CD39 expression defines cell exhaustion in tumor-infiltrating CD8$^+$ T cells

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Abstract

The ability of $CD8^+$ T lymphocytes to eliminate tumors is limited by their ability to engender an immunosuppressive microenvironment. Here we describe a subset of tumor-infiltrating $CD8^+$ T cells marked by high expression of the immunosuppressive ATP ecto-nucleotidase CD39. The frequency of $CD39^{high}CD8^+$ T cells increased with tumor growth but was absent in lymphoid organs. Tumor-infiltrating $CD8^+$ T cells with high CD39 expression exhibited features of exhaustion, such as reduced production of TNF and IL-2 and expression of co-inhibitory receptors. Exhausted $CD39^+CD8^+$ T cells from mice hydrolyzed extracellular ATP, confirming that CD39 is enzymatically active. Furthermore, exhausted $CD39^+CD8^+$ T cells inhibited IFNγ production by responder $CD8^+$ T cells. In specimens from breast cancer and melanoma patients, $CD39^+CD8^+$ 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 IFNγ, TNF, IL-2 and high expression of co-inhibitory receptors. Although T cell receptor engagement was sufficient to induce CD39 on human $CD8^+$ T cells, exposure to IL-6 and IL-27 promoted CD39 expression on stimulated $CD8^+$ 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 $CD8^+$ T cells, with implications for defining a biomarker of T cell dysfunction and a target for immunotherapeutic intervention.

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 $CD8^+$ T cells, which are essential for the elimination of tumors (1-3).

Regulatory immune cell populations, including $Foxp3^+CD4^+$ regulatory T cells (Tregs), myeloid-derived suppressor cells and tolerogenic dendritic cells (DCs) 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 which hydrolyzes extracellular ATP (eATP), a pro-inflammatory 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 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 $CD8^+$ T cells including anergy, exhaustion and senescence (10,11). $CD8^+$ 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 $CD8^+$ T cells are the gradual loss of effector cytokine production and sustained expression of multiple inhibitory receptors (iRs) such as PD-1, LAG-3 and Tim-3 (10). Exhausted $CD8^+$ T cells also show cell cycle arrest and display a particular pattern of key transcription factors (TFs), including T-bet, Eomesodermin (Eomes), Blimp-1 and others (15,16). Importantly, exhaustion of $CD8^+$ T cell contributes to poor immune control during chronic infections and cancer. Accordingly, current immunotherapies involve T cells' reinvigoration through the blockade of iRs (10,17).

It has been reported that human tumor-infiltrating $CD8^+$ 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 ecto-nucleotidases CD39 and CD73 in $CD8^+$ T cells in cancer models to help understand their biological role.

By using mouse cancer models we demonstrate that tumor-infiltrating $CD8^+$ T cells exhibit high expression of CD39. These $CD39^{high}$ cells display an exhausted phenotype and high capacity to hydrolyze eATP. Interestingly, we detected CD39-expressing $CD8^+$ 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 $CD39^+CD8^+$ 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 in order to restore the immune response against tumors.

Materials and Methods

Mice and human samples

C57BL/6 wild type, CD73KO (CD73KO-B6.129S1-$Nt5et^{m1Lft}$/J), 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 animals' protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the CIBICI-CONICET. CD73KO and CD45.1 mice were purchased from Jackson Laboratory (USA).

Human tumors and/or tumor-draining lymph nodes (dLNs) 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 non-invaded 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 enzimatically with liberase and DNase I (Roche). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Hypaque gradients (GE Healthcare).

Cell lines

B16F10-OVA melanoma cell line was kindly provided by Dr. K. Rock, and MCA-OVA (MCA101 fibrosarcoma cell line) was kindly provided by Dr. Clothilde Théry. B16F10-OVA and B16F10 were maintained in DMEM (GIBCO). 4T1 and MCA-OVA in RPMI-1640. 4T1 and B16F10 cell lines were purchased from ATCC. Media were supplemented with 10% Fetal Bovine Serum (GIBCO), 1mM L-Glutamine (GIBCO), 25 mmol/L Hepes (Cell Gro) and 40 ug/mL gentamicin sulfate (Richet). 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 (s.c.) with $1x10^6$ B16F10-OVA or B16F10 cells or $0.5x10^6$ MCA-OVA cells. Female BALB/c mice were inoculated s.c. in the mammary gland with $2x10^4$ 4T1 cells. B16F10-OVA and MCA-OVA tumors were excised in the majority of experiments at day 17 post-injection (p.i.) and 4T1 tumors at day 28 p.i. 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 monoclonal antibodies (mAb) against mouse and human antigens. For fluorochromes and clones refer to 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 manufacturer indications.

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 h at 37°C. OVA-specific $CD8^+$ T cells were detected with a PE-conjugated $OVA^{257-264}$ H-2Kb dextramer according to 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 µM $OVA^{257-264}$ peptide (SIINFEKL, Invivogen), or 5 µM SIY peptide (SIYRYYGL, Immudex) as control, in the presence of Brefeldin A and Monensin during 5 h 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.

$CD8^+$ T cell purification, co-culture and *in vitro* stimulation

Tumor-infiltrating $CD8^+$ 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^-Tim-3^-, PD-1^+Tim-3^- and PD-1^+Tim-3^+ subsets using a FACS Aria IIb Cell Sorter (BD Biosciences). *In vitro* stimulation was performed for 72 h 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 rIL-2 (eBioscience). Co-cultures: $5x10^4$ sorted tumor-infiltrating $CD8^+$ T cells were incubated in a 1:1 ratio with responder $CD8^+$ 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). CD39 induction: $CD8^+$ T cells magnetically purified from mice dLNs nodes were stimulated *in vitro* in the presence of mouse recombinant cytokines: IL-6 (20 ng/mL; eBioscience), IL-27 (10 ng/mL; eBioscience) or maintained the last 24 h in a hypoxic atmosphere (1.5% O_2). Human PBMCs were stimulated with T Cell Activation-Expansion Kit (Miltenyi) according to manufacturer´s indications. Human $CD8^+$ T cells were purified using the CD8 Microbeads kit (Miltenyi). Then $2x10^5$ cells were stimulated for 72 h in the presence or absence of human recombinant IL-6 (20ng/mL; ImmunoTools) and/or IL-27 (10 ng/mL; eBioscience).

ATP hydrolysis assay

For CD39 activity evaluation, $5x10^4$ sorted cells were cultured for 10 min in phenol red-free RPMI-1640 with 20 µM of exogenous ATP (SIGMA). Cell-free medium with ATP alone was used as control. For ATP hydrolysis inhibition cells were incubated for 16 h in presence or absence of 250 µM ARL67156 and ATP was added in the last 10 min. 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 previously described (18). Spleen $CD8^+$ T cells were magnetically purified from tumor-free mice.

Immunohistofluorescence

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 manufacturer´s indications.

Statistical analysis

GraphPad Prism software was used for statistical analysis. In experiments with mice, unpaired Student's *t*-test was used to compare two groups. For human samples paired Student´s *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$^+$ tumor-infiltrating T lymphocytes

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

Similar to the B16F10-OVA model, CD8$^+$ T cells from mice bearing 4T1 and MCA-OVA tumors also showed a high frequency of CD39$^+$ cells (Supplementary Fig. S1B), while in lymphoid organs CD39$^+$CD8$^+$ T cells were present at low frequencies. In these models a large proportion of CD39$^+$CD8$^+$ TILs also co-expressed CD73. These results allow us to conclude that CD39 expression on CD8$^+$ TILs is a common feature across tumors of different histological 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$^+$ T cells, conventional Foxp3$^-$CD4$^+$ T cells (Tconv) and Tregs from B16F10-OVA tumor-bearing mice. Surprisingly, the levels of CD39 on CD8$^+$ TILs were comparable to 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$^+$CD8$^+$ TILs could be further dissected into two distinct subsets, one with intermediate expression (CD39^{int}) and the other with high expression (CD39^{high}) (Fig. 1C and D). Interestingly, around 50% of CD39-expressing CD8$^+$ T cells in tumors were CD39^{high}, while in dLNs and spleens this population was absent. Consistent with our observations in B16F10-OVA tumors, CD39^{high}CD8$^+$ T cells were also

exclusively present in 4T1 and MCA-OVA tumors but not in lymphoid organs (Supplementary Fig. S1C). Interestingly, $CD8^+$ TILs from mice bearing B16F10-OVA tumors showed higher frequency of $CD39^{high}$ 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 $CD39^{high}$ cells within $CD8^+$ TILs increased by days 17 and 24 p.i., compared to day 10 p.i., while $CD39^-$ and $CD39^{int}$ $CD8^+$ TILs decreased (Fig. 1E). Notably, the frequency of $CD73^+CD8^+$ T cells decreased with tumor progression (Fig. 1F). These results reveal that the up-regulation of CD39 in $CD8^+$ TILs is associated with tumor burden.

