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Title : Autocrine Adenosine regulates tumor polyfunctional CD73⁺CD4⁺ effector T cells devoid of immune checkpoints

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Running title: Autocrine Ado regulates polyfunctional CD73⁺CD4⁺ T cells

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Abstract

The production of CD73-derived Adenosine (Ado) by Tregs, has been proposed as a resistance mechanism to anti-PD1 therapy in murine tumor models. We reported that

Human Tregs express the ecto-nucleotidase CD39, that generates AMP from ATP, but do not express the AMPase CD73. In contrast, CD73 defined a subset of effector CD4⁺ T cells (Teffs), enriched in polyfunctional Th1.17 cells characterized by expression of CXCR3, CCR6 and MDR1 and production of IL-17A/IFN- γ /IL-22/GM-CSF. CD39⁺ Tregs selectively targeted CD73⁺ Teffs through cooperative degradation of ATP into Ado inhibiting and restricting the ability of CD73⁺ Teffs to secrete IL-17A.

CD73⁺ Teffs infiltrating breast and ovarian tumors, were functionally blunted by Tregs expressing upregulated levels of CD39 and ATPase activity. Moreover, tumor-infiltrating CD73⁺ Teffs failed to express inhibitory immune checkpoints suggesting that CD73 might be selected under pressure from immune checkpoint blockade therapy and thus may represent a non-redundant target for restoring antitumor immunity.

Edited significance: Polyfunctional CD73⁺ T cell effectors lacking other immune checkpoints are selectively targeted by CD39 overexpressing Tregs that dominate the breast tumor environment.

Introduction

During infection or tumor development, adenosine triphosphate (ATP) is released into the extracellular space and can be found at high levels in inflamed tissues, including tumors (1). Extracellular ATP represents a pro-inflammatory alarmin for the immune system. It induces the chemo-attraction of dendritic cells (DC) and the activation of inflammasomes and IL-1 β secretion by monocytes/macrophages (M ϕ) through the engagement of P2 purinergic receptors (for review: (2)). Interestingly, the degradation of ATP into adenosine (Ado) by murine CD4⁺ regulatory T cells (Tregs), co-expressing CD39 and CD73, has been associated with immunosuppression (3), particularly within tumor environment (4, 5). Mechanistically, CD39 (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1) degrades ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP), which is then hydrolyzed by CD73 (5'-ectonucleotidase, NT5E) into Ado. Ado can favor tumor progression by inhibiting the function of immune cells (T cells, monocytes, DCs, M ϕ), through the engagement of its receptors (AdoR) A2a or A2b (for review: (6)). These AdoR induce an increased concentration of intracellular cyclic AMP, resulting in the inhibition of proliferation, cytotoxic functions and cytokine secretion (IL-2, TNF- α , IFN- γ and IL-13) of T

cells (for review: (6)). Ado can act also on innate immune cells by inhibiting IL-12 production by DCs (7, 8), and inducing IL-10 and VEGF secretion by M ϕ which favor immunosuppression and angiogenesis (9, 10). Moreover, Ado can act on non-immune cells, such as tumor cells, through A1 or A3 receptors coupled to G α i proteins, fostering tumor cell proliferation and migration (for review: (6)). The importance of Ado signaling in immunosuppression is evidenced in patients with Severe Combined Immunodeficiency (SCID), in whom accumulation of Ado, due to a lack of adenosine deaminase (ADA) enzyme that degrades Ado into Inosine, is observed (11). Remarkably, in CD73-deficient mice, in mice with CD73-deficient Tregs, or in A2a-deficient mice, the antitumor immune response is increased, leading to the tumor rejection and to the inhibition of metastases (12-15). Also, in murine models, targeting of CD73 alone or in combination with immunotherapy (anti-CTLA-4 or anti-PD-1) enhances tumor rejection and blocks metastases, demonstrating that endogenous Ado limits the efficacy of these immunotherapies (16, 17).

Studies investigating the expression of CD73 and CD39 on human T cells subsets remain scarce. In fact, despite the poor prognostic value of Tregs in breast and ovarian tumors (18, 19), the co-expression of CD39 and CD73 on human Tregs remains controversial (20-23). A recent report by Doherty *et al* also suggests the interconnection between CD73 expression on memory CD4⁺ T cells and a Th17 profile (24).

Th17 cells, implicated in the response to extracellular pathogens, are characterized by the secretion of IL-17A, IL-17F and IL-22. Recently, the involvement of a subset of Th17 cells, called Th1.17 co-producing IL-17A, IFN- γ and GM-CSF, in the development of autoimmune diseases, was unveiled (25), highlighting the polyfunctionality of these cells (26, 27). In tumor biology, a pro-tumoral role of Th17 was reported and mainly associated with the secretion of IL-17A, however, co-production of IFN- γ and GM-CSF was not considered, except in a recent report, demonstrating that Th1.17 cell subset contributes to tumor rejection (28).

In the present work, we demonstrate that CD73, not detected on human Tregs, defines a subpopulation of CD4⁺ effector T cells (Teffs) associated with cytokine polyfunctionality similar to that described in Th1.17 cells. CD73⁺CD4⁺ Teffs cooperate with CD39⁺ Tregs to degrade ATP into immunosuppressive Ado inhibiting and restricting function of CD73⁺CD4⁺ Teffs to IL-17A secretion. This cooperation does not require cell contact and, in a spaced-out microenvironment, induces specific inhibition of CD73⁺CD4⁺ Teffs through autocrine ATP-

derived Ado action without affecting distant CD73^{neg}CD4⁺ Tregs. Interestingly, we reveal that CD73⁺CD4⁺ Tregs are present in breast and ovarian tumor environment together with CD39^{high} Tregs and express only low levels of inhibitory immune checkpoints (i-ICPs) highlighting CD73 as a potential resistance mechanism to current immunotherapies, and as a non-redundant target for restoring antitumor immunity.

Material and methods

Human samples

HD-blood was purchased anonymously from the French Blood Service (EFS). In addition, blood and primary tumor samples were obtained from patients with non-pretreated breast tumors and chemotherapy-pretreated ovarian tumors. All of these samples were provided by the tissue bank (BRC) of Léon Bérard Cancer Center (CLB), after approval from the institutional review board and ethics committee (L-06-36 and L-11-26) and patients' written informed consent, in accordance with the Declaration of Helsinki. Human healthy colon endoscopic biopsies obtained during routine endoscopic check-ups of healthy donors selected on the basis that they were neither under chemotherapy nor taking antibiotics were obtained from the BRC of the CLB after approval from the institutional review board and ethics committee (French agreement number: AC-2013-1871) and donors' written informed consent.

Purification of the different cell subsets

PBMC were purified from blood of HDs or cancer patients by Ficoll density gradient. Memory CD4⁺ T cells were purified using MagniSortTM Human CD4⁺ Memory T Cell Enrichment Kit (eBioscience). CD73^{neg}CD4⁺ Tregs (CD4⁺CD45RA^{neg}CD127⁺CD25^{neg}CD39^{neg}CD73^{neg}), CD73⁺CD4⁺ Tregs (CD4⁺CD45RA^{neg}CD127⁺CD25^{neg}CD39^{neg}CD73⁺) and total Tregs (CD4⁺CD45RA^{neg}CD127^{neg}CD25⁺) or CD39⁺ and CD39^{neg} Treg subsets were sorted from purified memory CD4⁺ T cells by multi-parametric FC (FACS Aria III, BD Biosciences) using antibodies against CD25 (2A3, BD-Biosciences), CD45RA (2H4LDH11LDB9, Beckman-Coulter), as well as CD127 (eBioRDR5), CD39 (eBioA1) and CD73 (AD2) (all from eBioscience), alongside a viability marker (DAPI). Infiltrating CD4⁺ T cells from breast and ovarian tumors and healthy colonic tissues were isolated from single cell suspensions, obtained by enzymatic disaggregation (18), with the Dynabeads Human CD4⁺ Kit (Life Technologies).

Tumor-infiltrating CD4⁺ Teffs (CD127⁺CD25^{neg}) and Tregs (CD127^{neg}CD25⁺) were sorted from single cell suspensions by multi-parametric FC using antibodies against CD127, CD25, CD45RA, and a viability marker (Fixable Viability Dye, Biolegend).

Flow cytometry analyses

Multi-parametric FC analyses were performed on i) PBMC from HDs or cancer patients, or on ii) single cell suspensions derived either from primary breast and ovarian tumors or healthy colonic tissues. The FC panels used to assess T cell differentiation relied on the use of anti-human antibodies against CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), CD95 (DX2) and CD28 (CD28.2) (all from BD-Biosciences), CD27 (O323, eBioscience), CCR7 (G043H7, Biolegend), CD45RA, CD39 and CD73 (see above), while those used to evaluate T cell polarization relied on the use of the anti-human antibodies against CD3, CD4, CD45RA, CD127, CD25, CD39 and CD73 (see above), CCR6 (11A9, BD-Biosciences), CXCR3 (G025H7) and CRTH2 (BM16) from Biolegend, and a viability marker. The i-ICPs panel included antibodies against CD3, CD4, CD8 (see above), CD45 (HI30), PD-1 (EH12.1), TIM-3 (7D3) (all from BD-Biosciences), CD45RA (HI100) and CTLA-4 (L3D10) (Biolegend), TIGIT (MBSA43), CD39 and CD73 (eBioscience) and a fixable viability marker (Zombie Fixable Viability Dyes, Biolegend). The production of intracellular cytokines on CD73⁺CD4⁺ and CD73^{neg}CD4⁺ Teffs was analyzed using the test previously described (29) with some modifications, in particular the evaluation of IL-22 (22URTI, BD-Biosciences) instead of IL-21. Cells were analyzed on a LSR-Fortessa (BD Biosciences) and data were processed using the FlowJo Software (Tree Star). For the co-expression of i-ICPs, data were analyzed using the Boolean method on the FlowJo software and then represented using SPICE v5.3 software.

MDR1 staining and activity assay

Purified memory CD4⁺ T cells were incubated with Rh123 (1µg/ml, Sigma-Aldrich) for 30 min on ice. After washes in PBS, cells were incubated at 37°C for 2h efflux phase. Cells were then washed in PBS and stained with surface markers for FC analysis. In some conditions, a MDR1 inhibitor, Elacridar (1µM, Tocris), or vehicle (DMSO) were added to cells immediately before the efflux phase. MDR1 expression was assessed using an anti-human MDR1 antibody (UIC2, eBioscience) for 20 min at 37°C in the presence of Cyclosporin A (25 µM, R&D Systems), as described previously (30).

