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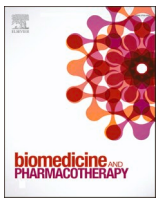

The CD73 is induced by TGF-β1 triggered by nutrient deprivation and highly expressed in dedifferentiated human melanoma

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ABSTRACT

CD73 is the key enzyme in the generation of extracellular adenosine, a mediator involved in tumor progression, tumor immune escape and resistance to anti-cancer therapeutics. Microenvironmental conditions influence the expression of CD73 in tumor cells. However how CD73 expression and activity is regulated in a stress condition of lower nutrient availability are largely unknown. Our results indicate that serum starvation leads to a marked up-regulation of CD73 expression on A375 melanoma cells in a time-dependent manner. The cell-surface expression of CD73 is associated with an increased release of TGF-β1 by starved cells. Blockade of TGF-β1 receptors or TGFβ/SMAD3 signaling pathway significantly reduce the expression of CD73 induced by starvation. Treatment of cells with rTGF-β1 up-regulates the expression of CD73 in a concentration-dependent manner, confirming the role of this pathway in regulating CD73 in melanoma A375 cells. The increased expression of CD73 is associated with enhanced AMPase activity, which is selectively reduced by inhibitors of CD73 activity, APCP and PSB-12489. Pharmacological blockade of CD73 significantly inhibits invasion of melanoma cells in a transwell system. Furthermore, using multiplex immunofluorescence imaging we found that, within human melanoma metastases, tumor cells at the dedifferentiated stage show the highest CD73 protein expression. In summary, our data provide new insights into the mechanism regulating the expression/activity of CD73 in melanoma cells in a condition of lower availability of nutrients, which is a common feature of the tumor microenvironment. Within human metastatic melanoma tissues elevated protein expression of CD73 is associated with an invasive-like phenotype.

1. Introduction

The CD73 is a cell-surface protein crucial for the generation of extracellular adenosine from adenosine monophosphate (AMP) [1]. This latter molecule derives from the hydrolysis of adenosine triphosphate (ATP), mainly via CD39 or CD203a, or from other substrates by alternative pathways [2]. Adenosine is a purine nucleoside which physiologically controls inflammation and immune response, protecting tissues from damage and auto-immunity via G-protein coupled receptor subtypes (A_1, A_{2A}, A_{2B} and A_3 receptors) [3].

In cancer, microenvironmental stress conditions, in response to hypoxia or inflammation, are associated with elevated levels of

Abbreviations: AMP, adenosine 5'-monophosphate; APCP, adenosine 5'α,β-methylene diphosphate; ATP, adenosine 5′-triphosphate; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; EDTA, ethylene diamine tetraacetic acid; EMC, extracellular matrix; EMT, epithelial-to-mesenchymal transition; HIF-1, hypoxia-inducible factor 1; MAPK, mitogen activated protein kinase; MITF, microphthalmia-associated transcription factor; NAPDH, nicotinamide adenine dinucleotide phosphate; NGFR, nerve growth factor receptor; PD-1, programmed cell death protein 1; TCEP, tris (2-carboxyethyl) phosphine; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor; s.d., standard deviation.

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adenosine, which markedly suppresses the antitumor immunity, favoring tumor progression and therapy resistance [4]. The gene expression landscape across different human cancers revealed that the expression of *NT5E,* the gene encoding the ecto-5'-nucleotidase CD73, is up-regulated compared with non-malignant tissues [4,5]. Several lines of evidence also suggest that the overexpression of CD73 is associated with poor clinical outcomes of cancer patients and resistance to anti-cancer treatments [4,5], emerging as a potential prognostic biomarker. Recently, we have also demonstrated that the expression and activity levels of extracellular CD73 are associated with the response to immunotherapy in patients with metastatic melanoma [6,7].

Melanoma is the most aggressive and deadliest type of skin cancer, characterized by high heterogeneity and remarkable ability to spread and metastasize [8,9]. Notably, the mutational burden in melanoma is the highest among all other cancer types [10–12]. In the last decades new therapeutic strategies to treat melanoma have been developed, including immunotherapies to boost cytotoxic T cell responses, such as antibodies against cytotoxic T lymphocyte protein 4 (CTLA4) or programmed cell death protein 1 (PD1) [13], and targeted-therapies, such as inhibitors of BRAF kinase [14]. However, still a large part of patients, especially those with metastatic disease, develop resistance and tumor relapse.

The cellular plasticity is a crucial factor associated with melanoma progression and therapy resistance [11]. Indeed, two major transcriptional programs have been described for melanoma, classically termed as "proliferative" and "invasive" phenotypes . The proliferative phenotype is characterized by enhanced proliferation of melanoma cells, that highly express the microphthalmia-associated transcription factor (MITF), responsible for the regulation of cell cycle and pigmentation [11]. The expression of MITF is associated with a concurrent expression of Melan-A and SOX10, involved in the pigmentation mechanism. On the other hand, the invasive phenotype is characterized by the absence of MITF expression and an enhanced expression of nerve growth factor receptor (NGFR) and mesenchymal markers [11]. Over the years, thanks to the advancement of genomic technology and knowledge, the scenario appeared to be more complex and additional cell states have been defined [11,12,15].

Within the tumor microenvironment the melanoma plasticity is linked to many factors, associated with inflammation, oxygen and nutrient availability, and the presence of non-cancer cells [16–21]. Therefore inflammatory mediators, such as tumor necrosis factor (TNF-α), transforming growth factor β (TGF-β) and other cytokines, hypoxia and nutrient deprivation, as well as different immune and resident cells, can influence the phenotype switching of melanoma cells toward a more aggressive and metastatic state, favoring in turn resistance to targeted-therapies and/or immunotherapies [12]. In addition, immunotherapy-induced inflammation as well as prolonged exposure to BRAF and/or MEK inhibitors promote melanoma phenotype switching, leading to acquired resistance [12]. It has been reported that inflammation-induced dedifferentiation in response to T cell immunotherapy is associated with increased expression of *NT5E* [22]. The expression of *NT5E* can be regulated by many transcription factors, including hypoxia-inducible factor 1 (HIF-1), SP1, SMAD and c-JUN/AP-1 [22–25], although an increased gene expression of CD73 does not necessarily correlate with protein expression and /or activity. Preliminary evidence also indicates that the expression of *NT5E* can be epigenetically regulated in malignant melanoma [26]. Of note, a detailed analysis of the CD73 protein expression profile in melanoma cells in tissues from patients based on their phenotype is still lacking.

Since CD73 plays a critical role in tumor progression and invasiveness as well as in tumor immune escape, understanding the mechanisms that regulate the expression and activity of CD73 within tumor microenvironment may be informative for the development of new therapeutic strategies.

Here, we demonstrate that the surface expression of CD73 is up-regulated in a condition of serum deprivation in melanoma cells that release TGF-β1. We also provide evidence on the CD73 protein expression signature in metastatic melanoma tissues from patients.