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

$CD39^{high}CD8^+$ TILs exhibit an effector memory phenotype and impaired IL-2 and TNF production

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

We next evaluated cytokine production and found that $CD39^+$ as well as $CD39^-CD8^+$ TILs were able to produce IFNγ when stimulated *ex vivo* with PMA/Ionomycin. Despite the fact that $CD39^{high}CD8^+$ TILs exhibited the highest percentage of $IFN\gamma^+$ cells, they showed a profound impairment in TNF and IL-2 production when compared to $CD39^-$ and $CD39^{int}CD8^+$ TILs (Fig. 2B). Furthermore, in accordance with their IFNγ-producer phenotype, $CD39^{high}CD8^+$ TILs exhibited the highest cytotoxic potential, evaluated by expression of Granzyme B, and CD107a mobilization (Fig. 2C). $CD39^{high}CD8^+$ TILs from B16F10-OVA tumor-bearing mice showed higher frequency of $IFN\gamma^+$ and Granzyme B^+ cells as well as higher proliferative potential than $CD39^{high}CD8^+$ TILs from B16F10 tumor-bearing mice, which may be attributed to the higher immunogenicity of the B16F10-OVA tumor. However, the frequency of TNF^+ and IL-2^+ cells within B16F10 $CD39^{high}CD8^+$ TILs was as low as that detected in the OVA-expressing melanoma (Supplementary Fig. S3A).

$CD39^{high}CD8^+$ TILs exhibit a phenotype associated with exhaustion

Exhausted $CD8^+$ T cells are characterized by early impairment in IL-2 and TNF secretion and expression of multiple iRs (10). Considering that $CD39^{high}CD8^+$ TILs exhibit significantly lower IL-2 and TNF production, we further evaluated iRs and TFs associated with exhaustion. Among $CD8^+$ TILs, $CD39^{high}CD8^+$ T cells exhibited the highest expression of all iRs tested (PD-1, Tim-3, LAG-3, TIGIT and 2B4) (Fig 3A). Similar profiles of iRs expression were also observed on $CD39^{high}CD8^+$ TILs in 4T1 and MCA-OVA tumors (Supplementary Fig. S3B). Consistent with the absence of $CD39^{high}CD8^+$ T cells in dLNs and spleens, no significant expression of iRs was observed on $CD39^-$ and $CD39^{int}CD8^+$ T cells from these organs (Supplementary Fig. S3C).

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

Analyzing antigen-specific $CD8^+$ TILs, we found that around 75% of OVA-specific $CD8^+$ TILs were $CD39^{high}$ and these cells exhibited higher expression of iRs than OVA-specific $CD39^{int}CD8^+$ TILs (Fig.

3B and C). After PMA/Ionomycin stimulation OVA-specific $CD39^{high}CD8^+$ TILs exhibited higher frequency of $IFN\gamma^+$ cells but reduced percentage of TNF^+ and $IL\text{-}2^+$ cells than OVA-specific $CD39^{int}CD8^+$ TILs (Fig. 3D). Upon $OVA^{257\text{-}264}$ specific peptide stimulation $CD39^{high}CD8^+$ TILs showed increased frequency of IFNγ-producing cells, but no significant induction of TNF and IL-2. When analyzing the proliferative potential of OVA-specific $CD8^+$ TILs, OVA-specific $CD39^{high}CD8^+$ TILs showed lower frequency of $Ki\text{-}67^+$ cells than $CD39^{int}CD8^+$ TILs after *in vitro* stimulation (Fig. 3E and F).

Intratumoral exhausted $CD8^+$ T cells show high expression of CD39 and ability to hydrolyze extracellular ATP

The co-expression of PD-1 and Tim-3 has been described as hallmark of $CD8^+$ T cell exhaustion in cancer and chronic infections (10,12). We identified $PD\text{-}1^+Tim\text{-}3^+CD8^+$ TILs in mice bearing B16F10-OVA tumors and determined that $PD\text{-}1^+Tim\text{-}3^+CD8^+$ TILs show higher expression of CD39 than $PD\text{-}1^+Tim\text{-}3^-$ and $PD\text{-}1^-Tim\text{-}3^-$ $CD8^+$ TILs, which exhibited intermediate and low expression of the ecto-enzyme, respectively (Fig. 4A). We further confirmed that $PD\text{-}1^+Tim\text{-}3^+CD8^+$ T cells ($CD39^{high}$) are functionally exhausted since, in contrast to the other $CD8^+$ T cell subsets, they showed cell cycle arrest (indicated by low frequency of $Ki\text{-}67^+$ cells), lower T-bet expression and decreased IFNγ production after *in vitro* re-stimulation with anti-CD3/anti-CD28 and rIL-2 for 72 h (Supplementary Fig. S4A and B). Collectively, our data support the concept that co-expression of PD-1 and Tim-3 along with high expression of CD39 mark the most exhausted population of $CD8^+$ TILs.

We next tested the ability of exhausted $CD8^+$ TILs to hydrolyze eATP. Consistent with their increased CD39 expression, sorted $PD\text{-}1^+Tim\text{-}3^+CD8^+$ TILs showed the highest capacity to hydrolyze eATP (Fig. 4B). In addition, $PD\text{-}1^+Tim\text{-}3^-CD8^+$ TILs, which have intermediate CD39 expression, exhibited higher eATP hydrolysis than $PD\text{-}1^-Tim\text{-}3^-CD8^+$ TILs and spleen $CD8^+$ T cells (Fig. 4B). In order to confirm that CD39 was responsible for ATP hydrolysis, exhausted $CD8^+$ TILs were pre-incubated 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 $CD8^+$ TILs is due to CD39 expression.

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

$CD39^+CD8^+$ 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 ecto-nucleotidases expression on $CD8^+$ T cells from human primary breast tumors, invaded/metastatic and non-invaded dLNs and peripheral blood. In primary tumors we noticed defined $CD39^+$ and $CD73^+$ $CD8^+$ T cell populations, although practically no cells co-expressed both ecto-enzymes. We observed a mean frequency of 18.5±4.3% $CD39^+CD8^+$ TILs (Fig. 5A, Supplementary Table S2), while a mean of 11.2±1.7% $CD8^+$ TILs exhibited CD73 expression. Interestingly, invaded/metastatic LNs (I-LNs) showed a higher frequency of $CD39^+CD8^+$ T cells than non-invaded LNs (NI-LNs) but reduced frequency of $CD73^+CD8^+$ T cells (Fig. 5B). Furthermore, $CD8^+$ T cells from peripheral blood from breast cancer patients did not exhibit CD39 expression (Fig. 5C). Our results associate the presence of $CD39^+CD8^+$ 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 $CD8^+$ T cells. Of note, we also observed that similar to the B16F10-OVA model, tumors from breast cancer patients are also infiltrated by $CD39^+CD4^+$ Tconv cells (Supplementary Fig. S5A).

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

We further evaluated the expression of iRs on $CD8^+$ T cells from tumors (Fig. 5D) and I-LNs (Fig. 5E). We first gated out naïve cells since they do not express iRs and may influence the results (gating out $CD45RA^+CD27^+$ cells). In comparison to their $CD39^-$ counterparts, $CD39^+CD8^+$ 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 $CD8^+$ TILs from other types of cancer (23-25).

Analysis of co-expression of iRs on $CD8^+$ T cell subsets revealed that the frequency of $CD8^+$ T cells that co-express two or more iRs was increased in $CD39^+$ population compared to $CD39^-CD8^+$ T cells (Supplementary Fig. S5C). The proportion of $CD39^+CD8^+$ 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 co-expressed iRs on human $CD8^+$ T cells depends on the anatomical localization (26). Alternatively, CD73 presence was not associated to iRs expression in LNs, in fact $CD39^-CD73^+CD8^+$ T cells did not express PD-1 (Supplementary Fig. S5D).