Transcriptomic analyses

CD73^{neg} and CD73⁺ CD4⁺ Tregs were sorted from the PBMC of six HDs blood samples. Cells were activated in the presence of anti-CD3/anti-CD28 beads (Expand beads, Life Technologies, ratio 1 bead to 4 cells) in complete RPMI medium (supplemented with antibiotics, L-glutamine (Life Technologies) and 5% of human serum AB⁺ (EFS)) at 37°C under 5% CO₂. Resting and short-term activated cells (mix of 6 hours and 24 hours activation), were lysed for mRNA extraction (miRNeasy micro Kit, Qiagen) and transcriptomic analyses (Human Gene-Expression 8x60K Microarray Kit AMADID 039494, Agilent Technologies) were performed.

Western blot analyses

Tregs, CD73^{neg} or CD73⁺ CD4⁺ Tregs (2x10⁶ cells) were isolated and lysed in RIPA-Buffer in the presence of proteinase inhibitors. Protein lysates were boiled, loaded on Mini-PROTEAN[®] TGX[™] Precast Gels 5-20% (Biorad) and transferred to Trans-Blot[®] Turbo[™] Mini PVDF membrane (Biorad). CD73 was detected using a mouse anti-hCD73 antibody (1D7, 1/500, Abcam). Pellets of MDA-MB231 cell line (LGC Standards) whose genetic profile has been verified (Eurofins Forensic Department) and negative for mycoplasma (MycoAlert mycoplasma detection kit, Lonza) and human purified HD blood monocytes were used as positive and negative control, respectively. ADA and A2b receptors were assessed either on purified CD73^{neg} or CD73⁺ CD4⁺ Tregs, or after 1 or 4 days activation with Expand beads (ratio 1:4), using a goat anti-A2bR antibody (ab40002, 1/1000, Abcam) and a mouse anti-hADA antibody (ab54969, 1/500, Abcam). The HRP-coupled secondary antibodies used were goat anti-mouse Ab (12-349, 1/5000, Upstate), rabbit anti-goat Ab (P 0449 1/2000, Dako). Membranes were revealed with Luminata Crescendo Reagent (Millipore) and analyzed on Chemidocs[™] system (Biorad).

Proliferation experiments

CD73^{neg}CD4⁺ and CD73⁺CD4⁺ Tregs were stained respectively with the carboxyfluorescein succinimidyl ester (CFSE) (2 μM, Life Technologies) and CellTrace Violet (CTV) (20 μM, Life Technologies) proliferation markers, while Tregs were co-stained with both CFSE and CTV according to manufacturer's instructions. CD73^{neg}CD4⁺ Tregs or CD73⁺ CD4⁺ Tregs (3x10⁴) alone, in co-culture with Tregs (total or sorted CD39⁺ and CD39^{neg} subsets) at a ratio 1:1 or co-culture of CD73^{neg}CD4⁺ Tregs, CD73⁺CD4⁺ Tregs and Tregs at physiological ratio

(70%:20%:10%) were incubated with Expand beads (ratio 1:4) in 96-round-bottomed-well plates (Falcon) in 200 μ l of complete RPMI medium for 4 days at 37°C under 5% CO₂. Ado, AMP, or ATP (Sigma-Aldrich) were added every day at the indicated concentration, whereas the CD73 inhibitor (APCP, 50 μ M, Sigma-Aldrich), CD39 inhibitor (ARL-67156, 250 μ M, Tocris) or rhADA (1 μ g/ml, R&D Systems) were pre-incubated for 30 minutes before the beginning of the culture. At the end of the experimental time-course, cells were harvested and stained with a viability marker (LIVE/DEAD® Fixable Dead Cell Stains) and fixed in 2% formaldehyde. Cell proliferation was analyzed under an inverted Zeiss microscope (Objective 4x) using an Axiovision 4 software (Zeiss), and by FC (LSR Fortessa, BD Biosciences) according to the CFSE and CTV dilution.

For experiments in Transwell Permeable Supports (polycarbonate membrane 0.4 μ m pore Corning), in the 6.5 mm inserts, 6×10^4 CD73⁺CD4⁺ Teffs alone or in co-culture with Tregs (at a ratio of 1:1) were activated using Expand beads (ratio of 1:4) in the presence, in the bottom wells, of 1.5×10^5 CD73^{neg}CD4⁺ Teffs alone, or in co-culture with Tregs (at a ratio of 1:4) and Expand beads (ratio 1:4), in a total volume of 800 μ l.

Cytokines analysis

CD73^{neg}CD4⁺ or CD73⁺CD4⁺ Teffs (5×10^4) were activated with either PMA (50ng/ml)/Ionomycin (1 μ g/ml) (Sigma-Aldrich) for 24 hours or Expand beads (ratio of 1:4) for 48 hours in complete RPMI at 37°C under 5% CO₂, and in the presence or not of Ado (75 μ M or 100 μ M). Supernatants were harvested and frozen, and cytokines were analyzed by ELISA using Luminex Multiplex Kits (eBioscience) (Multiplex 1: IL-2/TNF- α /IFN- γ /IL-22/IL-17A/IL-10/IL-13/IL-21; Multiplex 2: GM-CSF/IL-3).

Nucleotides and nucleosides quantification by HPLC coupled to LC-MS/MS

The capacity of 5×10^4 Tregs, CD73^{neg}CD4⁺ Teffs or CD73⁺CD4⁺ Teffs alone or co-culture of Tregs with either CD73^{neg}CD4⁺ Teffs or CD73⁺CD4⁺ Teffs (ratio 1:1), to degrade ATP or AMP was analyzed after 2 hours incubation at 37°C under 5% CO₂ with labeled ATP_{13C,15N} or AMP_{13C,15N} (all from Sigma- Aldrich) in 200 μ L of serum-free RPMI medium supplemented with antibiotics and L-glutamine (Life Technologies). In some cases, cells were pre-incubated with ARL-67156 (250 μ M) or APCP (50 μ M) for 30 minutes before the experiment. Cell supernatants were harvested, boiled at 65°C for 5 seconds and frozen at -20°C. ATP_{13C,15N},

AMP_{13C,15N}, Ado_{13C,15N} and Inosine_{13C,15N} were quantified in 50 µl of supernatant. Nucleotides and nucleosides were extracted using off-line Oasis-WAX[®] cartridges (60 mg; 3 cc). Briefly, cartridges were conditioned using 2 ml of methanol and 2 ml of water. After sample loading, cartridges were washed with 1 ml of water. Elution was performed thrice using 1 ml of the following mixture: NH₄OH 0.25% pH 10.0/water/acetonitrile (30/30/40, v/v). Eluates were pooled and gas dried under nitrogen at 37°C. The residue was suspended in 250 µl of 5mM HA-0.5% DEA in water, and 10 µl were injected into a liquid chromatograph.

Analysis was performed using liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS) as previously described by Machon *et al* (31). Quantification was conducted by adding standard solutions of labelled nucleotides (ATP_{13C}, AMP_{15N}, Ado_{13C} and Inosine_{15N}) to samples prior to the extraction step. ATP_{15N} and GMP_{13C,15N} were used as internal standards. Concentrations of nucleotides in the supernatants were calculated using calibration curves of the corresponding labelled nucleotides. We also verified that ARL-67156 (250 µM) and APCP (50 µM) did not interfere with the ATP, AMP, Ado and Inosine quantification.

Multi-immunofluorescence stainings on frozen tumor sections

Tissue-Tek[®] O.C.T. (Sakura[®] Finetek) embedded frozen human primary breast tumors were used to generate 6 µm frozen tissue sections with Cryotome[™] (Thermo Fisher Scientific). Sections were fixed in paraformaldehyde, permeabilized in Triton X-100 and stained with murine anti-human CD4-AF488 (Biolegend, Clone RPA-T4), rat anti-human FoxP3-APC (Ebioscience, Clone PCH101), and uncoupled rabbit anti-human CD73 (Cell signalling, Clone D7F9A). CD73 staining was revealed with secondary Donkey anti-rabbit antibody (Life Technologies). Immunofluorescence stainings were analyzed on Upright Microscope (Nikon Ni-E) using ImageJ free software.

Results

CD39 and CD73 are expressed by distinct memory CD4⁺ T cells in humans

We initially observed by flow cytometry (FC), that human CD4⁺ T cells in the blood of healthy donors (HD-blood) did not co-express CD39 and CD73 but exclusive CD39⁺ or CD73⁺ cells were observed among CD4⁺ T cell populations (**Fig. 1A**). While naive CD4⁺ T cells expressed no or very low levels of CD39 and CD73 (**Fig. 1A, B**), the expression of CD39 and CD73 was detectable on memory CD4⁺ T cells (**Fig. 1A**). Among memory CD4⁺ T cells, CD39, but not

CD73, was expressed at variable levels on human HD-blood Tregs defined by FoxP3 expression (**Fig. 1A, C**). The absence of CD73 expression on Tregs purified from blood was also confirmed by western blot analysis (**Fig. 1D**). In contrast, CD73, but also CD39, can be expressed on distinct populations of CD4⁺ T effs defined by the absence of FoxP3 expression (**Fig. 1A, E**). Of note, the intensity of CD73 expression on CD73⁺CD4⁺ T effs was significantly higher compared to naive CD73⁺CD4⁺ T cells (**Fig. 1F**). Moreover, the percentage and the intensity of CD39 expression was lower on CD4⁺ T effs compared to Tregs (Δ CD39 MFI Tregs/CD4⁺ T effs: 1.64 ± 0.33).

Our results demonstrate that in contrast to murine Tregs, human Tregs only express CD39. They also highlight the expression of CD73 on a subset of CD4⁺ T effs. Thus, we wondered whether the expression of CD73, favoring the generation of Ado, on a subset of CD4⁺ T effs may denote a functional specialization of this population.

CD73 identifies a population of CD4⁺ T effs enriched in polyfunctional Th1.17 cells

We analyzed by FC the expression pattern of the chemoattractant receptors CRTH2, CXCR3 and CCR6 on blood CD73⁺ versus CD73^{neg} CD4⁺ T effs in order to determine the relative percentage of Th1 (CRTH2^{neg}CXCR3⁺CCR6^{neg}), Th2 (CRTH2⁺CXCR3^{neg}CCR6^{neg}), Th17 (CRTH2^{neg}CXCR3^{neg}CCR6⁺) and Th1.Th17 (CRTH2^{neg}CXCR3⁺CCR6⁺) (26) (**Fig. S1A**). CD73⁺CD4⁺ T effs contained a similar proportion of Th1, Th17 and Th2 subsets as CD73^{neg} T effs, but exhibited an increased proportion of the Th1.17 subset, which accounted for 27.98 ± 7.99 % of total cells, at the expense of other Th cells (**Fig. S1B, Fig. 2A**). Consistent with this Th1.17 enrichment, a higher proportion of CD73⁺CD4⁺ T effs migrated toward a CXCL10 or CCL20 gradient in Transwell chemotaxis assays (**Fig. S1C**). Analysis of the transcriptome of purified CD73⁺ and CD73^{neg} CD4⁺ T effs after short term TCR stimulation revealed an overexpression of Th1.17-related genes (*CSF2, ABCB1, IL22, IL3, IFNG, GZMB, IL23R, TBX21, LGALS3*) (25, 27) in the former subset, while genes related to Th2 cells (*IL4, IL10, IL13, CCR4*) and Th17, Tfh or Tr1 (*LRMP, IKZF3, CXCR5, IL21, IL10*) were downregulated (**Fig. 2B**). IL-17A and IL-17F gene were not detected likely due to insufficient sensitivity of transcriptomic chip used in our conditions.