2. Materials and methods

2.1. Cell culture

Human melanoma cells A375 were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Euroclone S.p.A.) supplemented with 1% (v/v) L-glutamine (Euroclone S.p.A.), 10.000 units/mL of penicillin G and 10 mg/mL of streptomycin sulfate (Euroclone S.p.A.) and 10% (v/v) Fetal Bovine Serum (FBS) (Euroclone S.p.A.) (5% CO_2, 37 °C).

Human melanoma cells SK-MEL28 were cultured in Minimal Essential Medium (MEM) (Euroclone S.p.A.) supplemented with 1% (v/v) L-glutamine (Euroclone S.p.A.), 10.000 units/mL of penicillin G and 10 mg/mL of streptomycin sulfate (Euroclone S.p.A.), 1% (v/v) sodium pyruvate (Euroclone S.p.A.) and 10% (v/v) Fetal Bovine Serum (FBS) (Euroclone S.p.A.) (5% CO_2, 37 °C).

Melanoma cells were cultured both in a complete medium and in a serum - free medium.

Reagents used were: the human recombinant TGF-β1 protein (rh-TGF-β1) (AF-100–21 C; PeproTech) at different concentrations: 5 ng/mL, 10 ng/mL, 20 ng/mL; the antagonists of TβRI/ TβRII, such as LY2109761 5 μM (HY-12075 – MedChemExpress) and SB525334 1 μM (HY-12043 – MedChemExpress); the selective inhibitor of protein SMAD3, (*E*)-SIS3 10 μM (HY-13013 – MedChemExpress).

2.2. Western blotting analysis

Protein expression was examined by Western blotting, using total cell lysates obtained by using RIPA Buffer supplemented with protease inhibitors cocktail and phosphatase inhibitors. The protein content was determined by Bradford assay. For each sample, an amount of 25 μg of proteins was prepared in denaturing conditions, in Laemmli buffer, and loaded into a 10% polyacrylamide gel. Primary antibody used was: CD73 (1:1000; EPR6114; Abcam). Primary antibody was detected using the following secondary antibody: goat anti-rabbit antibody (1:2000; ImmunoReagents). As loading control, anti β-actin (1:1000; EAB30419; Elabscience) or anti β-tubulin (1:5000; CPA9128; CohesionBiosciences) antibodies were used. Signals were detected by using the LAS4000 Imaging System (GE Healthcare Life Sciences) and the intensity was analyzed by ImageJ software (NIH, Bethesda, MD, USA).

2.3. Flow cytometry analysis

For flow cytometry analysis cells were collected and each sample was divided into 2 aliquots (1×10^6 cells/mL) for the staining with the mouse anti-human CD73 FITC-conjugated antibody (Invitrogen, AD2, 11–0739–42) (0.250 μg/test) or the corresponding FITC mouse IgG1K isotype control (Invitrogen, 17–4714–42) (0.250 μg/test), or with the mouse anti-human CD73 allophycocyanin (APC)-conjugated antibody (Invitrogen, 17–0739–42) (0.125 μg/test) or the corresponding APC mouse IgG1K isotype control (Invitrogen, 17–4714–82) (0.125 μg/test).

Samples were acquired using a BD FACScalibur (Becton Dickinson) and analyzed by the BD CellQuest Pro Software. The results were expressed as mean fluorescence intensity (MFI).

2.4. ELISA assay

The TGF-β1 content was quantified in melanoma cells supernatants, previously concentrated by lyophilization and resuspended in RIPA Buffer (NaCl 150 mM; IGEPAL® (CA-630) 1% v/v; Sodium deoxycholate 0.5% w/v; SDS 0.1% w/v; Tris HCl 50 mM, pH 8; Sigma-Aldrich) supplemented with protease inhibitors cocktail (G135 – Microtech) and phosphatase inhibitors NaF (5 mM; Sigma-Aldrich), Na_3VO_4 (1 mM;

Sigma Aldrich). Commercially available ELISA kit (R&D Systems DuoSet ELISA #DY1679 or Invitrogen ELISA kit #88–8350) were used following the manufacturer's instructions. As indicated in the manufacturer's instructions, before testing samples have been acidified and the neutralized to activate latent TGF-β1 to the immunoreactive form. Cells were collected and the protein concentration was determined by Bradford assay. Results are expressed as pg/mg of total cell protein lysate.

2.5. Malachite green assay

The activity of cell-bound CD73 was evaluated in A375 cells cultured in a serum free condition or complete medium and measured by Malachite Green Assay, that allows quantifying the inorganic phosphate (Pi) produced during the hydrolysis of AMP into adenosine. Briefly, after 24 h, 48 h or 72 h of incubation in medium with or without serum, medium was removed and cells were incubated for 10 min at 37 °C in 200 μL of a phosphate free reaction buffer (10 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, 50 mM Tris-HCl, pH 7.4; Sigma-Aldrich). Afterwards CD73 inhibitors as adenosine 5'α,β-methylene diphosphate (APCP; 100 μM; Sigma-Aldrich) or PSB-12489 (1 μM) were added for 30 min at 37 °C before adding the substrate adenosine 5'-monophosphate (AMP; 220 μM; Sigma-Aldrich). The novel potent CD73 inhibitor PSB-12489 was kindly provided by Prof. Christa E. Müller (University of Bonn, Germany). The reaction was quenched with Trichloroacetic acid (TCA; 5% final concentration; Sigma-Aldrich) and the solution centrifuged at 3000 g for 10 min at 4 °C. The supernatants were tested by Malachite Green Phosphate Assay Kit (8118, ScienCell Research Laboratories) following the manufacturer's instructions. Cells were collected and the protein concentration was determined by Bradford assay. The results are expressed as nmol/mg of total cell protein lysate.

2.6. Transwell invasion assay

A375 cells were seeded in the upper chambers of a transwell system (6.5 mm diameter, 8.0-μm pore size, Corning Incorporeted) (2×10^4 cells/insert) in a serum free medium, after coating chambers with Matrigel (Corning). Complete medium (+10% FBS) was added in the lower chamber of the system as a chemoattractant. Cells were treated with APCP (100 μM; Sigma-Aldrich) or PSB-12489 (1 μM) in the upper chamber of the transwell system for 24 h. Then cells on the membrane were fixed in 4% p-formaldehyde and stained with Crystal Violet Solution (0.5% w/v crystal violet; 20% v/v methanol). Six random fields per insert were photographed by EVOS microscope. The cells into the lower surface were counted.

2.7. Human samples and clinicopathological characteristics of patients

To analyze the expression of CD73 in human melanoma, a tissue microarray (TMA) containing metastases from 12 patients was used (lymph nodes, n = 5; skin, n = 6, lung, n = 1). The TMA has been already described by others [27]. Samples were collected at Department of Dermatology and Allergy, University of Bonn (Germany) and were stored as formalin-fixed paraffin-embedded (FFPE) tissues, as tissue microarray including three cores for each patient. Slides of 4 μm thickness were cut and placed on poli-lysine coated coverslips, then used for the staining procedure. All the samples were collected before patients started the treatment with anti-PD-1 agent monotherapy (nivolumab or pembrolizumab). The clinicopathological characteristics of patients are summarized in the Supplementary file 1. Cases were selected for inclusion if there was sufficient archival FFPE tissue and clinical annotation for analysis.