CD39-expressing $CD8^+$ T cells from cancer patients exhibit an impairment in effector cytokine production

The effector function of $CD39^+CD8^+$ T cells from tumors and I-LNs was assessed analyzing their ability to produce IL-2, IFNγ and TNF upon activation. To compare the effector response between $CD39^-$ and $CD39^+CD8^+$ T cells, we focused our analysis on non-naïve cells (gating out

$CD45RA^+CD27^+$ cells). $CD39^+CD8^+$ T cells from tumors (Fig. 6A) and I-LNs (Fig. 6B) exhibited reduced frequency of TNF and IFNγ-producing cells compared to $CD39^-CD8^+$ T cells. Furthermore, $CD39^+CD8^+$ T cells from I-LNs also showed a compromise in IL-2 production. In addition, $CD39^+CD8^+$ T cells from tumors and I-LNs exhibited higher frequency of CD107a+ cells than their CD39- counterparts (Fig. 6A and B).

In addition, we observed that melanoma patients also exhibited a higher frequency of $CD39^+CD8^+$ T cells in I-LNs, compared to NI-LNs. Moreover, $CD39^+CD8^+$ T cells showed high PD-1 and TIGIT expression and impairment in TNF and IL-2 production (Supplementary Fig. S6A, B and C).

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

$CD8^+$ T cells up-regulate CD39 expression upon *in vitro* TCR stimulation plus IL-6 and IL-27.

We further evaluated signals that may influence CD39 up-regulation. $CD8^+$ 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 the CD39 expression observed *ex vivo*, however, IL-27 drove CD39 expression on stimulated cells, especially when combined with IL-6 (Fig. 7A). Phenotypic characterization showed that most of these induced $CD39^+$ 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 IL-27 but not with IL-6 or IL-6/IL-27 combination (Fig. 7B).

Stimulation of PBMCs from breast cancer patients with anti-CD3/anti-CD28 was sufficient to induce an impressive CD39 expression on $CD8^+$ T cells (Fig. 7C). Next, to better identify signals that induce CD39 on $CD8^+$ T cells and to rule out the interaction with other mononuclear cells, we stimulated purified $CD8^+$ T cells with anti-CD3/anti-CD28. We observed a lower increase in CD39 expression in comparison to total PBMCs, but it was further upregulated in the presence of IL-27 or the combination of IL-6 and IL-27. Moreover, TCR stimulated $CD8^+$ T cells in presence of IL-27 plus IL-6 exhibited

higher expression of PD-1 than $CD8^+$ T cells stimulated with anti-CD3/anti-CD28 alone (Fig. 7D and E and Supplementary Table S3).

Although murine and human $CD8^+$ T cells express CD39 upon *in vitro* stimulation, these cells showed high frequency of $IFN\gamma^+$ and TNF^+ cells when stimulated with PMA/Ionomycin (Supplementary Fig. S7A and B), 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, $CD39^+CD45^+$ cells, were mostly localized within hypoxic foci in tumor sections. However, CD39 was not induced in $CD8^+$ 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 D).

Discussion

Increasing evidence suggests that cancer and immune cells closely interact to generate an immunosuppressive environment which 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 $CD8^+$ T cells in different experimental mice models exhibits high CD39 expression. Interestingly, CD39 was associated with a specific subset of $CD8^+$ T cells with exhaustion features. Moreover, $CD39^+CD8^+$ 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 $CD8^+$ TILs from all tested experimental models as well as in $CD8^+$ T cells from cancer patients, CD73 was only co-expressed by $CD8^+$ 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 histological origin of tumors. Evidences demonstrate that expression and function of CD73 are both increased by TGFβ or under hypoxic conditions (31,32). In contrast, IL-4, IL-21, IL-12 and IFNγ prevent CD73 expression induced by TGFβ (31). Then, it is possible that each experimental model denotes a particular inflammatory microenvironment that differentially affects CD73 expression

on $CD8^+$ TILs. Our observations in B16F10-OVA tumor-bearing mice and cancer patients are in agreement with reports on HIV^+ and follicular lymphoma patients where $CD39^+CD8^+$ T cells did not co-express CD73 (18,33).

$CD39^+CD8^+$ 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 $CD45^+$ tumor-infiltrating cells expressing CD39 emphasizes the idea that mouse as well as human tumor microenvironment command CD39 expression. IL-6 and IL-27 have been linked to CD39 expression as well as induction of iRs and TFs related to exhaustion on DCs, $CD4^+$ T cells and other leukocytes (27,28,34,35). We showed that these cytokines induce CD39 on murine and human TCR-stimulated $CD8^+$ T cells which is more evident when combining IL-6 and IL-27, 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 $CD8^+$ T cells supporting the idea that signals provided by other mononuclear cells may work in synergy to up-regulate CD39.

In mice, $CD39^{high}CD8^+$ TILs exhibit high frequency of $IFN\gamma^+$, Granzyme B^+ and $CD107a^+$ cells, all features of a Tc1-*like* phenotype. These findings are in agreement with observations made by Bai *et al.* (36) who described $CD39^+CD8^+$ T cells with similar functionality in Chron's disease. However, the reduced production of IL-2 and TNF together with the expression of multiple inhibitory receptors define $CD39^{high}CD8^+$ TILs as exhausted cells. In fact, $CD39^{high}CD8^+$ T cells can be precisely identified on the basis of PD-1 and Tim-3 co-expression. Moreover, in models of chronic viral infections, terminally exhausted T cells display a T-$bet^{low}Eomes^{high}Blimp\text{-}1^{high}$ phenotype (15,16) that is shared by $CD39^{high}CD8^+$ 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 $CD39^{high}CD8^+$ T cells from B16F10-OVA experimental cancer model are not yet terminally exhausted, but reach terminal

exhaustion upon re-stimulation, as we observed *in vitro* with re-stimulated $CD39^{high}PD\text{-}1^{+}Tim\text{-}3^{+}CD8^{+}$ TILs.

Analyzing $CD39^{+}CD8^{+}$ 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 $CD39^{+}CD8^{+}$ T cells showed reduced production of IFNγ even without further *in vitro* re-stimulation. Altogether, our findings led us to conclude that CD39-expressing $CD8^{+}$ T cells exhibit a dysfunctional phenotype in mouse cancer models as well as in patients with breast cancer and melanoma. Recently, Gupta *et al*. (37) described that human HIV and HCV-specific $CD8^{+}$ T cells as well as mice LCMV-specific $CD8^{+}$ T cells express CD39. These cells also co-express 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 $CD39^{+}CD8^{+}$ T cells do not express CD73, which is in agreement with data that demonstrated that low expression of CD73 on $CD8^{+}$ T cells correlates with cell exhaustion in HIV^{+} 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 immunological switches that shift ATP-driven pro-inflammatory immune cell activity toward an anti-inflammatory state mediated by adenosine (39). Our findings show that mouse exhausted $CD8^{+}$ T cells express CD39 at levels comparable to tumor-infiltrating Tregs, and that vigorously hydrolyze eATP. This feature highlights the potential of exhausted $CD8^{+}$ T cells to contribute to the suppressive tumor microenvironment by modulating ATP/ADP balance even if these cells do not co-express CD73. In fact, Mascanfroni *et al.* (34) have shown that $CD39^{+}CD73^{-}$ Tr1 cells are able to produce adenosine in cooperation with other $CD73^{+}$ populations. Reinforcing our hypothesis, Parodi *et al.* (19) described *in vitro* suppressive functionality of $CD39^{+}CD8^{+}$ T cells obtained from human tumors, while other reports also described modulatory functions of *in vitro*-induced $CD39^{+}CD8^{+}$ T cells (40). In

addition, Bai *et al.* (36) demonstrated that *in vitro*-induced $CD39^{+}$Tc1 cells modulate IFNγ responses of $CD39^{-}CD8^{+}$ T cells via adenosine production. Accordingly, $CD39^{high}PD\text{-}1^{+}Tim\text{-}3^{+}CD8^{+}$ TILs showed *in vitro* capacity to decrease the frequency of IFNγ-producing cells, although in our settings the modulation was independent of CD39 or CD73. An alternative mechanisms by which $CD39^{high}CD8^{+}$ T cells may contribute to the immunosuppressive tumor environment is through their sustained IFNγ production that may promote PD-L1 expression on tumors as previously reported (41,42), and consequently, favor the negative regulation of $PD\text{-}1^{+}$ effector immune cells. It is tempting to speculate that the tumor immunosuppressive microenvironment drives the loss of effector functions on $CD8^{+}$ T cells, while simultaneously triggers the acquisition of regulatory molecules. These two processes may complement each other to divert $CD8^{+}$ T cell immunity from one with anti-tumoral 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 $CD8^{+}$ T cell effector functions and anti-tumor immunity.