We also performed short-term *in vitro* reactivation of PBMC with PMA/ionomycin to analyze cytokines production by intra-cytoplasmic staining in CD73⁺ versus CD73^{neg} CD4⁺ T effs. We found a higher proportion of cells producing IL-2, TNF- α , IL-17A, IFN- γ , IL-22, in CD73⁺ CD4⁺

Teffs (**Fig. 2C**). More precisely, CD73⁺CD4⁺ Teffs were enriched in cells co-producing IFN γ /IL-17A (**Fig. 2C**). A multiplex immunoassay performed with supernatants of these *in vitro* activated subsets confirmed the significantly increased production of all cytokines but also of IL-3 and GM-CSF by CD73⁺CD4⁺ Teffs (**Fig. 2D**) except IL-2 analyzed by FC (**Fig. 2C**). In addition, this assay revealed the low production of IL-10, IL-13 and IL-21 (**Fig. 2D**) compared to CD73^{neg}CD4⁺ Teffs in accordance with Th1.17 gene signature (**Fig. 2B**).

One of the most upregulated genes differentiating CD73⁺ and CD73^{neg} CD4⁺ Teffs (**Fig. 2B**) after *CSF2* gene (coding for GM-CSF) is the *ABCB1* gene, coding for the multidrug transporter MDR1 and recently proposed as a Th1.17 specific marker (27). A significantly increased expression of MDR1 on CD73⁺CD4⁺ Teffs compared to CD73^{neg}CD4⁺ ones was observed at the protein level by FC (**Fig. 2E**) and at the functional level with the Rh123 exclusion assay (**Fig. 2E, Fig. S1D**).

Taken together, our results demonstrate that CD73 identifies a subset of CD4⁺ Teffs enriched in polyfunctional Th1.17 cells. Consistent with this conclusion, a high proportion of CD73⁺CD4⁺ Teffs was observed in the colon of healthy individuals (**Fig. 2F**), a mucosal tissue known to be enriched in Th17 and Th1.17 populations (32).

CD73⁺CD4⁺ Teffs cooperate with CD39⁺ Tregs for Ado production

Next, we evaluated the capacity of CD73⁺CD4⁺ Teffs to generate Ado from AMP through the measurement of nucleotides and nucleosides by HPLC (31). AMP degradation and generation of Ado were observed after 2-hours incubation with AMP and were abrogated by a specific CD73 inhibitor (**Fig. 3A**). Whereas purified human CD73⁺CD4⁺ or CD73^{neg}CD4⁺ Teffs were not able to degrade ATP (**Fig. 3B**), purified human Tregs were able to degrade 48.24 \pm 6.20% of ATP into AMP without generation of Ado (**Fig. 3B**). We confirmed that degradation of ATP results from CD39 expression by Tregs as only purified CD39⁺ Tregs but not CD39^{neg} Tregs generated AMP and that ATP degradation was blocked by a CD39 inhibitor (**Fig. 3C**). The addition of purified CD73⁺CD4⁺ Teffs with Tregs favored the generation of Ado following ATP degradation (**Fig. 3B**).

Ado suppresses proliferation and restricts polyfunctional CD73⁺CD4⁺ T effs to IL-17A secretion

The capacity of CD73⁺CD4⁺ T effs to generate Ado suggests that these cells may be particularly sensitive to Ado. This immunosuppressive molecule exerts its immunosuppressive functions by engaging A2a or A2b receptors but can be degraded into Inosine by the enzyme ADA. Our transcriptomic data revealed that *ADORA2A*, but not *ADORA2B*, was expressed on resting CD73⁺ and CD73^{neg} CD4⁺ T effs, and that both receptors were upregulated following short-term TCR triggering (**Fig. 4A**). In contrast, *ADORA1* (*A1R*) and *ADORA3* (*A3R*) were not detected as described in the literature (6). *ADA* mRNA expressed at steady state, decreased after activation on both subsets (**Fig. 4A**). The modulation observed for *ADORA2B* and *ADA* at mRNA level was confirmed at the protein level, which gradually increased for A2bR and decreased for ADA until 4 days of activation (**Fig. 4B**). These data demonstrated the acquisition of an Ado-sensitive phenotype by CD73⁺ and CD73^{neg} CD4⁺ T effs upon TCR triggering, as observed in the murine model (3). Otherwise, no significant difference in the cell-proliferation capacities of both subsets were observed using same proliferation markers evaluated by FC (**Fig. S1E**).

The metronomic addition of exogenous Ado (75 μM/day) inhibited the proliferation of both subsets as shown by the reduction of cell clusters size (round bottomed well observation, (**Fig. 4C**) and dilution of the CTV proliferation marker (**Fig. S2**) compared to the medium condition. The addition of recombinant ADA (rhADA, 1 μg/ml), restored the proliferative capacity of both T cell subsets (**Fig. 4C, Fig. S2**).

The impact of Ado was also analyzed on the cytokines production capacity of both purified CD4⁺ T effs subsets. To avoid any confounding effects of Ado on proliferation, cytokine secretions were analyzed after 2 days of TCR triggering, determined as the optimal time lapse for cell activation without proliferation. Of great interest, Ado significantly inhibited the secretion of most of the cytokines produced by CD73⁺CD4⁺ T effs except IL-17A and only a slight effect was observed on IL-22 secretion (**Fig. 4D**).

These results indicate that Ado suppresses the proliferation of both subsets in the same manner but dramatically restricts the functionality of CD73⁺CD4⁺ T effs to IL-17A production.

CD39⁺ Tregs make CD73⁺CD4⁺ Teffs sensitive to ATP-derived Ado in spaced-out environment.

We previously observed that CD73⁺CD4⁺ Teffs were able to generate Ado from AMP produced by the ATP degradation from CD39⁺ Tregs (**Fig. 3**). Moreover, we found that functional CD73 on CD4⁺ Teffs favored their suppression mediated by AMP-derived Ado similar to that observed in presence of exogenous Ado (**Fig.S3A, S3B**). We then analyzed the biological impact of the cooperation between CD39⁺ Tregs and CD73⁺CD4⁺ Teffs, by co-culturing Tregs with purified CD73⁺ or CD73^{neg} CD4⁺ Teffs in presence or absence of exogenous ATP. As previously reported (18, 33), Tregs did not present any sign of proliferation, due to their anergic status. Conversely, the proliferation of purified CD73⁺ or CD73^{neg} CD4⁺ Teffs was unaltered by the addition of Tregs, due to the strong TCR triggering signal. Although ATP alone did not modulate the proliferation of either CD4⁺ Teffs subpopulations, combination of Tregs and ATP strongly suppressed the proliferation of CD73⁺CD4⁺ Teffs, but not CD73^{neg}CD4⁺ Teffs. This inhibition was reversed by the addition of inhibitors of CD73 (APCP) or CD39 (ARL67156) (**Fig. 5A, Fig. S4**).

Similar suppression experiments performed with Treg subsets sorted based on CD39 expression confirmed that CD39⁺ Tregs but not CD39^{neg} ones suppressed specifically the proliferation of CD73⁺CD4⁺Teffs in presence of exogenous ATP (**Fig. 5B**).

Transwell experiments enabled us to address the importance of the co-localization of CD73⁺CD4⁺ Teffs and Tregs in this Ado-mediated suppression. CD73⁺CD4⁺ Teffs were cultured in the upper chamber and CD73^{neg}CD4⁺ Teffs in the lower one, whereas Tregs were added in either one or the other. The addition of exogenous ATP, whatever the localization of Tregs, induced a strong inhibition of CD73⁺CD4⁺ Teffs proliferation, indicating that the co-localization of CD73⁺CD4⁺ Teffs and Tregs is not necessary to obtain ATP-mediated inhibition of CD73⁺CD4⁺ Teffs (**Fig. 5C**).

Of importance, these experiments revealed that the proliferation of CD73^{neg}CD4⁺ Teffs localized in the lower chamber was not altered by Ado generated by CD73⁺CD4⁺ Teffs in the upper one demonstrating that CD73⁺ cells among CD4⁺ Teffs were preferentially inhibited by Tregs through Ado generation (**Fig. 5C**). As Ado similarly inhibited purified CD73^{neg} or CD73⁺ CD4⁺ Teffs in 'U' wells (**Fig. 4C**) and in Transwell (**Fig. 5C**), we addressed the importance of both CD4⁺ Teffs populations co-localization for Ado-mediated inhibition resulting from ATP degradation. In cultures where Tregs, CD73⁺CD4⁺ Teffs and CD73^{neg} CD4⁺ Teffs, mixed at

physiological ratio observed in blood (10%/20%/70%), were co-localized altogether in “U” wells, the CD73⁺CD4⁺ Teffs subset was suppressed by autocrine generation of Ado through collaboration with Tregs, but the CD73^{neg} population was also suppressed by paracrine action of generated Ado (**Fig. 5C, D, E**). This demonstrated that, in a culture system favoring close interactions between T cell subsets, cooperation of Tregs and CD73⁺CD4⁺ Teffs degrading ATP into Ado, inhibited proliferation of CD73⁺CD4⁺ Teffs but also surrounding CD73^{neg}CD4⁺ Teffs.

Collectively, these data highlight that the cooperation between Tregs and CD73⁺CD4⁺ Teffs does not require cell-contact. Furthermore, Ado, acting in an autocrine manner on CD73⁺CD4⁺ Teffs, does not affect the proliferation of CD73^{neg}CD4⁺ Teffs except if they are co-localized at high cell density, through collateral paracrine effect. Altogether our data suggest that the CD73⁺ population among CD4⁺ Teffs is selectively targeted by CD39⁺ Tregs through autocrine Ado production.