All tissue were collected after obtaining written informed consent in accordance with participating hospitals/research institute Human Research Ethics Committee procedures and guidelines and conforming to the Declaration of Helsinki.

2.8. Multiplexed immunofluorescence imaging (Co-detection by indexing tissue imaging)

Co-detection by indexing experiments were performed following the protocol recently published [28], that was slightly adapted as follows.

FFPE tissues were heated at 55 °C, for 30 min, on a slide warmer heating plate and were then let cool down at room temperature (RT). Tissues were rehydrated in the following solutions: 100% xylene (5 min, two times), 100% ethanol (5 min, two times), 90% ethanol (5 min), 70% ethanol (5 min), ddH_2O (5 min, two times). Heat-induced antigen retrieval was performed at high-pressure, at 97 °C, for 20 min, using a pressure cooker (PC), in which tissues were immersed into a beaker containing the antigen retrieval solution (Target Retrieval Solution, pH 9, Agilent, S2367). Tissues were then let cool down at RT.

To reduce autofluorescence of tissues, samples were immersed into a bleaching solution (20 mM NaOH, 4.5% (v/v) H_2O_2, in PBS), then placed between two LED lamps for 45 min. This step was repeated once more, and tissues were washed in 1X TBS IHC buffer containing Tween 20 (Supplementary file 2) for 10 min at RT. Then tissues were covered with 100 μL of blocking solution (Supplementary file 2) and incubated for 1 h, at RT, in a humidity chamber, as described by Black and colleagues [28]. At the end of incubation, the blocking solution was removed and 100 μL of the mix containing the conjugated antibodies, at the proper dilution (Supplementary file 2) were added on the tissues. Samples were incubated overnight at 4 °C, in a humidity chamber. The following antibodies were used CD73 (clone AF5795 R&D), SOX10 (clone SOX10/1074, Abcam ab212845; working dilution: 1:50), MITF (clone D5, Abnova MAB14360), Melan-A (clone A103 Santa Cruz Biotechnology sc-20032), NGFR (clone D4B3, Cell Signalling Technology 8238BF). Cell nuclei were stained using DAPI (Biolegend, #422801).

Tissues were then washed in staining buffer 2 (S2) (Supplementary file 2), fixed for 10 min in 1.6% paraformaldehyde solution (Supplementary file 2) and then washed in PBS (2 min, 3 times). Samples were incubated in ice-cold 100% methanol, for 5 min at 4 °C and washed again three times in PBS. Samples were placed in the humidity chamber and 100 μL of BS3 fixative solution (Supplementary file 2) were added on each tissue. After 20 min of incubation, tissues were washed again three times in PBS and stored in S4 buffer until the image acquisition.

The cyclic acquisition of the images was enabled using the Phenocycler® instrument (Akoya Biosciences). Specifically, during each cycle, the instrument enables the acquisition of the signal of up to three fluorescent oligonucleotides, corresponding to three individual antibodies, and the nuclear stain DAPI. The fluorescent oligonucleotides were diluted in a total volume of 250 μL of plate buffer (5 mg/mL sheared salmon sperm, DAPI nuclear stain 1:300, in H2 buffer, Supplementary file 2), at a concentration of 400 nM, in a Corning black 96-well plate. Each well corresponds to one cycle and contains up to three spectrally distinct fluorescent oligonucleotides, ATTO550 (Absorption max. 554 nm, Emission max. 579 nm), DY647P1 (Absorption max. 653 nm, Emission max. 672 nm), DY747P1 (Absorption max. 747 nm, Emission max. 769 nm), in addition to DAPI (Absorption max. 358 nm, Emission max. 461 nm) for nuclear detection. The first and the last cycles of the cyclic run do not contain fluorescent oligonucleotides, but only DAPI (1:300).

In the Supplementary file 3, the pairs of antibody-DNA oligonucleotides and the fluorescent DNA-oligonucleotides chosen for the experiments are summarized. The DNA oligonucleotides were purchased from Biomers. The antibodies were conjugated to the corresponding DNA oligonucleotides, following the protocol already published by others [28], that we adapted as follows. Briefly, 100 μg of each purified antibody were added into a 50 kDa filter column and concentrated by centrifuging at 12000g, for 8 min, at RT, and the flow through was discarded. Then, 360 μL of Tris (2-carboxyethyl) phosphine (TCEP) + Ethylene diamine tetraacetic acid (EDTA) solution (Supplementary file 4) were added into each column and antibodies were incubated for 30

min at RT, then centrifuged again at 12000*g*, for 8 min, at RT. To stop the reduction reaction and to remove any TCEP residues, antibodies were washed three times, by adding 400 μL of Buffer C (Supplementary file 4) and spinning at 12000*g*, for 8 min, at RT, and discarding the flow through each time.

Finally, 200 μg of DNA oligonucleotide solution (0.5 μg/μL, in 1X PBS) were added to the respective antibody and the mixtures were incubated 2 h at RT. At the end of the incubation, tubes were centrifuged at 12000*g*, for 8 min at RT and antibodies were washed three times by adding 450 μL of high-salt PBS to each column, then centrifuged at 12000*g*, for 8 min at RT.

After the last centrifugation, antibodies were resuspended in 200 μL of antibody stabilizer buffer (Supplementary file 4), collected in new tubes and stored at 4 °C.

2.9. Image acquisition and data analysis

Images were acquired using a Zeiss Axio Observer 7 inverted microscope, equipped with Colibri 7 as the LED Light source, the Plan-Apochromat 20X/0,8 M27 (a=0,55 mm) as objective and the Prime BSI PCIe camera.

Once acquired, the images were converted to TIF files by using Codex Manager Instrument® (Akoya Biosciences).

Then, TIF files were processed by using CODEX® Processor 1.8.2, and the following methods were applied: image stitching, shading correction, tile processing (cycle alignment, background subtraction, deconvolution, extended depth of field), region processing (shading correction, tile registration, overlap cropping).

Images were analyzed using Halo® v3.3.2541.256 (Indica Labs). Cell segmentation was performed using the Nuclei Segmentation Classifier, while the algorithm HighPlex FL v4.1.3 was used to analyze cell phenotypes. The TMA tool was used to run the analyses.

The expression of CD73 was analyzed in NGFR+ cells, SOX10+ cells, and MITF+Melan-A+ cells.

2.10. Statistical analyses

Statistical analysis was performed using GraphPad Prism V.9.0. Data are expressed as mean ± s.d. P values < 0.05 were considered statistically significant.

Statistical significance was determined by one way or two way ANOVA test, followed by Tukey's or Šidák's Multiple Comparison test, as appropriate.