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Figure Legends:

Figure 1. **B16F10-OVA tumor-infiltrating CD8$^+$ T cells exhibit high expression of CD39**. **A** to **D**, cells obtained at day 17 p.i. (D17) from tumors, dLNs and spleens. **A**, Representative density plots and graphs show frequency of CD39$^+$ and CD73$^+$ cells in CD3$^+$CD8$^+$ T cells. **B**, Histograms and graphs show CD39 expression on Tregs, CD4$^+$ Tconv and CD8$^+$ T cells. **C**, Frequency of CD39$^-$, CD39^{int} and CD39^{high} (CD39hi) CD8$^+$ T cells. **D**, Frequency of total CD39$^+$ (CD39^{int} + CD39^{high}) and CD39^{high} cells in CD3$^+$CD8$^+$ T cells. **E**, Frequency of CD39$^-$, CD39^{int} and CD39^{high} CD3$^+$CD8$^+$ TILs during tumor progression, mean values and statistical analyses (D10 vs. D17 and D24) are shown. **F**, Frequency of CD73$^+$ cells in CD3$^+$CD8$^+$ TILs at different days p.i. All results are representative of 3 to 5 independent experiments (*n*=6-15 mice per experiment). **A**, **B**, **D**-**F**: data presented as mean ± SEM. ns, non significant; * or # *P*≤0.05; ***P*≤0.01; *** or ### *P*≤0.001; **** or †††† *P*≤0.0001.

Figure 2. **B16F10-OVA CD39^{high}CD8$^+$ TILs exhibit an effector/memory phenotype and a particular profile of effector functions**. TILs obtained at day 17 p.i. **A**, Frequency of cells expressing CD44 and CD62L within CD3$^+$CD8$^+$ TILs. **B** and **C**, Frequency of CD3$^+$CD8$^+$ TILs showing different expression of CD39 that produce IFNγ, TNF and IL-2 (**B**) and express Granzyme B and CD107a (**C**) after PMA/Ionomycin stimulation. All results are representative of 3 to 5 independent experiments (*n*=6-15 mice per experiment). Data presented as mean ± SEM. ns, non significant; **P*≤0.05; ***P*≤0.01; ****P*≤0.001; *****P*≤0.0001.

Figure 3. **High expression of CD39 identifies CD8$^+$ T cells that express iRs associated with exhaustion.** TILs obtained at day 17 p.i. **A**, Expression of iRs in total CD39$^-$, CD39^{int} and CD39^{high}

$CD8^+$ TILs. **B**, Representative plots show frequency of OVA-specific $CD8^+$ TILs and plot and graph shows frequency of $CD39^+$ cells within OVA-specific $CD8^+$ 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 $CD39^{int}$ and $CD39^{high}$ OVA-specific $CD8^+$ TILs. **D**, Frequency of $IFN\gamma^+$, TNF^+ and $IL\text{-}2^+$ cells within OVA-specific $CD39^{high}CD8^+$ TILs versus $CD39^{int}CD8^+$ TILs after stimulation with PMA/Ionomycin. **E**, Frequency of cytokine-producing $CD39^{high}CD8^+$ TILs after stimulation with $OVA^{257\text{-}264}$ peptide or SIY peptide (control). **F**, Frequency of $Ki\text{-}67^+$ cells within $CD39^{int}$ and $CD39^{high}$ OVA-specific $CD8^+$ TILs after anti-CD3/anti-CD28 stimulation. All results are representative of 2 to 5 independent experiments (*n*=10-15 mice per experiment). Data presented as mean ± SEM. ns, non significant; **P*≤0.05; ***P*≤0.01; ****P*≤0.001; *****P*≤0.0001.

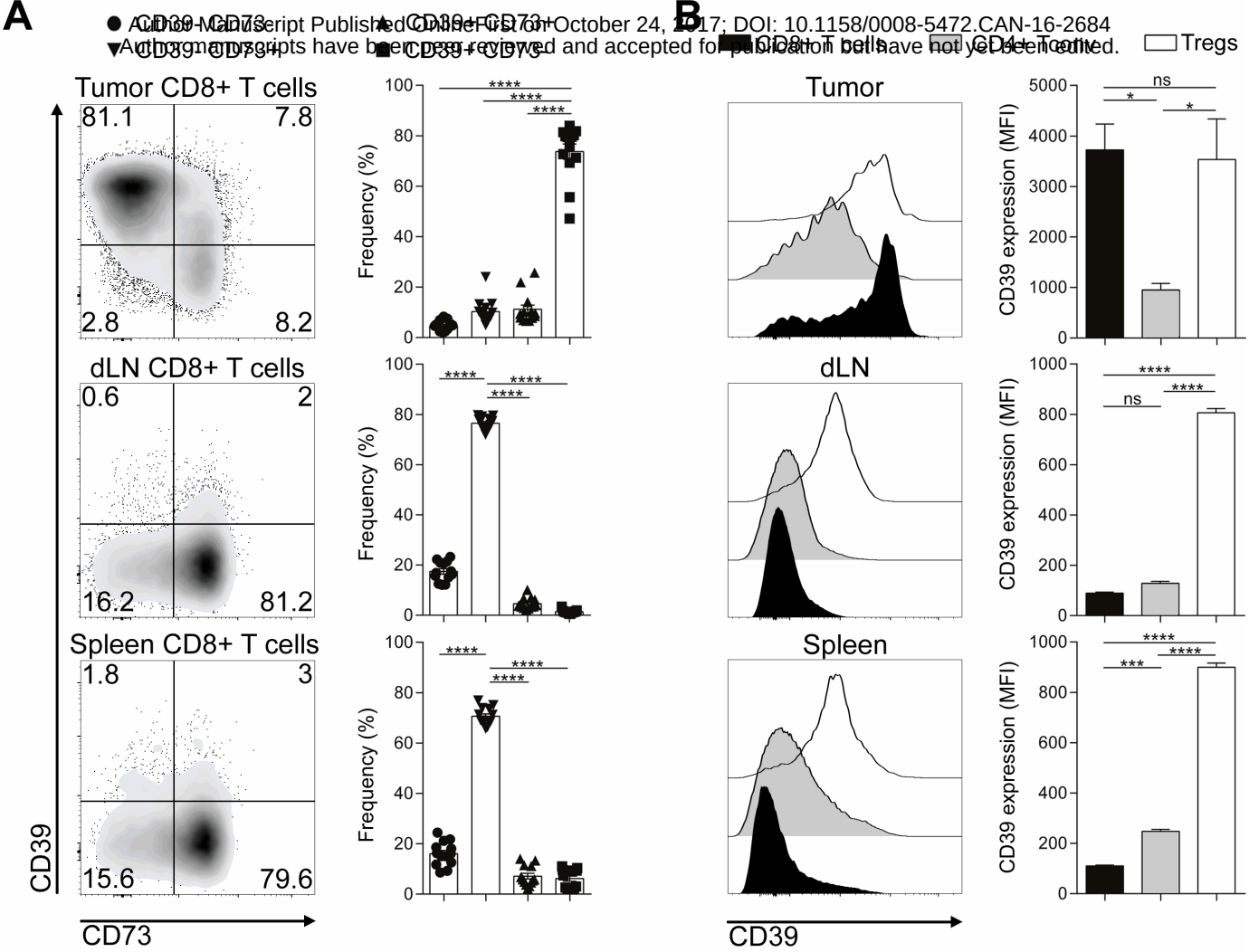
Figure 4. **Exhausted $CD8^+$ TILs exhibit high expression of enzymatically active CD39 and modulate IFNγ production by spleen $CD8^+$ T cells.** TILs obtained at day 17 p.i. **A**, CD39 expression on $PD\text{-}1^-Tim\text{-}3^-$, $PD\text{-}1^+Tim\text{-}3^-$ and $PD\text{-}1^+Tim\text{-}3^+$ $CD8^+$ TILs. **B**, Percentage of ATP hydrolysis after culture of sorted $CD8^+$ TIL subsets incubated with exogenous ATP (*n*=3, representative from two independent experiments). **C**, Percentage of eATP hydrolysis by $PD\text{-}1^+Tim\text{-}3^+CD8^+$ TILs pre-incubated or not with ARL67156 (*n*=2, data from two independent experiments performed with pools of cells from 6 mice per pool). **D**, Frequency of $IFN\gamma^+$ responder cells ($CD8^+$ T cells from CD45.1 tumor-free mice), stimulated alone or in co-culture with $CD45.2^+$ $PD\text{-}1^-Tim\text{-}3^-$ or $PD\text{-}1^+Tim\text{-}3^+CD8^+$ TILs (1:1 ratio). **E**, Frequency of $IFN\gamma^+$ responder cells described in **D** (*n*=11, data from 6 independent experiments). **A**: results are representative of 5 independent experiments (*n*=6-15 mice per experiment). **A**-**C**, **E**: data presented as mean ± SEM. ns, non significant; **P*≤0.05; ***P*≤0.01; ****P*≤0.001; *****P*≤0.0001.