CD73⁺CD4⁺ Teffs are present in the tumor microenvironment enriched in CD39⁺ Tregs

We analyzed CD73⁺CD4⁺ Teffs and CD39⁺ Tregs in patients with primary Breast (BT) or Ovarian (OT) tumors. The frequency of memory cells among CD4⁺ T cells (**Fig. S5A**), Tregs among memory CD4⁺ T cells (**Fig. S5B**), and the CD39/CD73 expression pattern on Tregs and CD4⁺ Teffs in BT- or OT-blood were similar to that observed in healthy donors (**Fig. 6A, B**). However, in contrast to that observed on CD4⁺ Teffs, the percentage and intensity of CD39 on Tregs tended to increase in BT-blood (62.31 ± 14.94%) and OT-blood (62.79 ± 14.93%), compared to HD-blood (50.01 ± 24.88%). Conversely, there was no modulation of CD73⁺CD4⁺ Teffs proportion (**Fig. S5C**), cytokine production pattern (**Fig. S5D**) or Rh123 efflux capacity (*Bossennec, in preparation*), in the blood of cancer patients.

In breast and ovarian tumor tissues, we observed a strong enrichment of memory cells among CD4⁺ T cells (**Fig. S5A**) and of Tregs among memory CD4⁺ T cells, which frequency reached roughly 3-times that found in patient’s blood as previously reported by our team (18) and others (19). In the BT and OT microenvironment, as observed in blood, Tregs expressed CD39 but not CD73 (**Fig. 6A**). Interestingly, the percentage of CD39⁺ Tregs was also increased, leading to an increase of CD39⁺ Tregs frequency among total CD4⁺ T cells of about four fold compared to blood. Finally, the intensity of CD39 expression was also

significantly higher in BT and OT compared to matched blood (**Fig. 6C, D**). In addition, CD39 expression on tumor-infiltrating Tregs was higher than on CD4⁺ Tregs in terms of percentage and intensity of expression (**Fig. 6E, F**). The upregulated CD39 expression on tumor Tregs compared to blood Tregs was associated to an increased capacity to degrade exogenous ATP into AMP (**Fig. 6G**). In contrast, the proportion of CD73⁺CD4⁺ Tregs was not significantly modulated in the tumor microenvironment (BT: 4.74 ± 2.64%; OT: 4.73 ± 2.71%) compared to blood (HD-blood: 7.37 ± 3.21%) (**Fig. 6B, H**). Tumor-infiltrating CD73⁺CD4⁺ Tregs, activated with PMA/ionomycin, presented a higher percentage of IL-2, TNF- α , IFN- γ , IL-17A producers, and IFN- γ /IL-17A co-producers compared to the CD73^{neg}CD4⁺ Tregs (**Fig. 6I**), as observed in BT- and OT-blood (**Fig. S5D**) and HD blood (**Fig. 2D**). Multi-Immunofluorescence stainings on primary BT frozen sections confirmed the co-detection of Tregs (FoxP3⁺) and CD73⁺CD4⁺ Tregs within the stromal immune infiltrate (**Fig. 6J**).

The presence of CD73⁺CD4⁺ Tregs and the enrichment of CD39⁺ Tregs in BT and OT support the idea that in breast and ovarian tumor environments, CD39/CD73-mediated Ado generation can occur and suppress CD4⁺CD73⁺ Tregs activation.

Tumor-infiltrating CD73⁺CD4⁺ Tregs have lower expression of inhibitory immune checkpoints

To determine whether the intrinsic capacity to transform AMP into Ado may constitute the main functional regulatory pathway of CD73⁺CD4⁺ Tregs, we analyzed, by FC, the co-expression of i-ICPs namely TIGIT, CTLA-4, TIM-3 and PD-1 on CD4⁺ Tregs (**Fig. 7A**). i-ICPs expression was mostly observed on tumor-infiltrating CD4⁺ T cells compared to paired blood. The relative percentage of TIGIT⁺ or PD-1⁺ cells were significantly lower in CD73⁺ cells compared to CD73^{neg} ones among CD4⁺ Tregs from tumor tissues (**Fig. 7A, B**). Indeed, tumor-infiltrating CD73⁺CD4⁺ Tregs contained higher proportion of cells devoid of i-ICPs and fewer cells co-expressing 2, 3 or 4 i-ICPs, compared to CD73^{neg}CD4⁺ Tregs (**Fig. 7C**). Moreover, the intensity of PD-1 expression was lower on tumor-infiltrating CD73⁺CD4⁺ Tregs compared to CD73^{neg}CD4⁺ Tregs (**Fig. 7D**).

These findings, associated with the previous evidence that CD73⁺CD4⁺ Tregs are functionally suppressed by autocrine Ado generated through cooperation with CD39⁺ Tregs, strongly suggest that the CD39/CD73/Ado pathway might constitute the main pathway controlling the Th1.17 potency of human CD73⁺CD4⁺ Tregs.

Consistent with this conclusion, analyses done on PD-1 expressing CD4⁺ tumor-infiltrating T cells in BT and OT unveils that, the proportion of cells devoid of i-ICPs was reduced to 7% among BT-infiltrating PD1⁺CD4⁺ Teff (and 15,5% for OT) contrasting with more than 63% among BT-infiltrating CD73⁺CD4⁺ Teffs (and 46,8 for OT) (**Fig. S5E**).

Discussion

We have herein demonstrated that CD73 expression on CD4⁺ T cells delineates polyfunctional memory CD4⁺ Teffs enriched in Th1.17 cells whereas Tregs are devoid of CD73 expression. CD73⁺CD4⁺ Teffs are targeted by CD39⁺ Tregs through ATP degradation into Ado inhibiting and restricting their functionality to IL-17A secretion. This contact independent cooperation induces specific inhibition of CD73⁺ cells among CD4⁺ Teffs in a spaced-out environment. In breast and ovarian tumor environments, the increased proportion of Tregs overexpressing CD39 and the presence of CD73⁺CD4⁺ Teffs almost devoid of i-ICPs strongly suggest that CD73-mediated generation of autocrine Ado represents an essential regulatory mechanism of these potent CD4⁺ Teffs.

Our results highlight that, compared to CD73^{neg}CD4⁺ Teffs, the CD73⁺ subset secretes higher levels of pro-inflammatory cytokines (IL-17A, IFN- γ , GM-CSF, IL-22, TNF- α , IL-2, IL-3), and lower levels of anti-inflammatory ones (IL-10, IL-13, IL-21). Of importance, FC analyses reveal the higher propensity of CD73⁺CD4⁺ Teffs to co-produce IFN- γ and IL-17A and co-express CXCR3/CCR6, in line with Th1.17 compared to CD73^{neg}CD4⁺ Teffs. Our results extend the work of Doherty *et al* (24), who identified the memory CD73⁺CD4⁺ T cells as Th17 cells. Indeed, the exploitation of the transcriptome data from Ramesh *et al* (27), comparing MDR1⁺ and MDR1^{neg} CD4⁺ memory populations (Gene Expression Omnibus (GEO) repository, accession ID: GSE49702), reveals that *nt5e* mRNA, coding for CD73, is one of the most significantly upregulated genes in the MDR1⁺ population (Log2-fold change (FC) = 0.867; p value = 0.004). Moreover, CD73⁺CD4⁺ Teffs present a number of features of Th1.17 cells recently described by Ramesh *et al* (27). They express high level of functional MDR1, secrete IL-3 and GM-CSF and present higher expression of IL-23R in contrast to non-pathogenic Th17 that produce only IL-17A, IL-22, IL-10 and IL-21 (26, 27, 34). Altogether, our data emphasize the poly-functionality of CD73⁺CD4⁺ Teffs and their similarity with Th1.17.

Th1.17 cells are known to play a pathogenic role in autoimmune diseases through their production of IFN- γ and GM-CSF in addition to IL-17A (for review: (35)). While, Th1.17 cells,

also called pathogenic Th17 induced by Gram⁺ bacteria influence gut anti-tumor immune response in murine models (36), information on the impact of this population in breast and ovarian tumor remains limited. In fact, in patients with invasive breast carcinoma, IL-17A produced by CD4⁺ T cells positively correlates with a high histological grade and with triple negative molecular subtype, and represents an independent prognostic factor for shorter disease-free survival (37, 38). In line with these observations, neutralization of IL-17 in murine mammary tumor models inhibits tumor growth (39). In contrast, in ovarian carcinoma, the detection of IL-17 within the tumor microenvironment (40), and more precisely the presence of CD4⁺ T cells co-expressing IL-17 and IFN- γ (41), is associated with a good prognosis. Owing to their capacity to produce IFN- γ , these cells can exert a potent tumor suppressive activity in synergy with IL-17 in order to reprogram recruited neutrophils and myeloid-derived suppressor cells into potent antitumor effectors, and to neutralize the pro-angiogenic effects of IL-17 through the production of the CXCR3 ligand (CXCL10) (41). This is in line with the strong antitumor response associated to Th1.17 cells in murine ovarian tumor model (28, 41).

Of importance, we observe that Ado blocks all cytokines produced by CD73⁺CD4⁺ Teffs, except IL-17A. In this context, the analysis of cytokine pattern in 102 human breast and ovarian tumor mechanic disaggregation milieu (STM: soluble tumor milieu) with sensitive method highlight that 51% (n=52) STM were negative for both IL-17A and IFN γ , 28.4% (n=29) contained only IL-17A whereas 18.6% (n=19) contained both IL-17A and IFN γ and 2.9% (n=3) IFN γ only. As CD73⁺CD4⁺ Teffs were detected in most tumors at frequency similar to blood and preserve their capacity to co-produce IL-17A and IFN γ upon PMA+Ionomycin activation (**Fig. 6**), these STM data suggest that the level of endogenous Ado production may vary from tumor to tumor, a high level of Ado production leading to IL-17A only, while a low Ado level allowing both IL-17A and IFN γ co-production. Moreover, 87.5% of STM producing both IL-17A and IFN γ contained high concentrations of CXCL10 (>8ng/ml) whereas only 12.5% of those with IL-17A only and none of the other contained significant levels of CXCL10. This represents another argument that IL-17/IFN γ found in STM may be co-produced by CCR6⁺CXCR3⁺ Th1.17 CD73⁺ Teffs. Further analyses remain necessary to confirm this.

Importantly, we demonstrate that in the presence of exogenous ATP, CD39⁺ Tregs potently inhibit the proliferation and cytokine production of purified CD73⁺CD4⁺ Teffs through CD73-dependent Ado generation without impacting CD73^{neg}CD4⁺ Teffs. These results support the

concept that the potent CD73⁺ Th1.17 cells are selectively inhibited by CD39⁺ Tregs, through the cooperative production of Ado in their local microenvironment. The localized effect of Ado can be explained by its short half-life in tissues due to the presence of ADA and specific membrane transporters (42).

High ATP concentrations released in the tumor microenvironment (1) play an important role in the initiation of the immune response, whereas Ado, also found in human tumor environment (43), fosters tumor progression through its immunosuppressive functions (for review: (2)).