3. Results

3.1. CD73 expression enhances in a condition of nutrient deprivation

Increased expression of CD73 and accumulation of adenosine are features of the tumor microenvironment [4]. We investigated the expression of CD73 in malignant human melanoma A375 cells cultured in a condition of serum starvation, to mimic a condition of nutrient deprivation. In this condition, the A375 cells metabolic activity is not affected (Supplementary file 5A), and cells show a low capacity to proliferate (Supplementary file 5B). To assess the nutritional stress in cells, the expression of Light Chain 3 (LC3) was evaluated as indicator of autophagy, that can be induced by nutritional starvation [29]. The expression levels of LC3 II increased in starved cells compared with that in control cells (Supplementary file 5C). In addition, the A375 melanoma cells exposed to nutrient deprivation display decreased expression of E-cadherin and increased expression of N-cadherin, twist and vimentin, markers linked to epithelial-to-mesenchymal transition (EMT)-like features, and a fibroblast-like morphology (Supplementary file 6). Treatment with recombinant TGF-β, a known inducer of EMT [30,31] was used as control (Supplementary file 6).

Serum deprivation significantly increased the CD73 expression at 24 h compared to control cells, reaching the highest level at 48 h up to 72 h (Fig. 1A). These data were confirmed by flow cytometry analysis showing that CD73 is upregulated on membrane surface of melanoma cells in starvation in a time-dependent manner (Fig. 1B and 1C). Similar results were also obtained in the malignant mouse melanoma cells B16. F10 (Supplementary file 7A and 7B). In contrast, the SK-MEL28 melanoma cells in a condition of serum deprivation (Supplementary file 8) show the CD73 expression profile unaltered at 24–48 h and 72 h compared to control (cultured in complete medium) (Fig. 2A). These data were confirmed by flow cytometry analysis showing no change in the expression of cell surface CD73 in SK-MEL28 cultured with or without serum (Fig. 2B and 2C). SK-MEL28 cells display a characteristic elongated morphology and low expression of E-cadherin along with high expression of N-cadherin and vimentin both in control cells and starved cells (Supplementary file 9).

Overall, these data indicate that in a condition of serum deprivation the expression of CD73 enhances in A375 melanoma cells but not in SK-MEL28.

3.2. In condition of serum deprivation melanoma cells release high amount of TGF-β1

Based on our results on CD73 over-expression in both mouse and human highly aggressive melanoma cells under serum deprivation, we hypothesized that tumor-derived factors could regulate the expression of CD73 on cell membrane in an autocrine-manner. Serum deprivation induces the release of inflammatory mediators [18,32]. In response to inflammation, it is known that CD73 can be up-regulated in many cell types, including cancer cells [33]. As shown in Fig. 3A, levels of TGF-β1 in the supernatants of A375 cells collected after 6–24–48–72 h of starvation result increased compared to control cells (+ serum) in a time-dependent manner (data are expressed as pg TGF-β1 in supernatant per mg protein producer cells). These observations indicate that in a condition of serum deprivation TGF-β1 is secreted by A375 cells. Similar results were also obtained in B16. F10 melanoma cells that produce TGF-β1 in a condition of serum starvation (Supplementary file 7C). Conversely, we found that levels of TGF-β1 produced by serum-starved SK-MEL28 are low and not different from those measured in control cells (Fig. 3B). Therefore, we set to investigate whether TGF-β1 triggered by nutrient deprivation in A375 melanoma cells might be responsible for CD73 up-regulation.

3.3. TGF-β1 triggered by serum starvation up-regulates the expression of CD73 on melanoma cells membrane via TGF-β receptors TβRI/TβRII -SMAD2/3 signals

As previous studies have demonstrated that TGF-β can induce the expression of CD73 in various lymphoid and myeloid cell types [34–36] and in some cancer cells, such as breast cancer cells [37] and cervical cancer cells [38], we investigated whether the induction of CD73 expression on A375 melanoma cells was dependent on TGF-β released from melanoma cells themselves in condition of nutrient deprivation.

The treatment with SB525334, a selective inhibitor of TGF-β receptor I [39], or LY2109761, the selective dual inhibitor of TGF-β receptor type I/II (TβRI/II) [40], that inhibit the TGF-β/SMAD signaling pathway (Supplementary file 10), significantly reduces the cell surface expression of CD73 in starved A375 cells (Fig. 4A and Supplementary file 11A). These effects indicate that in a condition of nutrient deprivation the increased cell surface expression of CD73 is sustained by enhanced TGF-β1 levels via TGF- β receptors.

The TGF-β signaling involves the canonical activation of SMAD2/3 transcription factors through transmembrane serine/threonine kinases (type I and type II receptors, TβRI and TβRII) [41]. A previous study demonstrated that SMAD proteins bind to the rat *NT5E* promoter [24], suggesting that CD73 is a transcriptional target of SMADs. Therefore, to further investigate the role of the canonical TGF- β/SMADs pathway in