Figure 5. **$CD39^+CD8^+$ T cells infiltrate human breast tumors and invaded/metastatic lymph nodes and exhibit high expression of iRs**. **A**, Representative dot plot shows frequency of $CD39^+$ and $CD73^+$ cells in $CD3^+CD8^+$ TILs (one out of nine breast tumor samples). **B**, Frequency of $CD39^+$

and $CD73^+$ cells in $CD3^+CD8^+$ T cells from NI-LNs and I-LNs from breast cancer patients (*n*=11). **C**, Representative dot plot shows frequency of $CD39^+$ and $CD73^+$ cells in peripheral blood (PB) $CD3^+CD8^+$ T cells (one out of seven samples). **D** and **E**, Expression of PD-1, TIGIT and BTLA on $CD39^-$ and $CD39^+$ $CD8^+$ T cells from breast tumors (**D**) and I-LNs from breast cancer patients (**E**) (*n*=3-7). Lines indicate that data are paired.

Figure 6. **Human $CD39^+CD8^+$ T cells from tumors and invaded/metastatic lymph nodes exhibit impaired production of effector cytokines.** Frequency of cytokine-producing cells (IL-2, TNF and IFNγ) and $CD107a^+$ cells in $CD39^-$ and $CD39^+$ $CD8^+$ 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 $CD45RA^+CD27^+$ cells). Lines indicate that data are paired.

Figure 7. **CD39 expression is promoted on $CD8^+$ T cells by TCR stimulation plus IL-6 and IL-27.** **A**, $CD8^+$ T cells from dLNs from B16F10-OVA tumor-bearing mice (D17) were stimulated in the presence of recombinant IL-6 and/or IL-27. Frequency (density plots and graph) of $CD39^+CD8^+$ T cells induced in each condition. **B**, Frequency of $CD73^+$, PD-1^+ and Tim-3^+ cells within mouse $CD39^+CD8^+$ T cells induced in each condition. **C**, Frequency of $CD39^+$ and $CD73^+$ $CD3^+CD8^+$ T cells from PBMCs from breast cancer patient stimulated with anti-CD3/anti-CD28 or unstimulated. **D**, Frequency of $CD39^+$ and $CD73^+$ cells within $CD8^+$ T cells purified from PBMCs from breast cancer patient after anti-CD3/anti-CD28 stimulation in presence or absence of IL-6 and/or IL-27. **E**, Representative histograms show PD-1 expression (MFI indicated) on $CD39^+CD8^+$ T cells in each condition. **A** and **B**: data presented as mean ± SEM. ns, non significant; **P*≤0.05; *****P*≤0.0001.