In this study, we confirm the increase in Tregs proportion in breast and ovarian tumor environment (18, 19) and demonstrate an overexpression of CD39 on these tumor-infiltrating Tregs (frequency of CD39⁺ Tregs within total CD4⁺ T cells, about 4 fold in tumor compared to blood) which efficiently degrades exogenous ATP into AMP. Consequently, CD73⁺CD4⁺ Teffs will be inhibited through autocrine Ado production. These results support the concept that the Th1.17 potency of CD73⁺CD4⁺ effectors can be selectively inhibited by tumor-infiltrating CD39⁺ Tregs through cooperative Ado production. Others CD39-expressing cells such as Mφ or B cells may also contribute to the regulation of this CD73⁺CD4⁺ Teffs (44, 45).

Of further relevance for therapy, CD73⁺CD4⁺ Teffs from blood or tumor present a high expression of the functional multidrug transporter MDR1 suggesting their protection from deleterious effects of chemotherapy, thus strengthening the role they could play in the antitumor immunity. This key property could be evaluated through the analysis of the proportion of these CD73⁺CD4⁺ Teffs following neo-adjuvant chemotherapy. Furthermore, CD73⁺CD4⁺ Teffs express very low levels of i-ICPs (PD-1, TIM-3, TIGIT, CTLA-4), suggesting the non-redundancy of i-ICPs and the adenosinergic pathway in the regulation of these cells. These results corroborate recent reports, in murine tumor models, demonstrating that Ado limits the efficacy of immunotherapeutic interventions targeting CTLA-4 or PD-1/PDL-1, and that CD73 neutralization enhances the efficacy of these therapies (16, 17). Thus, CD73-mediated generation of Ado is an important mechanism of regulation of these polyfunctional CD73⁺CD4⁺ Teffs, and may represent a critical resistance mechanism to current immunotherapies (anti-PD1/L1, CTLA-4). These findings should promote the development of combined therapies based on immunotherapies targeting i-ICPs and the neutralization of the enzymatic function of CD73 (46, 47).

Furthermore, clinical investigations are required to evaluate the presence of CD73⁺CD4⁺ Tregs in primary or chemotherapy-treated tumors and their clinical impact on the survival of the patients in association with the presence of CD39⁺ cells such as Tregs or Mφ.

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Figures legends

Figure 1: CD39 and CD73 are expressed on distinct subsets of memory CD4⁺ T cells. (A) Representative expression of CD39 and CD73 on CD4⁺ T cell subsets from HD-blood analyzed by FC. (B, C, E) Cumulative data from seventeen donors of the different subsets defined by the expression of CD39 and CD73 among naive CD4⁺ T cells (B) CD4⁺ Tregs (C) and CD4⁺ Teffs (E) (statistical analysis: Friedman Test). (D) Tregs were analyzed for CD73 expression by Western Blot analysis (CD73: 70 kDa). (F) Intensity of CD73 staining on naive CD4⁺ T cells and CD4⁺ Teffs (statistical analysis: Wilcoxon Test).

Figure 2: CD73⁺CD4⁺ Teffs are enriched in poly-functional Th1.17 cells. (A) HD-blood CD73⁺ and CD73^{neg} CD4⁺ Teffs were analyzed by FC for their composition in T helper subsets. Data represented mean of seven donors (B) Transcriptomic analysis was performed on resting or short term activated CD73⁺ or CD73^{neg} CD4⁺ Teffs (n = 6), (p-value < 0.05 for the genes presented) and genes related to Th profiles were extracted. (C) Percentage of IL-2 or TNF- α , IFN- γ , IL-22, IL-17A producing cells and IFN- γ /IL-17A co-producing cells was assessed by FC on CD73⁺ (black) and CD73^{neg} (white) CD4⁺ Teffs from HD-blood after short-term PMA/ionomycin reactivation (statistical analysis: Wilcoxon Test). (D) Quantification of cytokines produced by purified HD-blood CD73⁺ (black) and CD73^{neg} (white) CD4⁺ Teffs activated for 24h with PMA/ionomycin (n=16 for IL-2, TNF α , IFN γ , IL17A, IL10, IL13, and IL21

with exception due to out of range quantification, and n=10 for IL-22, GM-CSF and IL-3) (statistical analysis: Wilcoxon test). **(E)** MDR1 expression and MDR1 functionality was assessed by FC on CD73⁺ (black) and CD73^{neg} CD4⁺ (white) Tregs (statistical analysis: Wilcoxon Test). **(F)** CD73⁺CD4⁺ Tregs frequency in HD-blood and healthy colonic tissues (statistical analysis: Mann-Whitney Test).

Figure 3: CD73⁺ CD4⁺ Tregs cooperate with Tregs to degrade ATP into Ado. **(A)** The capacity of HD CD73⁺ or CD73^{neg} CD4⁺ Tregs to degrade AMP into Ado, was analyzed after a 2-hour incubation with the stable AMP_{13C,15N} isotope (37.5 μM). Residual and generated metabolites were quantified by mass spectrometry coupled-HPLC and the role of CD73 was confirmed by pre-incubation of cells with the CD73 inhibitor (APCP, 50 μM) for 30 minutes before AMP addition. Experiments were performed in duplicate (n = 3 donors) (statistical analysis: Friedman Test). **(B)** The capacity of HD-blood Tregs alone or co-cultured with CD73⁺ or CD73^{neg} CD4⁺ Tregs (ratio 1:1) to degrade ATP into Ado were analyzed after 2 hours' incubation with ATP_{13C,15N} isotope (37.5 μM), residual and generated metabolites were quantified by mass spectrometry coupled-HPLC. The role of CD39 and CD73 were confirmed by pre-incubation of cells with the CD39 inhibitor (ARL-67156, 250 μM) and/or the CD73 inhibitor (APCP, 50 μM) for 30 minutes before ATP addition. Experiments were performed in duplicate (n = 3 donors) (statistical analysis: Friedman Test). **(C)** Cell-sorted CD39^{neg} and CD39⁺ Treg capacity to degrade ATP_{13C,15N} isotope (37.5 μM) was analyzed by the quantification by mass spectrometry coupled-HPLC of ATP and metabolite (AMP) generated. The role of CD39 was confirmed by pre-incubation of cells with the CD39 inhibitor (ARL-67156, 250 μM). Experiments were performed on 3 donors.

Figure 4: Ado suppresses proliferation and restricts polyfunctional CD73⁺CD4⁺ Tregs to IL-17A secretion. **(A)** Gene expression profiles of ADA and Ado receptors (AdoRa2a, AdoRa2b) were extracted from transcriptomic analyses of purified resting CD73⁺ or CD73^{neg} CD4⁺ Tregs (statistical analysis: two-way ANOVA). **(B)** AdoRa2b and ADA proteins levels in CD73⁺ and CD73^{neg} CD4⁺ Tregs before and after 1 or 4 days activation with Expand beads were quantified by Western blot. **(C)** The impact of Ado (75 μM/day) on the proliferation of purified CD73⁺ or CD73^{neg} CD4⁺ Tregs after activation using Expand beads (1:4), was assessed by microscopy after 4 days. In some conditions cells were pre-incubated for 30 minutes with

rhADA (1 $\mu\text{g}/\text{ml}$), before adding Ado. Data are representative of three donors. **(D)** The impact of metronomic doses of Ado (37.5 μM or 75 $\mu\text{M}/\text{day}$) on cytokine secretion (IL22, IL-17A, IFN- γ , GM-CSF, IL-10, IL-13, IL-21, IL-3) by purified CD73⁺CD4⁺ Teffs was quantified by Multiplex Luminex assay in the supernatant of cells after a 48h-culture period with Expand beads (1:4) (statistical analysis: Friedman Test).

Figure 5: Isolated CD73⁺ CD4⁺ Teffs are preferential target of CD39⁺ Tregs through the generation of Ado. (A-B) The impact of ATP (18.75 $\mu\text{M}/\text{day}$) on **(A)** the proliferation of purified CD73⁺ or CD73^{neg} CD4⁺ Teffs alone, or in co-culture with total Tregs **(A)** or cell sorted CD39⁺ and CD39^{neg} Tregs **(B)** at a ratio of 1:1, was assessed by microscopy **(A)** or by CTV dilution **(B)** 4 days after their activation with Expand beads (ratio 1:4). **(C)** Similar experiments were performed using Transwell plates. CD73⁺ (black) and CD73^{neg} (white) CD4⁺ Teffs were cultured in the upper and lower chamber, respectively, in the presence of with Expand beads (ratio 1:4), with or without ATP (18,75 $\mu\text{M}/\text{day}$) or Ado (75 $\mu\text{M}/\text{day}$), and total Tregs (R) were added either in the upper or lower chamber. **(D-E)** The impact of ATP was also assessed on the proliferation of CD73⁺ and CD73^{neg} CD4⁺ Teffs, after the addition of Tregs, all present at a physiological ratio (20%/70%/10%), by microscopy and by FC, by measuring the dilution of proliferation markers (CTV: CD73⁺CD4⁺ Teffs; CFSE: CD73^{neg}CD4⁺ Teffs). The impact of CD73 and CD39 was assessed by pre-incubating cells with inhibitors of CD73 (APCP: 50 μM) and CD39 (ARL-67156: 250 μM) for 30 minutes.

Figure 6: Breast and ovarian tumors contain highly functional CD39⁺ Tregs and CD73⁺ CD4⁺ Teff with Th1.17 characteristics. (A) Representative FC expression of CD39 and CD73 on Tregs from paired BT and blood sample. **(B)** Representative example of CD39 and CD73 expression on CD4⁺ Teffs from paired BT and OT and blood samples, by FC. The proportion **(C)** and MFI **(D)** of CD39 expression on Tregs from paired blood and BT (n=11) or OT (n=4) samples were analyzed by FC (statistical analysis: two-way ANOVA). **(E-F)** Comparison of CD39% **(E)** and MFI **(F)** of CD4⁺ Tregs and Teffs within breast (n=11) and ovarian (n=4) tumor environment. **(G)** Purified tumor-infiltrating Tregs were assessed for ATP (37.5 μM) degradation by HPLC, and compared to HD-blood Tregs. Thirty minutes pre-incubation with the CD39 inhibitor (ARL-67156, 250 μM), was used to validate the role of CD39.. **(H)** The proportion CD73⁺CD4⁺ Teffs from paired blood and BT (n=11) or OT (n=4) samples analyzed

by FC (statistical analysis: two-way ANOVA). **(I)** Single cell suspensions of BT (n=12) and OT(n=7) were reactivated with PMA/ionomycin, and single cytokine production (TNF- α , IL-2, IFN- γ and IL-17A) and co-production of IFN- γ and IL-17A were analyzed by FC after gating on CD73⁺ (black circles) or CD73^{neg} (white circles) Teffs (statistical analysis: two-way ANOVA). **(J)** Localization of Tregs (FoxP3⁺) and CD73⁺CD4⁺ Teffs was analyzed in frozen human breast tumor section by multi-IF stainings.