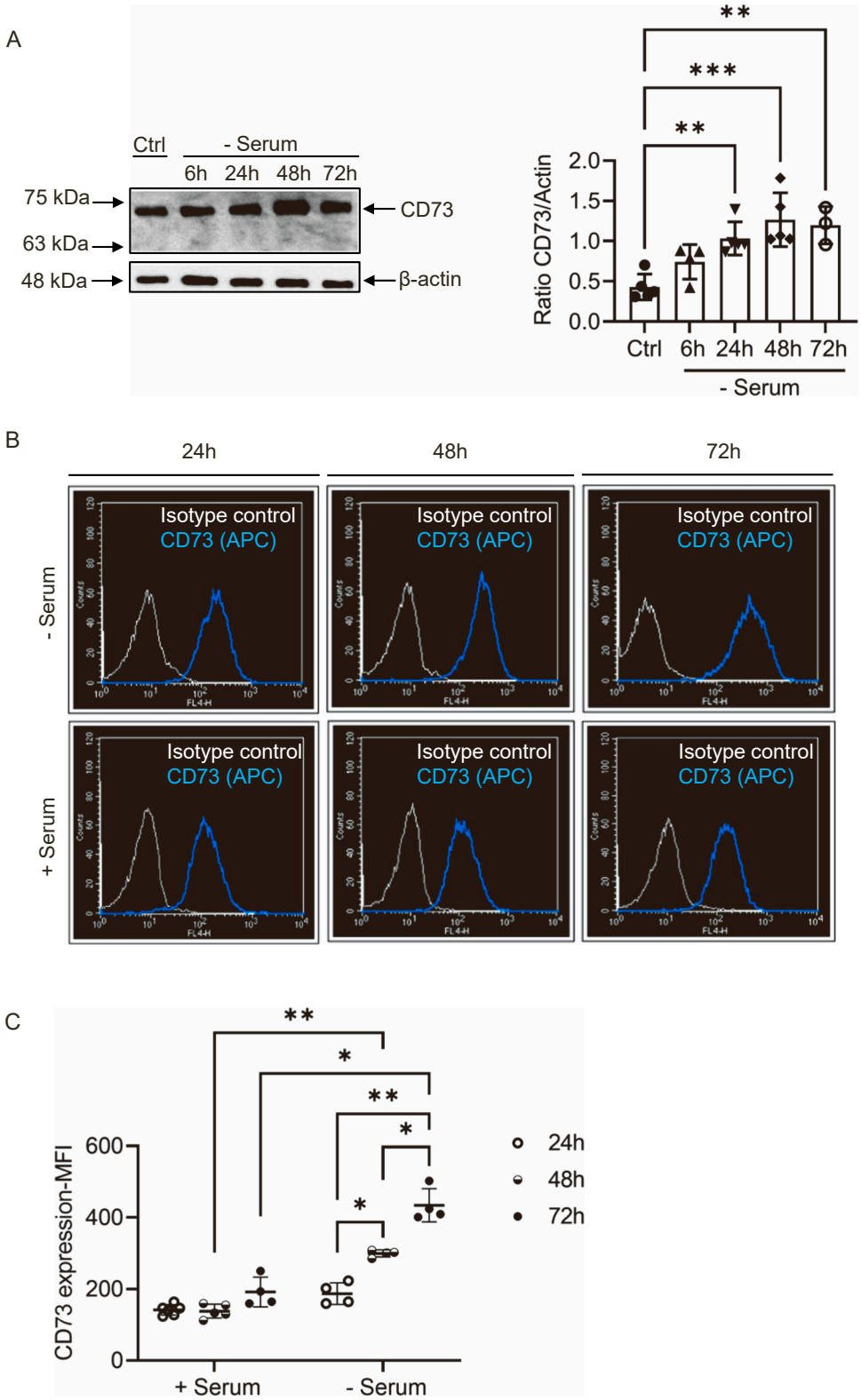

Fig. 1. CD73 expression in A375 melanoma cells. A) Representative Western blot and quantification of CD73 protein level in A375 cells cultured in a serum free condition (- *serum*; n = 3–5 independent experiments) at different time points (6–24– 48–72 h), compared with cells cultured in complete growth medium (Ctrl). β-actin expression was evaluated as protein loading control. Data are expressed as mean ± sd. P value is from a one-way ANOVA test followed by Tukey's multiple comparison test. **p < 0.01; ***p < 0.001. B) Representative flow cytometry histograms of CD73 expression in A375 cells cultured in a condition of serum deprivation (- *serum*) or in complete growth medium (+ *serum*), using an antibody anti-CD73-APC (in blue) and the corresponding control isotype IgG (in white), at different time points (24–48–72 h). C) CD73 protein expression by flow cytometry as MFI values corresponding to the experiments described in B. Data are expressed as mean ± sd. (n = 4–6 independent experiments). P value is from two-way ANOVA test followed by Tukey's multiple comparison test. *p < 0.05; **p < 0.01.

the up-regulation of CD73 on the melanoma cell surface, we examined the expression of CD73 in our experimental conditions in the presence of a known SMAD3 inhibitor, (*E*)-SIS3 [42] (Supplementary file 10). Blockade of SMAD3 reduces the cell surface expression of CD73 in starved A375 cells (Fig. 4B and Supplementary file 11B), suggesting that SMAD2/3 pathway is involved in the TGF- β-mediated up-regulation of CD73 in melanoma cells.

In support of the critical role of TGF-β in inducing the expression of CD73 on A375 cells, treatment with human recombinant TGF-β1 (rhTGF-β1) up-regulates the expression of CD73 in A375 cells in a concentration-dependent manner, reaching significance at concentrations of 10 ng/mL and 20 ng/mL of rhTGF-β (Fig. 4 C and 4D). This effect is completely abrogated by addition of the selective inhibitors of TGF-β receptors, SB525334 or LY2109761 (Fig. 4E and Supplementary