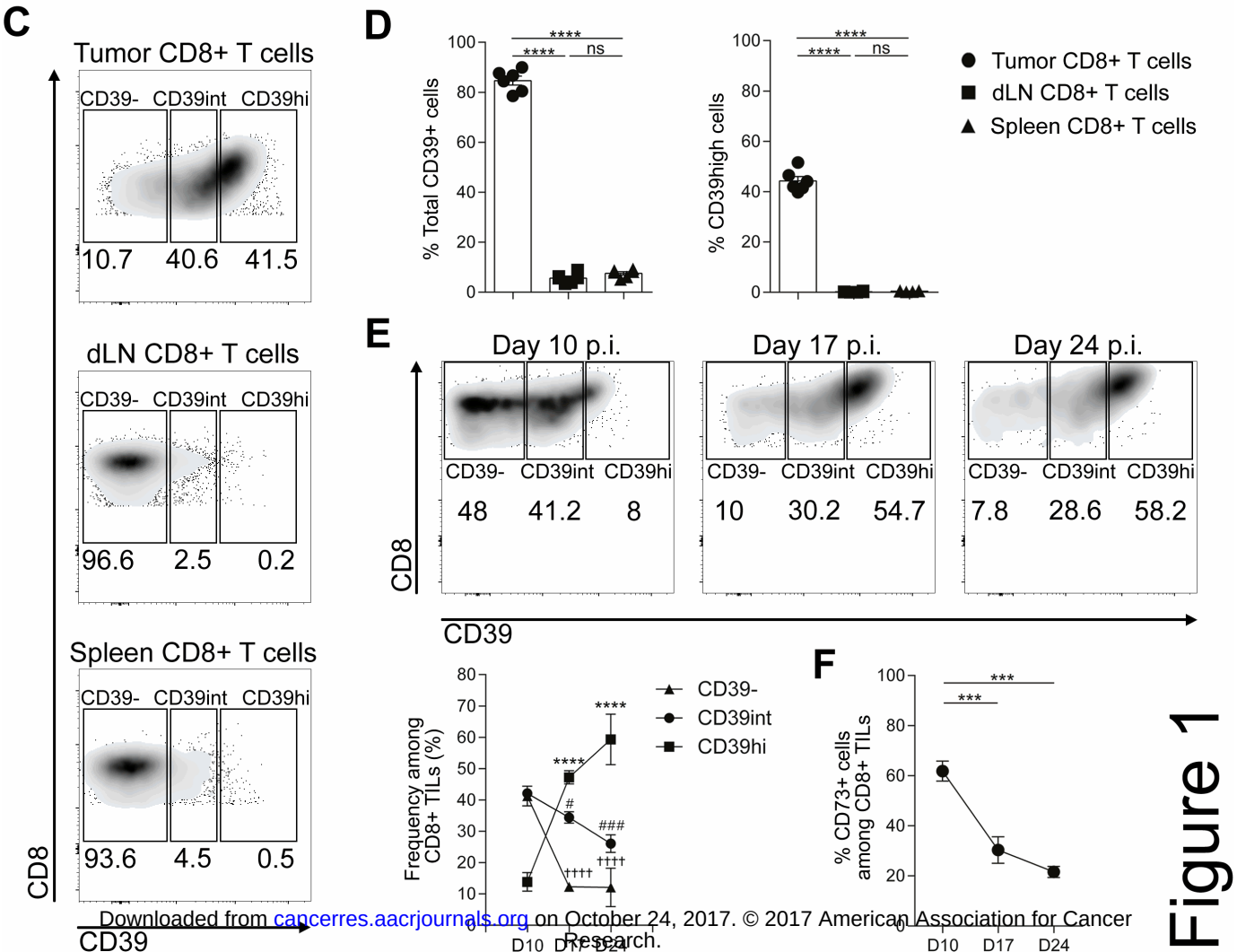


Figure 1

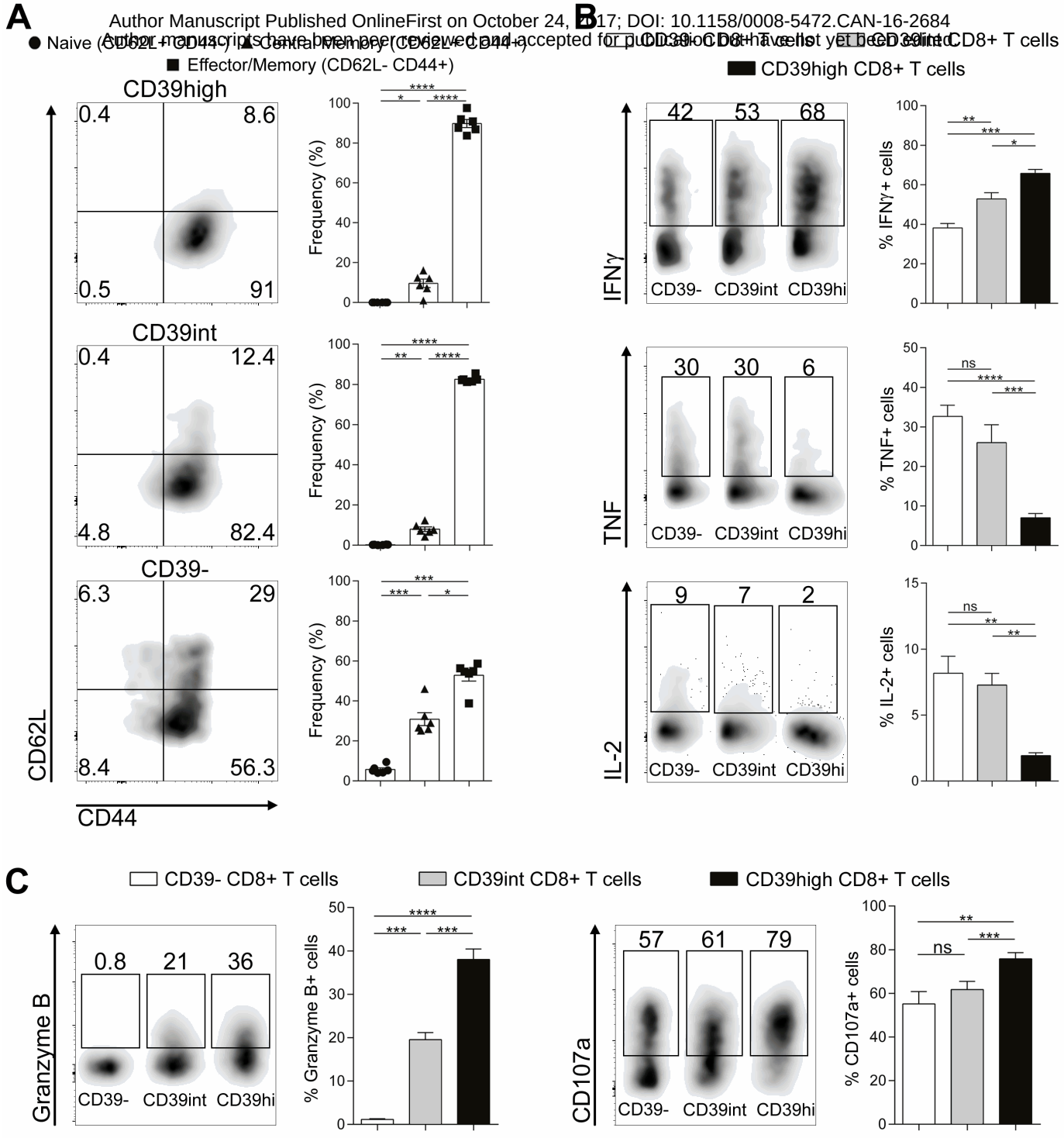


Figure 2

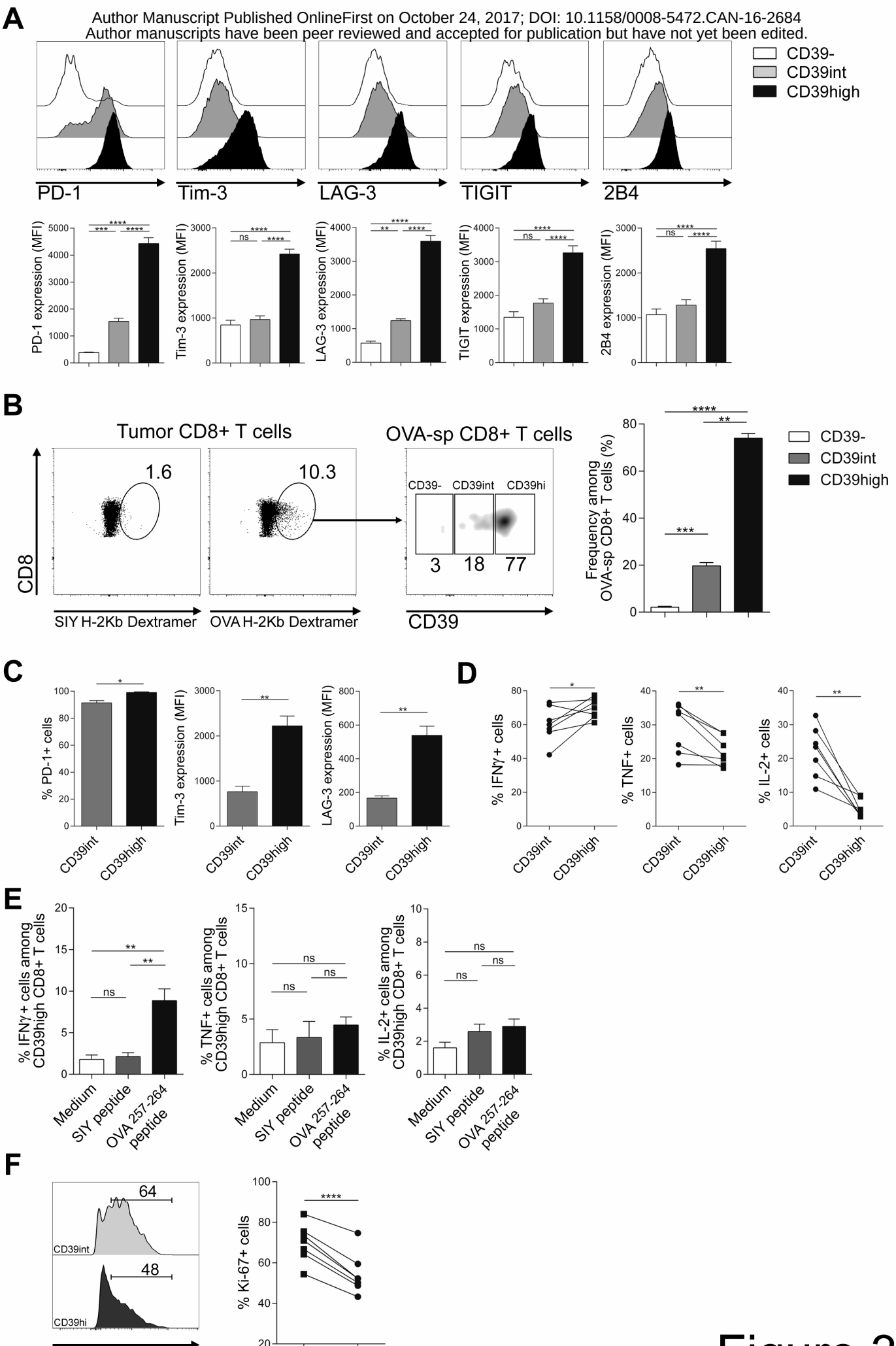