Figure 7: CD73⁺ CD4⁺ Teffs express less i-ICPs. **(A)** Representative expression of i-ICPs (TIGIT, CTLA-4, TIM-3, PD-1) and CD73 on CD4⁺ Teffs from cancer patients blood (Blood-Teff) and paired Tumor-infiltrating CD4⁺ Teffs (Ti- CD4⁺ Teff), analyzed by FC. **(B)** Expression of i-ICPs on CD73⁺ (black) and CD73^{neg} (white) CD4⁺ Teffs (statistical analysis: Two-way ANOVA) **(C)** Pie chart representing the expression and co-expression of i-ICPs (TIGIT, CTLA-4, TIM-3, PD-1) on CD73⁺ and CD73^{neg} CD4⁺ Teffs from BT (n = 10) and OT (n = 10) samples. **(D)** MFI of i-ICPs (CTLA-4, PD-1, TIGIT, TIM-3) expressed on CD73^{neg} versus CD73⁺ CD4⁺ Teffs from BT and OT samples (statistical analysis: Two-way ANOVA).

Figure 1

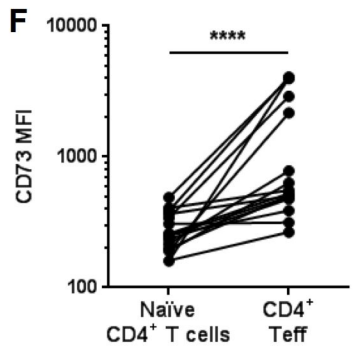
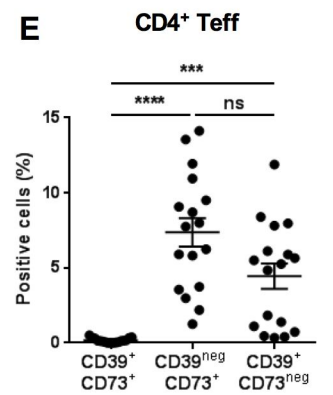
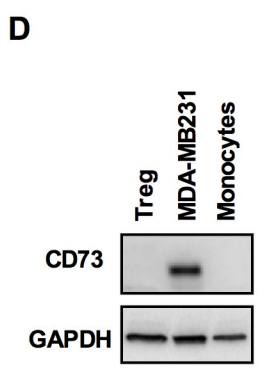
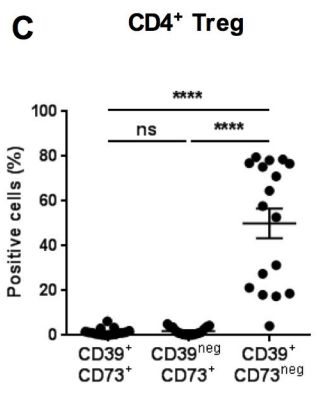
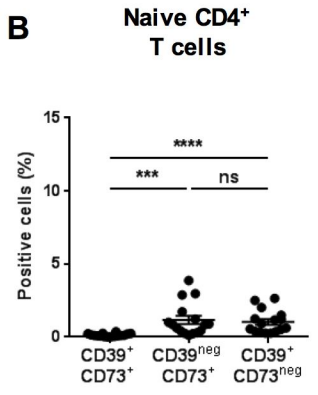
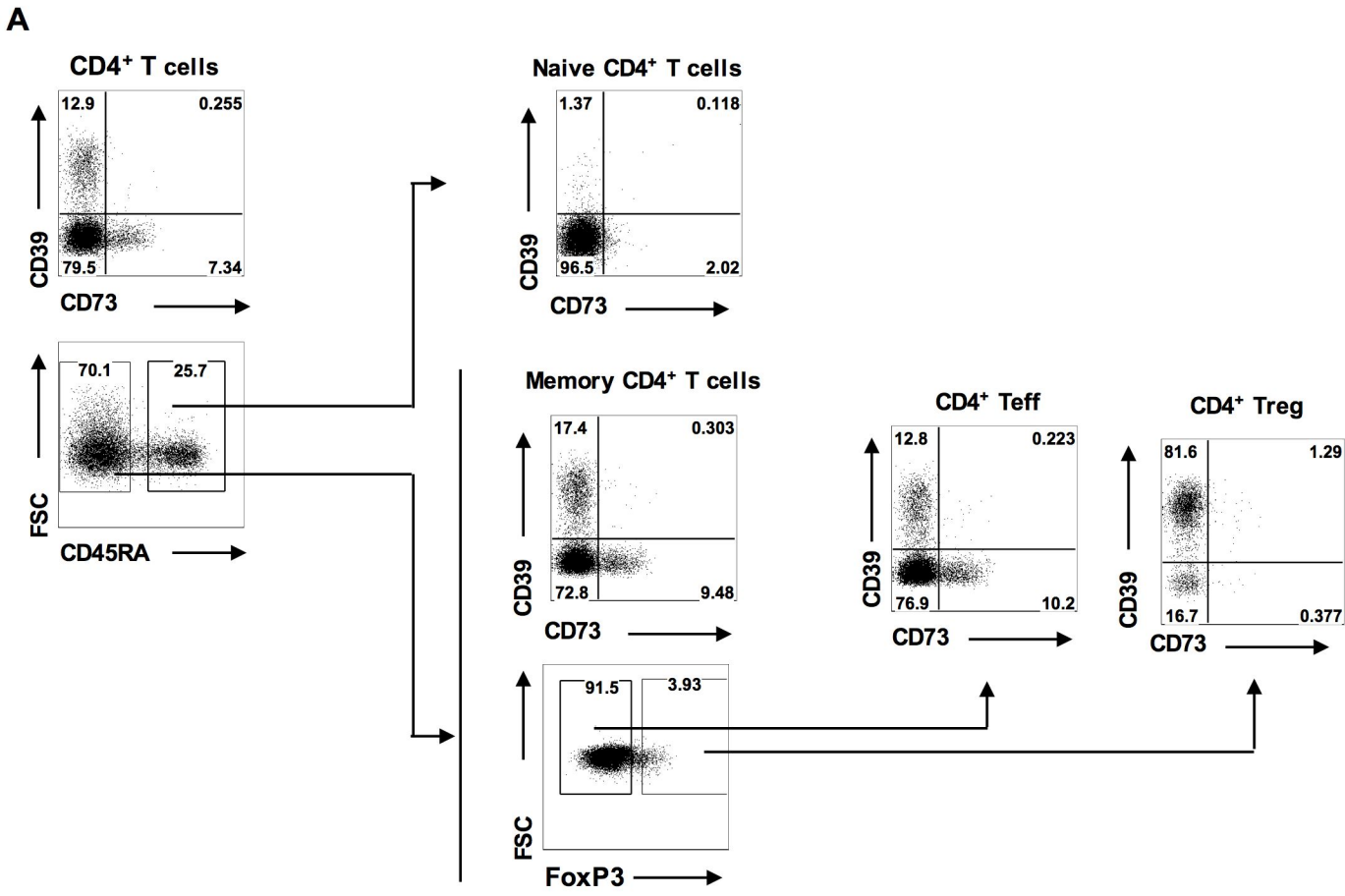