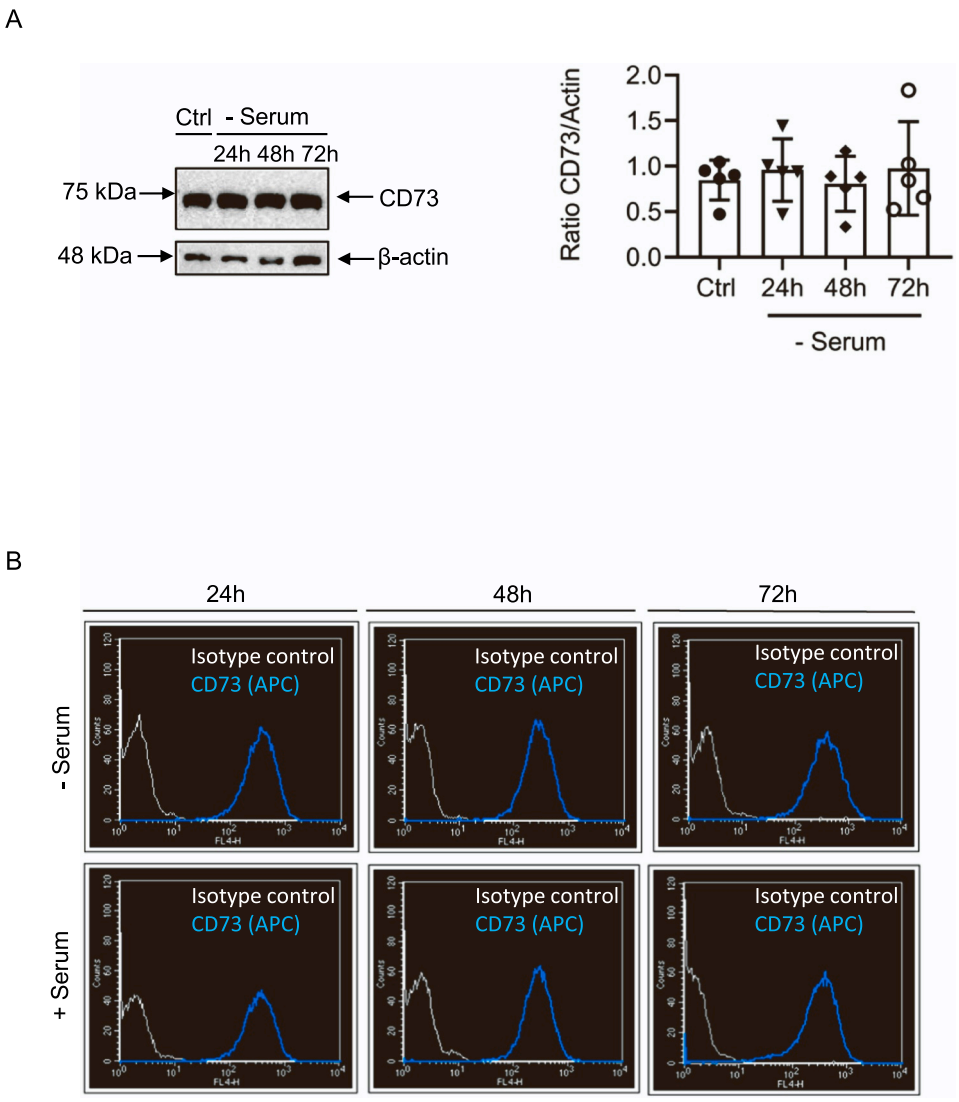

Fig. 2. CD73 expression in SK-MEL28 melanoma cells. A) Representative immunoblot and quantification of CD73 protein level by Western blot analysis in SK-MEL28 cells cultured in a serum free condition (- *serum*) at different time points (24–48–72 h) compared with cells cultured in a complete growth medium (Ctrl). β-actin expression was evaluated as protein loading control. Data are expressed as mean ± sd from n = 4 independent experiments. B) Representative flow cytometry histograms of CD73 expression on SK-MEL28 cells membrane, cultured in a condition of serum deprivation (- *serum*) and in complete growth medium (+ *serum*), using an antibody anti-CD73-APC (blue line) and the corresponding control isotype IgG (white line), at different time points (24–48–72 h). C) CD73 protein expression by flow cytometry as MFI values corresponding to the experiments described in B. Data are expressed as mean ± sd from n = 3 independent experiments.

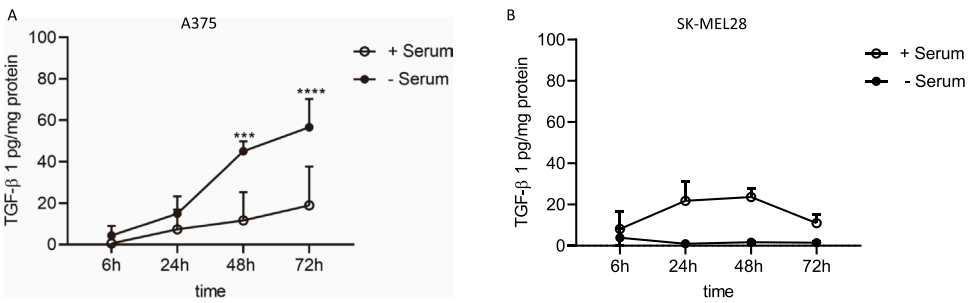

Fig. 3. Analysis of TGF-β1 levels in A375 and SK-MEL28 melanoma cells. A) TGF-β_1 levels were measured by ELISA assay in the supernatants of A375 cells cultured in complete growth medium (+ *serum*) or in serum free medium (- *serum*) at different time points (6–24– 48–72 h). Data are expressed as pg TGF-β1 in supernatant per mg protein producer cells and as mean ± s. d. from n = 4 independent experiments. P value is obtained from a two-way ANOVA test followed by Šidák's multiple comparison test. ***$p < 0.001$; ****$p < 0.0001$. B) TGF-β1 levels were measured by ELISA assay in the supernatants of SK-MEL28 cells cultured in complete growth medium (+ *serum*) or in a serum free medium (- *serum*) at different time points (6–24– 48–72 h). Data are expressed as mean ± sd from n = 3 independent experiments.

file 11 C).

Overall these results suggest that TGF-β triggered by nutrient deprivation up-regulates in an autocrine manner the expression of CD73 on cell membranes.

3.4. The increased expression of CD73 in starved cells is associated with increased AMPase activity

CD73 is able to generate extracellular adenosine, from AMP, which derived from the hydrolysis of ATP (in a canonical way) or from NADPH (in a non-canonical way) [2]. To evaluate the activity of CD73 over-expressed in melanoma cells in our experimental conditions, we analyzed the AMPase activity in A375 cells cultured in serum deprivation at 24–48–72 h. Therefore, after starvation at each time point, cells were incubated with AMP to measure the levels of inorganic phosphate by using the malachite green assay. The levels of inorganic phosphate derived from AMP hydrolysis are markedly increased compared with basal levels (control) at any time points tested (Fig. 5A). We observed that the levels of inorganic phosphate increase over time in cells in presence of AMP, being 12.23 ± 5.97 nmol/mg of protein at 24 h, 20.47 ± 8.02 nmol/mg of protein at 48 h and 31.58 ± 10.1 nmol/mg of protein at 72 h, although differences were not statistically significant. To verify that the AMPase activity observed in starved cells was dependent on the activity of CD73 we used the adenosine 5'-α,β-methylene diphosphate (APCP), a selective competitive inhibitor of the enzymatic activity of CD73. In presence of APCP the hydrolysis of AMP results significantly reduced (Fig. 5A). These experiments have been performed also in presence of a novel more potent, more selective and metabolically stable CD73 inhibitor, PSB-12489 [43]. In presence of PSB-12489 the hydrolysis of AMP results significantly inhibited (Fig. 5A). The AMP hydrolysis in cells cultured in complete medium instead remains low at each time point, being 3.22 ± 0.65 nmol/mg of protein at 24 h; 3.77 ± 1.35 nmol/mg of protein at 48 h and 2.94 ± 0.53 nmol/mg of protein at 72 h.

These results indicate that, in a condition of serum deprivation, CD73 is strongly up-regulated on the membrane of melanoma cells in a time-dependent manner. This effect is associated with an increased AMPase activity, which is blocked by the selective inhibitors APCP or PSB-12489.

The effect of CD73 inhibitors was analyzed on invasion of A375 cells through the coating of Matrigel in transwells. Treatment of cells, seeded in the upper chamber of a transwell system, with APCP or PSB-12489 significantly reduces the ability of cells to directionally respond to chemoattractant and adhere to matrigel (Fig. 5B), supporting the potential of targeting CD73 to reduce tumor cells invasion [44,45].

Given the crucial role of CD73-derived adenosine in regulating the production of tumor-promoting factors as well as immunosuppressive mediators, we also tested the potential effect of APCP in affecting the release of TGF-β1 from melanoma cells triggered by nutrient deprivation. We observed that CD73 inhibition by APCP does not affect the release of TGF-β1 from A375 melanoma cells (Supplementary file 12).

3.5. CD73 is highly expressed by tumor cells showing a dedifferentiated phenotype within human melanoma metastases

Finally, we assessed CD73 in melanoma cells from patients. The expression of CD73 was analyzed in melanoma metastases from 12 patients, by multiplexed immunofluorescence imaging (co-detection by indexing tissue imaging, CODEX) [28]. Tumor cells were clustered in three major groups, identifying different stages of differentiation: pigmented stage (MITF$^+$, Melan-A$^+$), intermediate stage (SOX10$^+$) and dedifferentiated stage (NGFR$^+$). Among these groups, the expression of CD73 was analyzed (Fig. 6). We observed that CD73 is more frequently expressed by melanoma cells that are negative for MITF, Melan-A or SOX10, but positive for NGFR (Fig. 6). This observation is in line with what already observed by Reinhardt and colleagues, who indicated that *CD73* gene expression is associated with both a nascent and fully established dedifferentiated melanoma phenotype [22].