Figure 3

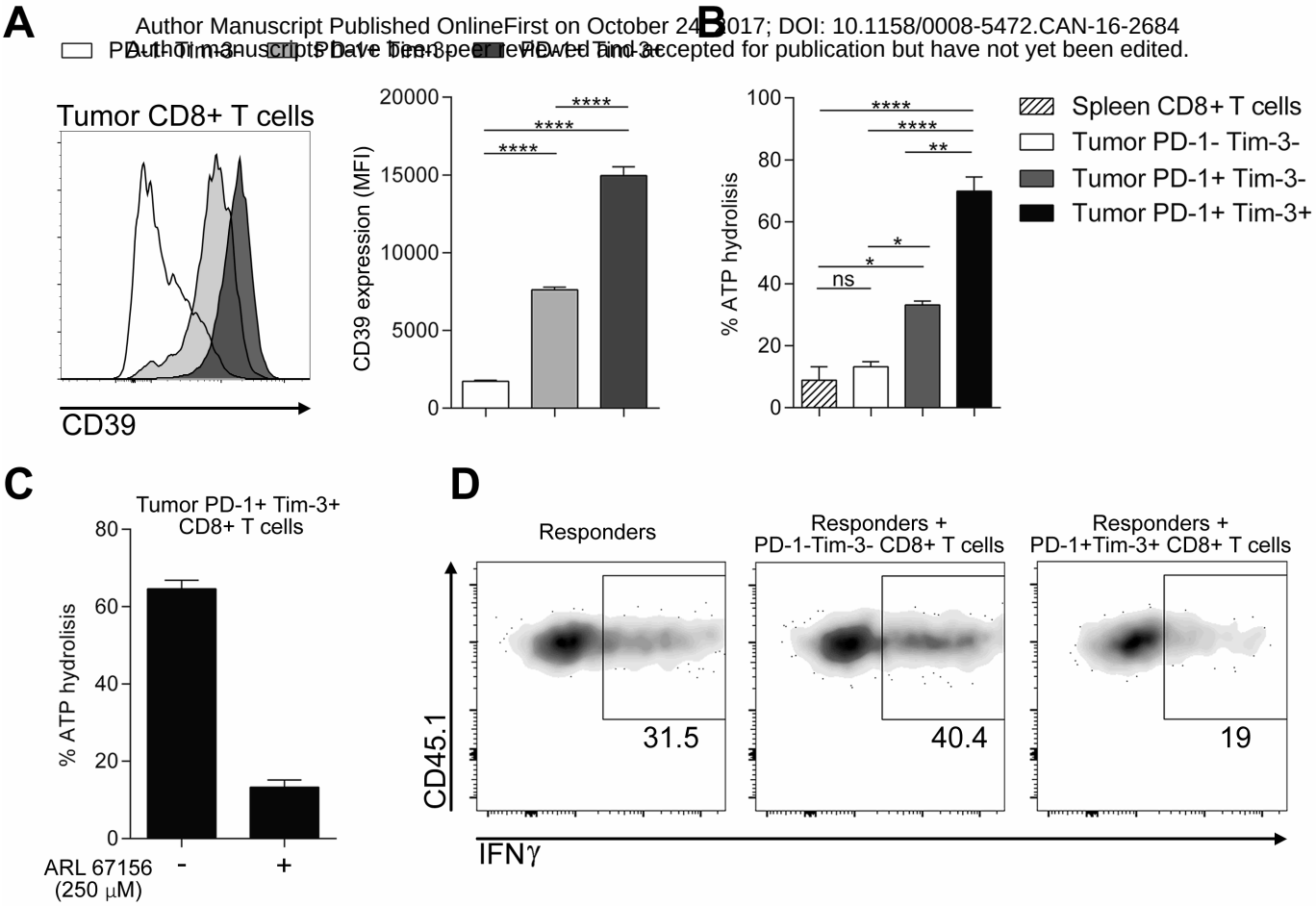


Figure 4

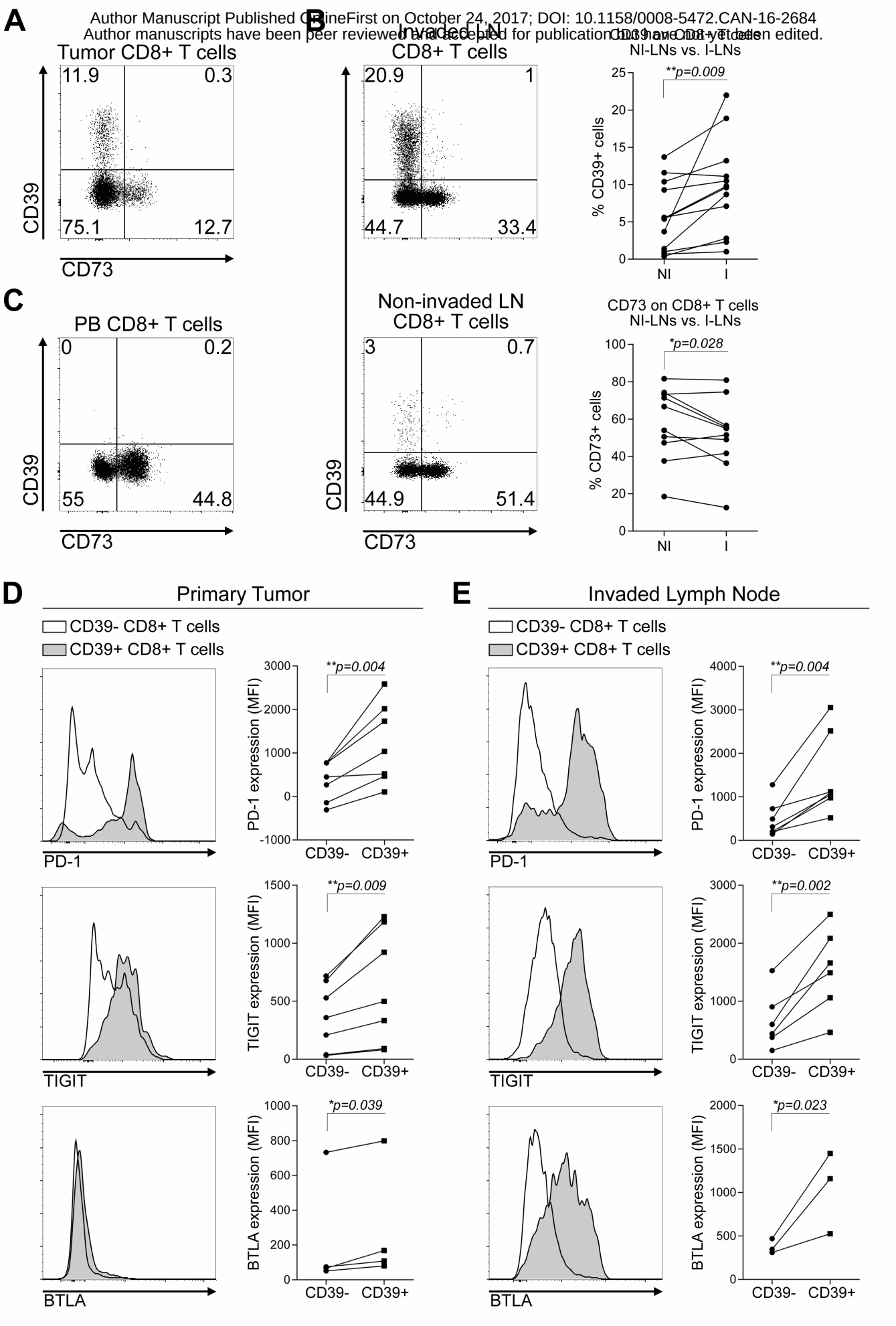
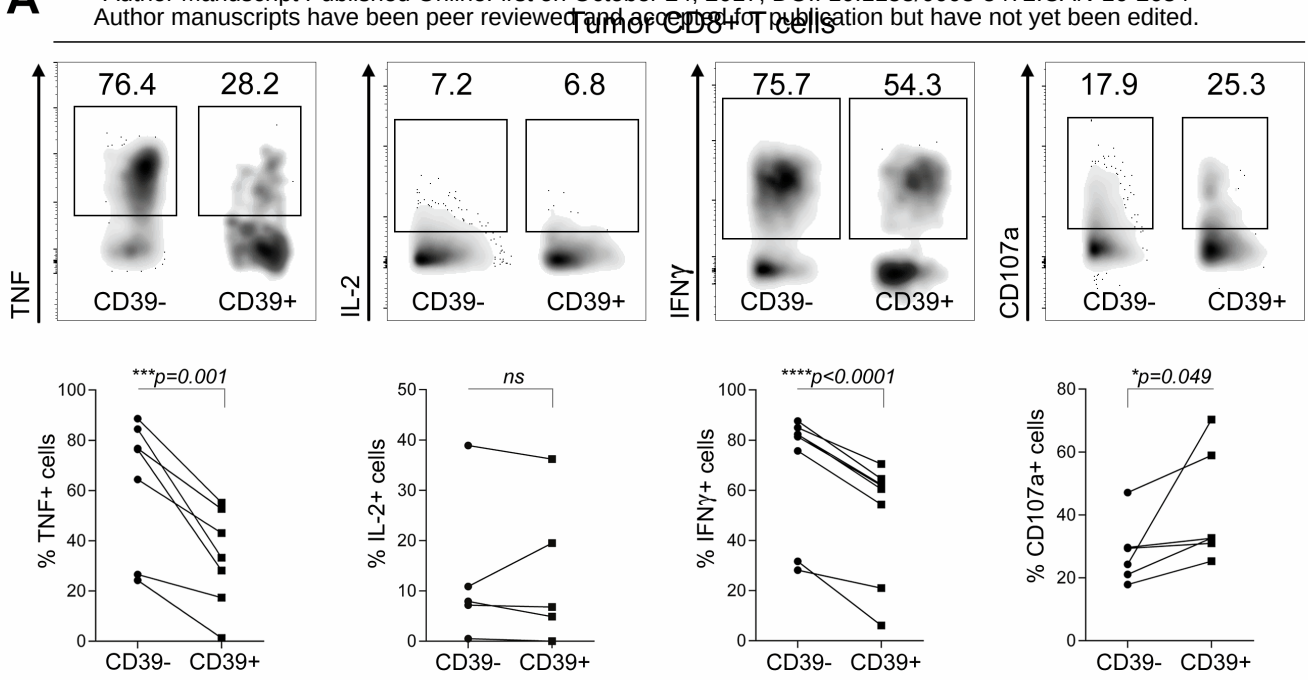