Figure 2

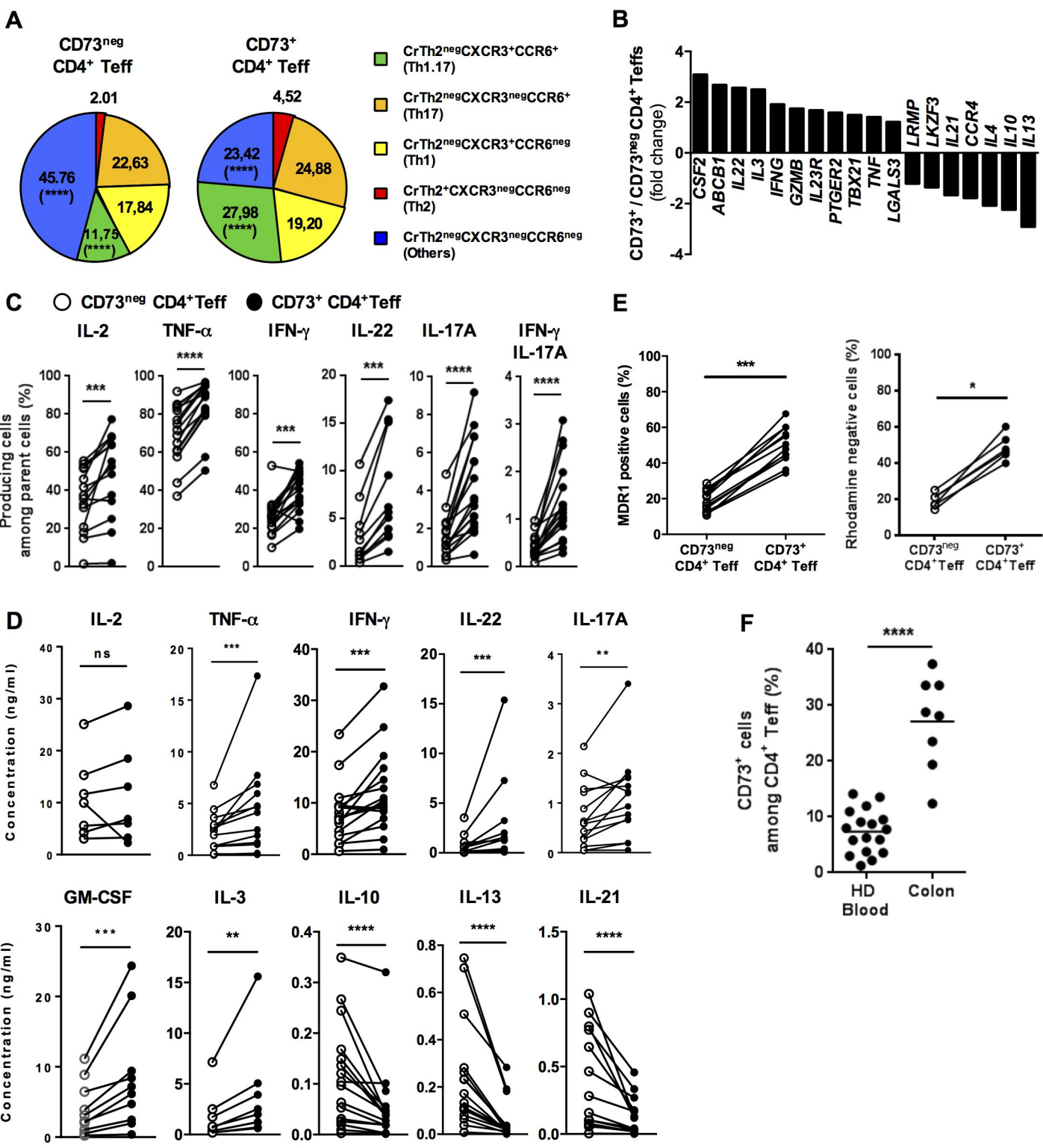


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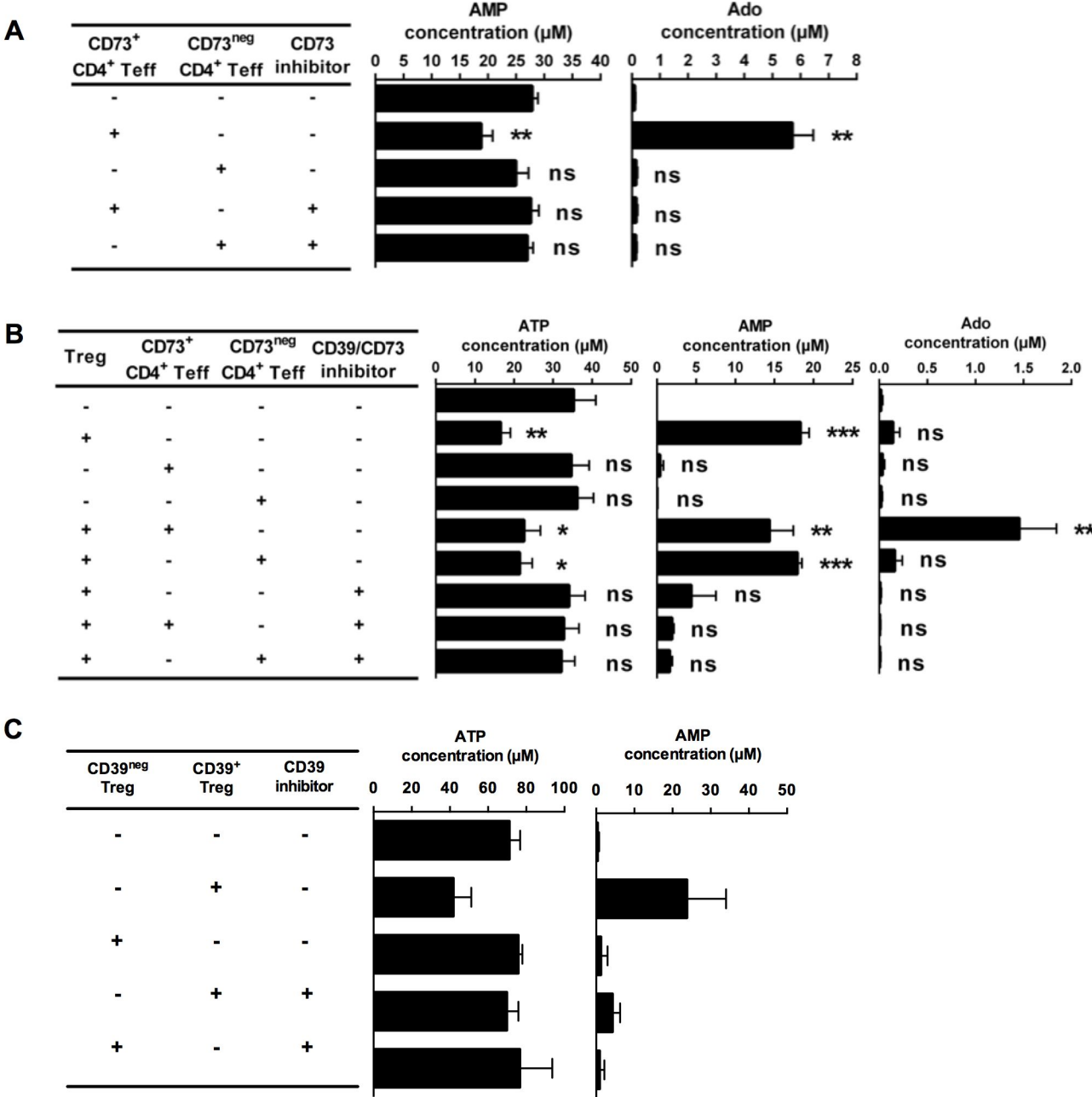


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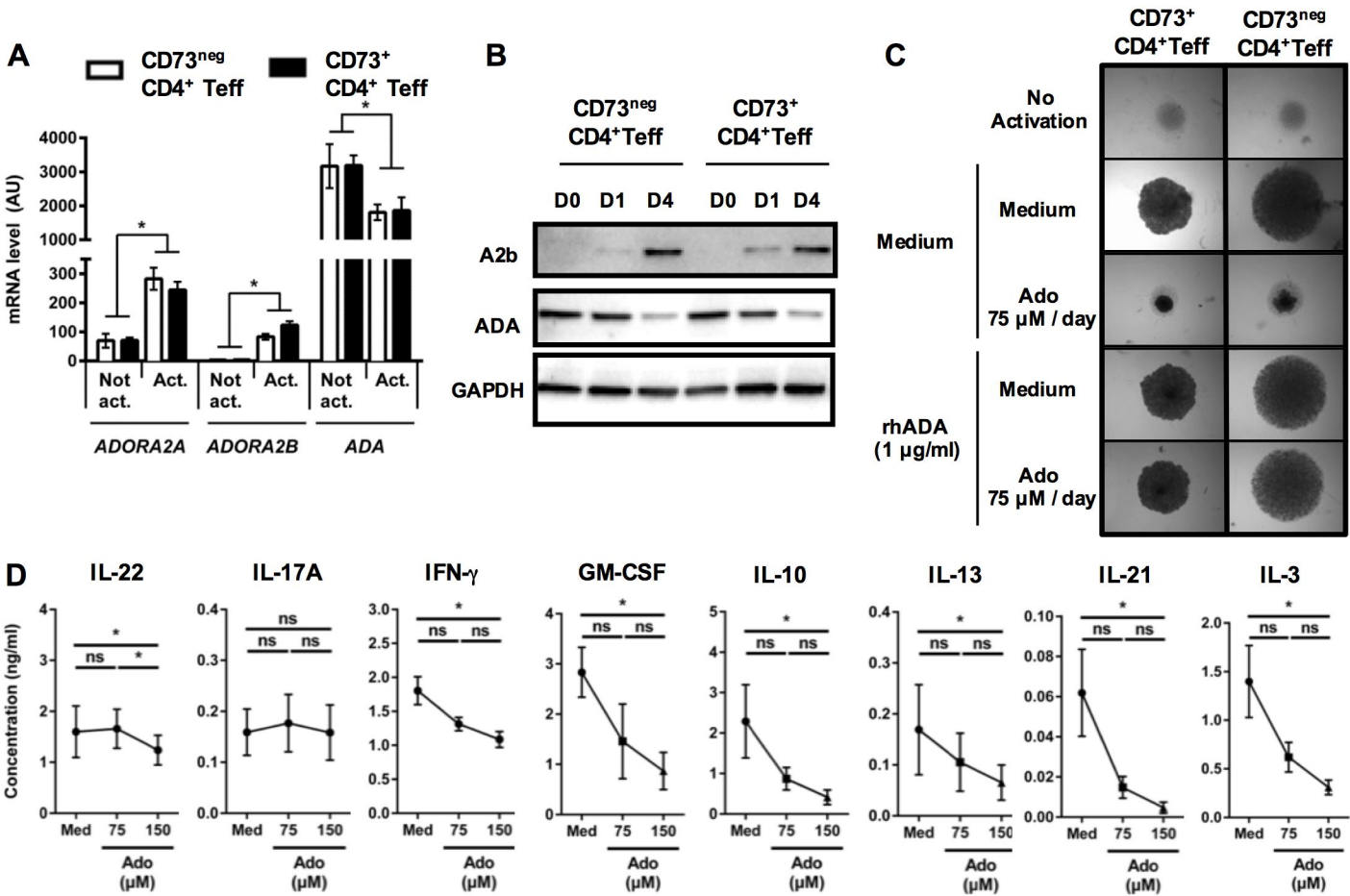


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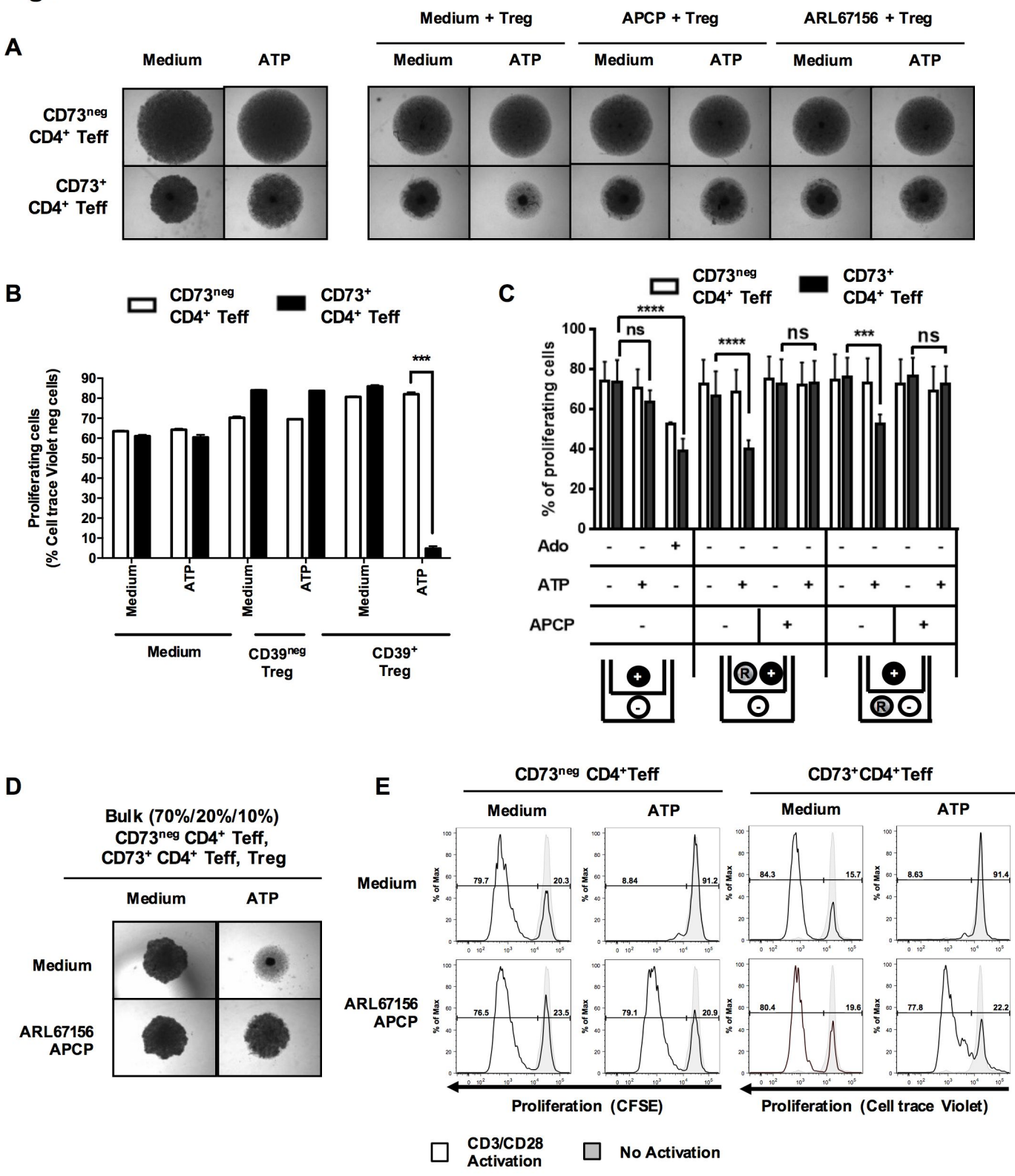