4. Discussion

CD73 plays a pivotal role in tumor biology, as increased expression and activity of CD73 are associated with tumor immune escape, neoangiogenesis and metastasis [46]. Likely, changes in CD73 expression during tumor progression are dependent on environmental factors and nutrients / oxygen supply in tumor lesions. In this study we found that CD73 expression was increased in A375 melanoma cells, through TGF-β triggered by nutrient deprivation. Cells treated with TGF-β up-regulate the surface expression of CD73, confirming the role of this pathway in inducing CD73 expression. We observed that high levels of CD73 are associated with increased AMPase activity in starved cells. We further demonstrate that elevated protein expression of CD73 is associated with an invasive-like phenotype within human melanoma metastases.

The rapid growth of the tumor mass leads to the establishment of a poorly vascularized and hypoxic environment, in which cancer cells are exposed to nutrient deprivation. This condition has been proposed to modulate the aggressiveness of cancer cells and invasion [47]. Within the tumor microenvironment the condition of nutrient deprivation is coupled to several inflammatory signaling molecules, such as TGF-β, that contributes in generating phenotypic heterogeneity in tumor tissue. The role of TGF-β in tumor microenvironment is complex: during the years, many results have been published, proposing TGF-β either as tumor suppressor or as tumor promoter [48,49]. As tumor promoter, TGF-β signaling facilitates the immunosuppression within tumor microenvironment, the proliferation of cancer cells, metastasis, and directs the epithelial-to mesenchymal transition (EMT) [49]. The EMT is a process that characterize the phenotype plasticity in cancer, promoting progression and metastasis. In melanoma, many EMT-related factors can drive the phenotype switching between differentiated and invasive phenotypes, contributing to therapeutic resistance [50,51].

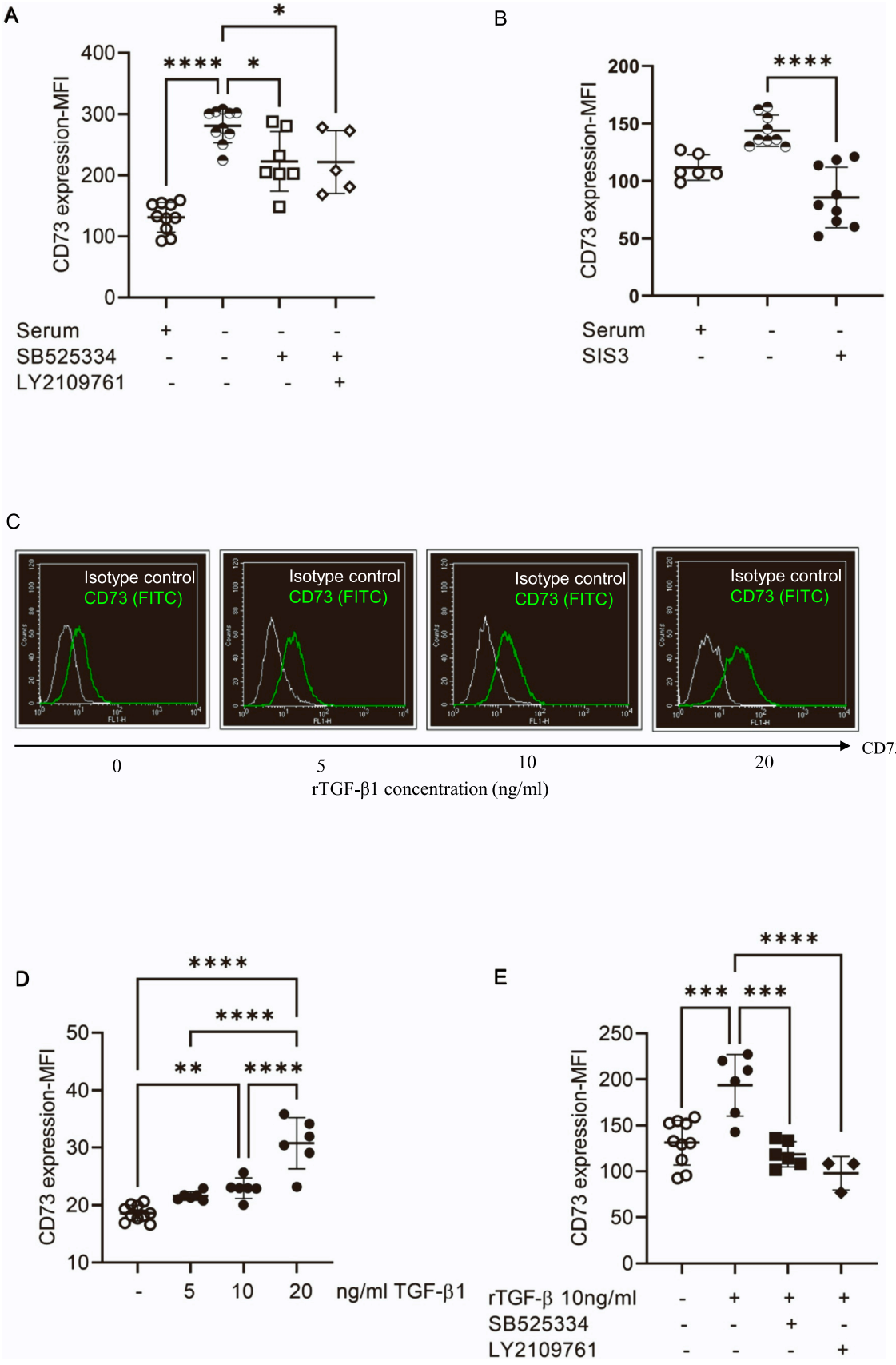

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Fig. 4. Analysis of TGF-β1$_1$ pathway involved in CD73 expression. A) CD73 protein expression by flow cytometry as MFI values on A375 cells membrane cultured in serum free medium for 48 h and treated with TβRI/TβRII antagonists SB525334 (1 μM) or LY2109761 (5 μM). Cells cultured in complete medium were used as control (n = 3 independent experiments). B) CD73 protein expression by flow cytometry as MFI values on A375 membrane cultured in a serum free medium and treated with (*E*)-SIS3 (10 μM), a selective SMAD3 inhibitor, for 24 h. Cells cultured in complete medium were used as control (n = 3 independent experiments). C) Representative flow cytometry histograms of CD73 expression on A375 cells membrane, cultured in complete medium and treated with the rhTGF-β1 at different concentrations (5 ng/mL, 10 ng/mL, 20 ng/mL) for 48 h, using an antibody anti-CD73-FITC (green line) and the corresponding control isotype IgG (white line). D) CD73 protein expression by flow cytometry as MFI values corresponding to the experiments described in C (n = 3 independent experiments). E) CD73 protein expression at 48 h on A375 cells membrane cultured in complete medium and treated with the rhTGF-β1 protein (10 ng/mL) in presence or not of SB525334 (1 μM) or LY2109761 (5 μM) (n = 3 independent experiments). Data on graphs are expressed as mean ± sd. P value is obtained from a one-way ANOVA test, followed by Tukey's multiple comparison test (A,B,D,E). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

