD73 expression could be explained by the genetic background of mouse strains and the histologic origin of tumors. Evidences demonstrate that expression and function of CD73 are both increased by TGF $\beta$  or under hypoxic conditions (31, 32). In contrast, IL4, IL21, IL12, and IFN $\gamma$  prevent CD73 expression induced by TGF $\beta$  (31). Then, it is possible that each experimental model denotes a particular inflammatory microenvironment that differentially affects CD73 expression on CD8<sup>+</sup> TILs. Our observations in B16F10-OVA tumor-bearing mice and cancer patients are in agreement with reports on HIV<sup>+</sup> and follicular lymphoma patients where CD39<sup>+</sup>CD8<sup>+</sup> T cells did not coexpress CD73 (18, 33).

CD39<sup>+</sup>CD8<sup>+</sup> T cells are increased specifically in tumors, and I-LNs from patients, while in peripheral tissues these cells are present in lower frequencies or even absent. This observation together with high percentage of CD45<sup>+</sup> tumor-infiltrating cells expressing CD39 emphasizes the idea that mouse as well as human tumor microenvironment command CD39 expression. IL6 and IL27 have been linked to CD39 expression as well as induction of iRs and TFs related to exhaustion on DCs, CD4<sup>+</sup> T cells, and other leukocytes (27, 28, 34, 35). We showed that these cytokines induce CD39 on murine and human TCR-stimulated CD8<sup>+</sup> T cells, which is more evident when combining IL6 and IL27, indicating that CD39 expression may result as a consequence of the integration of different signals. Unexpectedly, stimulation of PBMCs from breast cancer patients with anti-CD3/anti-CD28 was sufficient to trigger CD39 expression even higher to that observed by stimulated purified CD8<sup>+</sup> T cells supporting the idea that signals provided by other mononuclear cells may work in synergy to upregulate CD39.

In mice, CD39<sup>high</sup>CD8<sup>+</sup> TILs exhibit high frequency of IFN $\gamma$ <sup>+</sup>, granzyme B<sup>+</sup>, and CD107a<sup>+</sup> cells, all features of a Tc1-like phenotype. These findings are in agreement with observations made by Bai and colleagues (36) who described CD39<sup>+</sup>CD8<sup>+</sup> T cells with similar functionality in Crohn disease. However, the reduced production of IL2 and TNF together with the expression of multiple inhibitory receptors define CD39<sup>high</sup>CD8<sup>+</sup> TILs as exhausted cells. In fact, CD39<sup>high</sup>CD8<sup>+</sup> T cells can be precisely identified on the basis of PD-1 and Tim-3 coexpression. Moreover, in models of chronic viral infections, terminally exhausted T cells display a T-bet<sup>low</sup>Eomes<sup>high</sup>Blimp-1<sup>high</sup> phenotype (15, 16) that is shared by CD39<sup>high</sup>CD8<sup>+</sup> TILs, with the only exception of T-bet, which is still expressed by this subset and may be responsible for their sustained IFN $\gamma$  production.

Considering that exhaustion is a progressively acquired state, we presume that CD39<sup>high</sup>CD8<sup>+</sup> T cells from B16F10-OVA experimental cancer model are not yet terminally exhausted, but reach terminal exhaustion upon restimulation, as we observed *in vitro* with restimulated CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs.

Analyzing CD39<sup>+</sup>CD8<sup>+</sup> T cells from cancer patients, we also found impairment in the production of effector cytokines together with high expression of iRs. Remarkably, unlike mouse counterparts, human CD39<sup>+</sup>CD8<sup>+</sup> T cells showed reduced production of IFN $\gamma$  even without further *in vitro* restimulation. Altogether, our findings led us to conclude that CD39-expressing CD8<sup>+</sup> T cells exhibit a dysfunctional phenotype in mouse cancer models as well as in patients with breast cancer and melanoma. Recently, Gupta and colleagues (37) described that human HIV and HCV-specific CD8<sup>+</sup> T cells as well as mice LCMV-specific CD8<sup>+</sup> T cells express CD39. These cells also coexpress PD-1 and exhibit a signature of exhaustion. Accordingly, the authors proposed CD39 as marker of exhausted T cells in chronic viral infections. Our results further extend these findings and support the association between CD39 expression and T-cell exhaustion as a general phenomenon in chronic inflammatory conditions. In addition, we determined that human exhausted CD39<sup>+</sup>CD8<sup>+</sup> T cells do not express CD73, which is in agreement with data that demonstrated that low expression of CD73 on CD8<sup>+</sup> T cells correlates with cell exhaustion in HIV<sup>+</sup> patients (33).