Figure 6

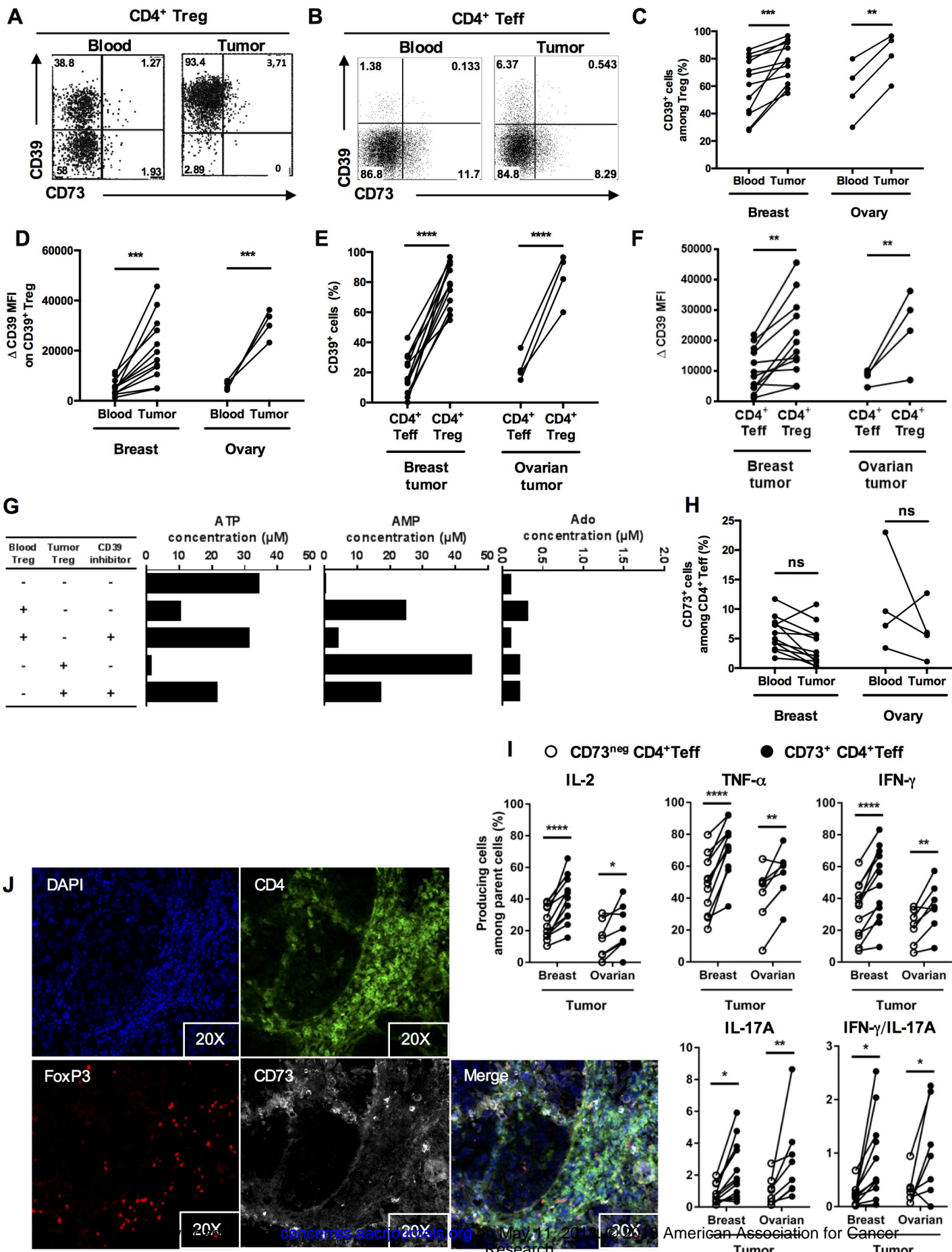
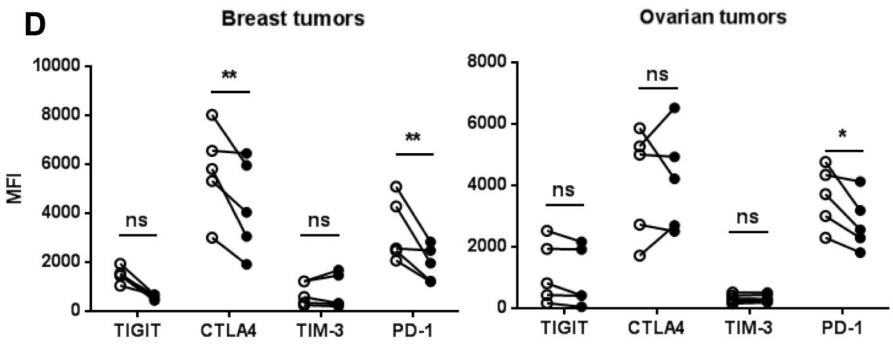
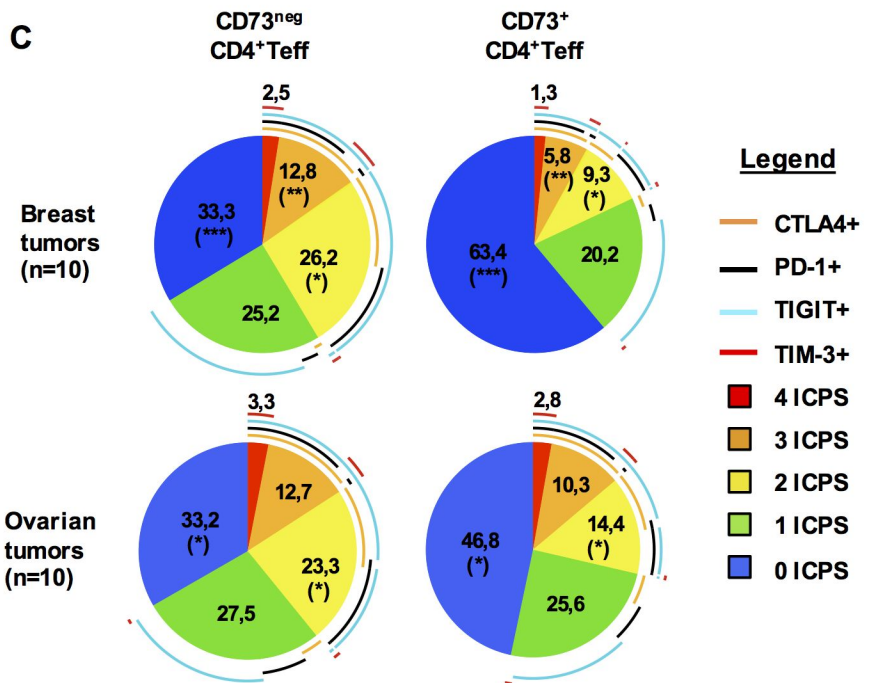
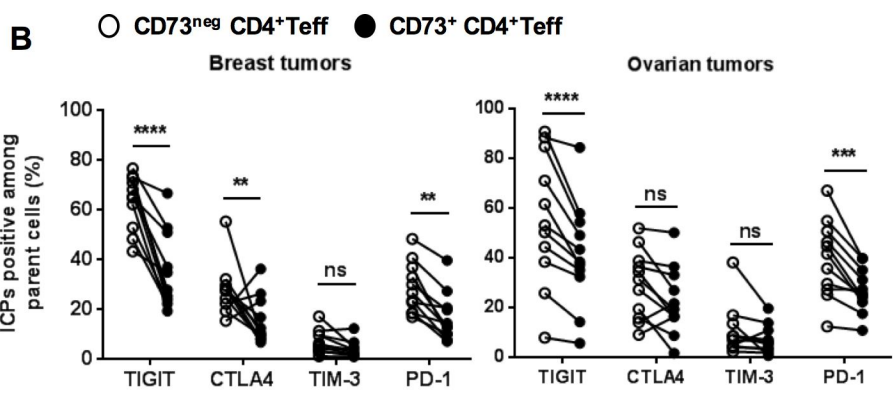
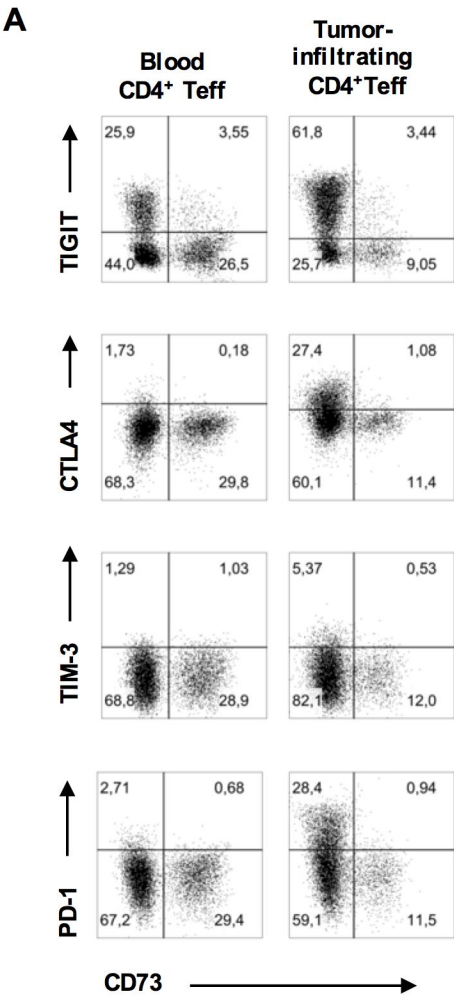


Figure 7



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Autocrine Adenosine regulates tumor polyfunctional CD73+CD4+ effector T cells devoid of immune checkpoints

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