Figure 5



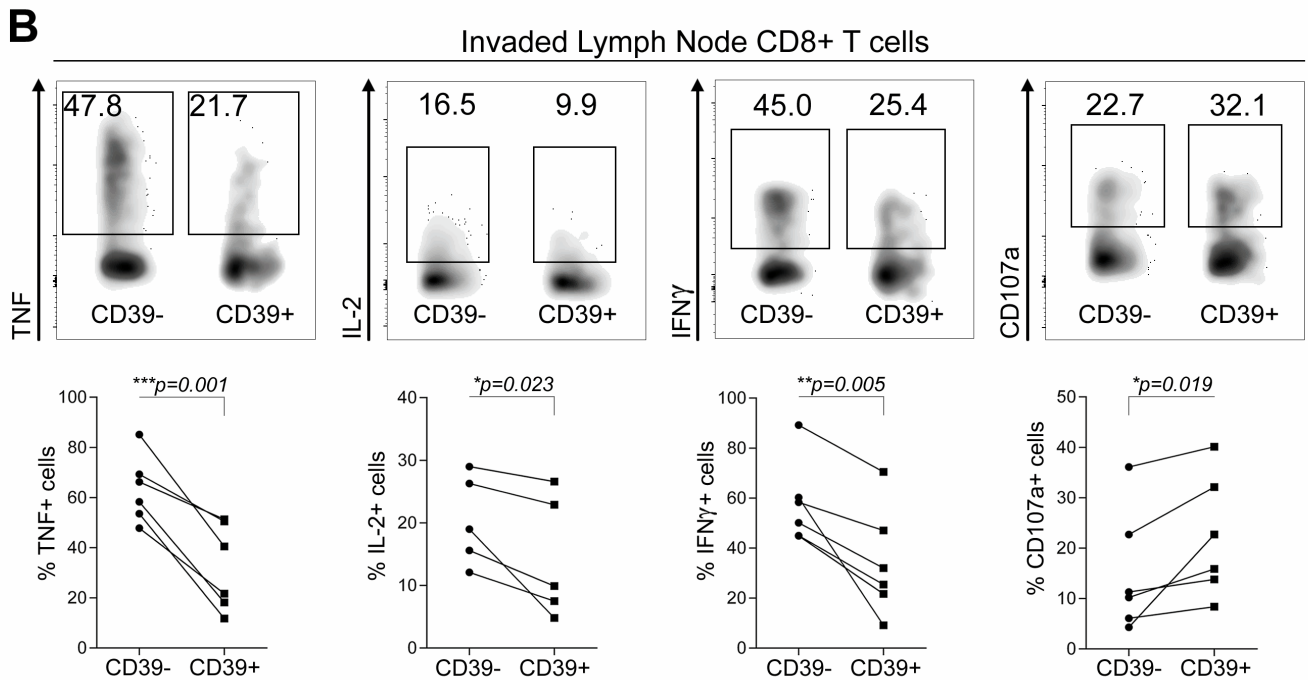


Figure 6

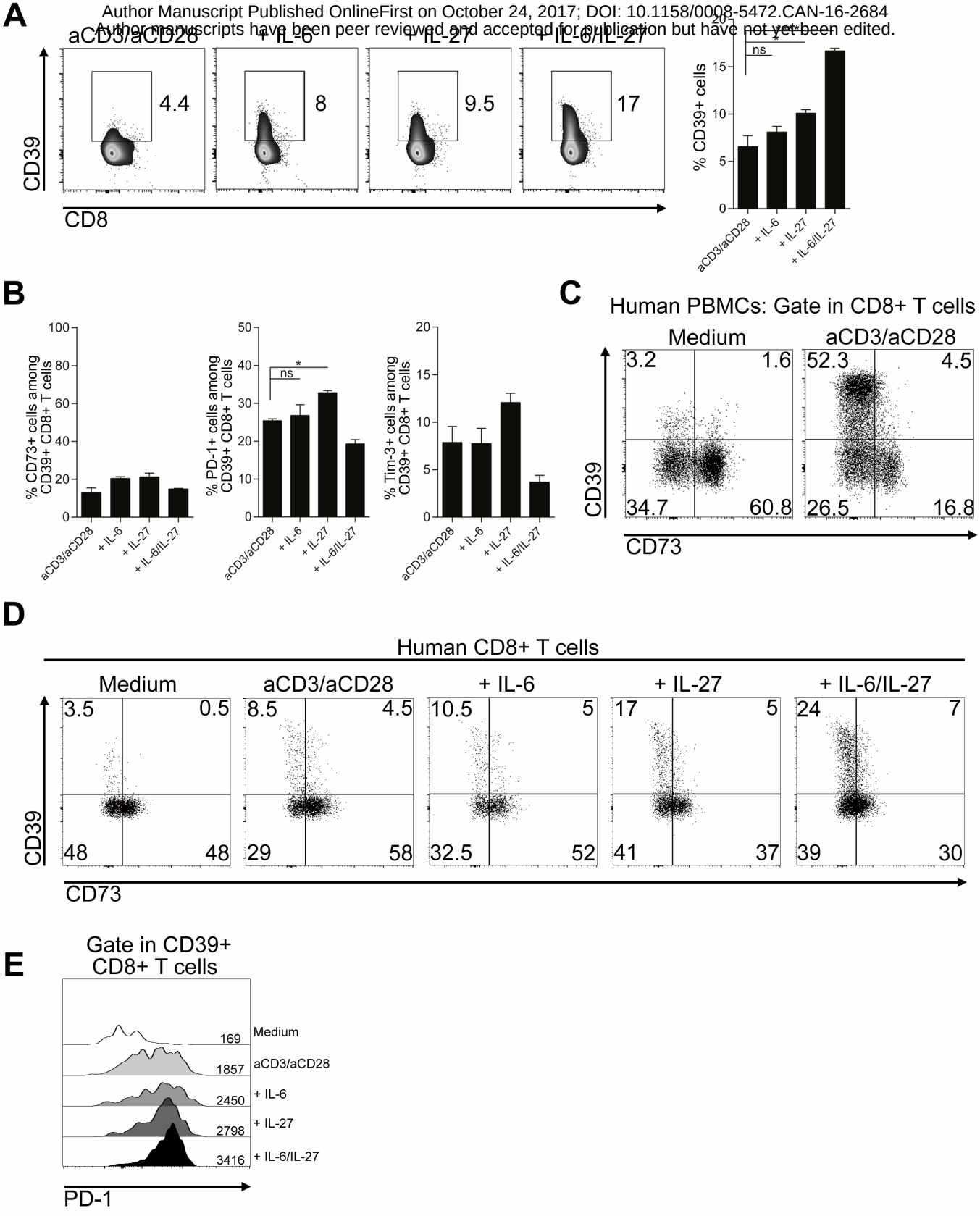


Figure 7

AACR American Association for Cancer Research

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

CD39 expression defines cell exhaustion in tumor-infiltrating CD8+ T cells

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