Previous reports describe that eATP is necessary for proper T-cell activation (7), while AMP and adenosine are able to suppress immune responses by several mechanisms (7, 38). Given that the combination of CD39 and CD73 degrades ATP to adenosine, these molecules can be considered as immunologic switches that shift ATP-driven proinflammatory immune cell activity toward an anti-inflammatory state mediated by adenosine (39). Our findings show that mouse exhausted CD8<sup>+</sup> T cells express CD39 at levels comparable with tumor-infiltrating Tregs, and that vigorously hydrolyze eATP. This feature highlights the potential of exhausted CD8<sup>+</sup> T cells to contribute to the suppressive tumor microenvironment by modulating ATP/ADP balance even if these cells do not coexpress CD73. In fact, Mascanfroni and colleagues (34) have shown that CD39<sup>+</sup>CD73<sup>-</sup> Tr1 cells are able to produce adenosine in cooperation with other CD73<sup>+</sup> populations. Reinforcing our hypothesis, Parodi and colleagues (19) described *in vitro* suppressive functionality of CD39<sup>+</sup>CD8<sup>+</sup> T cells obtained from human tumors, while other reports also described modulatory functions of *in vitro*-induced CD39<sup>+</sup>CD8<sup>+</sup> T cells (40). In addition, Bai and colleagues (36) demonstrated that *in vitro*-induced CD39<sup>+</sup>Tc1 cells modulate IFN $\gamma$  responses of CD39<sup>-</sup>CD8<sup>+</sup> T cells via adenosine production. Accordingly,

CD39<sup>high</sup>PD-1<sup>+</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> TILs showed *in vitro* capacity to decrease the frequency of IFN $\gamma$ -producing cells, although in our settings the modulation was independent of CD39 or CD73. An alternative mechanisms by which CD39<sup>high</sup>CD8<sup>+</sup> T cells may contribute to the immunosuppressive tumor environment is through their sustained IFN $\gamma$  production that may promote PD-L1 expression on tumors as previously reported (41, 42), and consequently, favor the negative regulation of PD-1<sup>+</sup> effector immune cells. It is tempting to speculate that the tumor-immunosuppressive microenvironment drives the loss of effector functions on CD8<sup>+</sup> T cells, while simultaneously triggers the acquisition of regulatory molecules. These two processes may complement each other to divert CD8<sup>+</sup> T-cell immunity from one with antitumoral features into another that favors tumor progression.

Considering that CD39-blocking antibodies have been proposed as a therapeutic tool to inhibit suppressive functions of Tregs in cancer (21), our and others' results (19, 36, 37) strongly suggest that CD39 blockade could target additional CD39-expressing cell populations. Moreover, it would be interesting to investigate the possible synergistic effect of combining CD39 blockade together with inhibitory receptors to reinvigorate CD8<sup>+</sup> T-cell effector functions and antitumor immunity.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** F.P. Canale, M.C. Ramello, E. Piaggio, C.L. Montes  
**Development of methodology:** F.P. Canale, M.C. Ramello, C.L. Araujo Furlan, S.N. Bossio

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A.D. Castillo, M. Ledesma, E. Piaggio

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#### Acknowledgments

We thank Dr. P. Abadie and Dr. M.P. Crespo for Flow Cytometry technical support and F. Navarro, D. Lutti, V. Blanco, I. Novotny, and C. Florit for animal care at CIBICI-CONICET. We also thank Z. Maciorowski and the Institut Curie Flow Cytometry facility; A. Salomon from the Center of Clinical Investigations CIBT507 IGR/Curie and the Department of Pathology; D. Loirat from the Medical Oncology Department and M. Millder from the Clinical Immunology Laboratory, Institut Curie, for collection of human samples. We thank Dr. Gabriel Rabinovich for helpful discussion of data. This work was supported by fundings from MINCYT (PICT 2012-0480 and PICT 2015-1954 to C.L. Montes), SECYT 2012-2016 (to C.L. Montes), Labex DCBIOL (ANR-10-IDEX-0001-02 PSL and ANR-11-LABX0043 to E. Piaggio and N. Núñez), F.P. Canale, M.C. Ramello, C.L. Araujo Furlan, M. Gorosito Serrán, J. Tosello Boari, and S.N. Bossio are supported by fellowships from CONICET. N.G. Núñez is supported by a fellowship from Ligue Nationale Contre le Cancer. A. Gruppi, E.V. Acosta Rodríguez, and C.L. Montes are members of the Scientific Career in CONICET.

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Received October 2, 2016; revised April 10, 2017; accepted October 18, 2017; published OnlineFirst October 24, 2017.

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