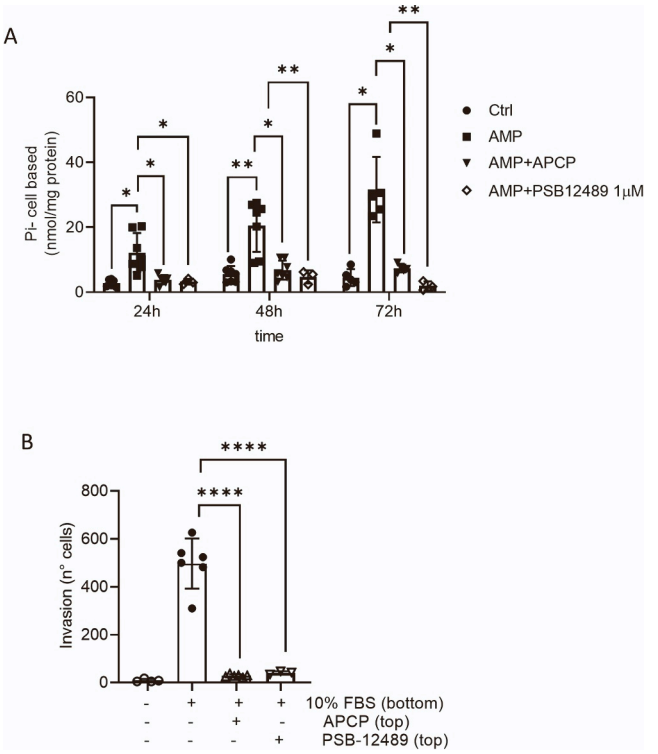

Fig. 5. Enzymatic activity of CD73 in A375 cells in a condition of nutrient deprivation. A) Levels of inorganic phosphate were determined by malachite green assay on starved A375 cells at different time point (24–48–72 h) in presence of the substrate AMP (220 μM) and/or the selective CD73 inhibitor APCP (100 μM) or PSB-12489 (1 μM). The levels of inorganic phosphate are normalized by total cell protein content (nmol/mg of protein). Data are expressed as mean ± sd (n = 3 independent experiments). P value is from a two-way ANOVA test followed by Tukey's multiple comparison test. *p < 0.05; **p < 0.01. B) Number of invading cells by a transwell invasion assay, in presence or not of the selective CD73 inhibitor APCP (100 μM) or PSB-12489 (1 μM) for 24 h. The FBS 10% (v/v) was used as a chemoattractant agent. Top= upper chamber of the transwell system; bottom= lower chamber of the transwell system. Data are expressed as mean ± sd, (n = 3 independent experiments). P value is obtained from a one-way ANOVA test, followed by Tukey's multiple comparison test. ****p < 0.0001.

Environmental signals, other than TGF-β, could direct this reversible process, including hypoxia, inflammatory mediators and nutrient starvation (reviewed by others [49]). In our study we show for the first time, to the best of our knowledge, that the expression of CD73 is up-regulated in aggressive melanoma cells in a condition of lower nutrient availability. In particular, the up-regulation of CD73 on cell surface of melanoma cells relies on their capacity to produce elevated levels of TGF-β1 triggered by nutrient deprivation. The up-regulation of CD73 in A375 cells is accompanied by acquisition of an EMT-like signature in response to nutrient deprivation, an observation supported by previous reports on increased EMT-related genes expression by nutritional stress in different cancer cells [52–54]). In SK-MEL28 cells with mesenchymal-like characteristics the expression of CD73, that is already high at basal level, remains unaltered in response to nutrient deprivation. In this context, it is possible that nutrient starvation increased the expression of CD73 in A375 cells during EMT. Nevertheless, whether CD73 might be an effector in starvation-induced EMT-like process in melanoma cells remains to be explored.

Consistent with a role of TGF-β in inducing the CD73 expression, we observed that the TGF-β receptors/SMAD3 signaling pathway blockade reverses the CD73 expression in starved cells. Therefore, we propose that in a condition of nutritional stress tumor cells that are able to release high levels of TGF-β may up-regulate the cell surface expression of CD73 in an autocrine manner. In support, the highly aggressive triple negative breast cancer MDA-MB-231 cells that release TGF-β in a condition of serum starvation show increased levels of CD73 (data not shown in this manuscript). In contrast, the expression of CD73 remains unaltered in starved human breast cancer MCF-7 cells that do not release TGF-β in response to nutritional stress (data not shown in this manuscript). In this scenario, the up-regulation of CD73 in response to a lower nutrient availability in tumor cells characterized by an aggressive behavior might contribute to increase invasion and immunosuppression via adenosine signaling. It would be of interest to deepen this phenomenon in other cancer cell types in future studies.

Likewise, within the tumor microenvironment it is reasonable to suppose that TGF-β released from melanoma cells in response to nutrient deprivation may induce the expression of CD73 also on tumor-infiltrating immune cells. At this regard, previous studies have demonstrated that TGF-β sustains the expression of CD73 on T cells, dendritic cells, peritoneal macrophages [34,55] and myeloid cells in tumors [35,36]. The induction of CD73 expression on both cancer cells and immune cells, in a TGF-β rich tumor tissue, by producing adenosine can markedly affect the anti-tumor immunity, limiting the efficacy of anti-cancer therapeutics [37,39]. In further support of the critical role of adenosine signaling in contributing to the immunosuppression driven by TGF-β, a study published by Sidders and co-workers demonstrates that enhanced adenosine signaling is associated with mutation of TGF-β superfamily members and worse overall survival [56]. High adenosine signature score is ultimately associated with reduced efficacy of anti-PD-1 therapy in patients with a variety of solid tumors and anti-CTLA4 therapy in metastatic melanoma patients [56]. As such, increased CD73 protein expression in cancer cells is clinically relevant in patients developing resistance to anti-cancer therapies. In this regard, targeting CD73/adenosine pathway represents an attractive and powerful therapeutic strategy to improve the efficacy of anti-cancer treatments. The main enzymatic activity of CD73 is in generating from AMP adenosine, which through its receptors is able to modulate signalling pathways involved in tumor progression and immune escape. As demonstrated in many preclinical tumor models, blockade of CD73-adenosine signaling markedly limits tumor growth and metastasis, and improve the anti-tumor immunity especially in combination with other immune-therapeutics [57–62]. These experimental evidence have prompted the development of monoclonal antibodies against CD73 as well as inhibitors of A_{2A}/A_{2B} receptors that have entered clinical trials in patients with solid tumors in combination with anti-PD1/PD-L1 inhibitors or chemotherapy [4,63]. In support of the therapeutic potential of targeting CD73 in cancer therapy we observed that blockade of the

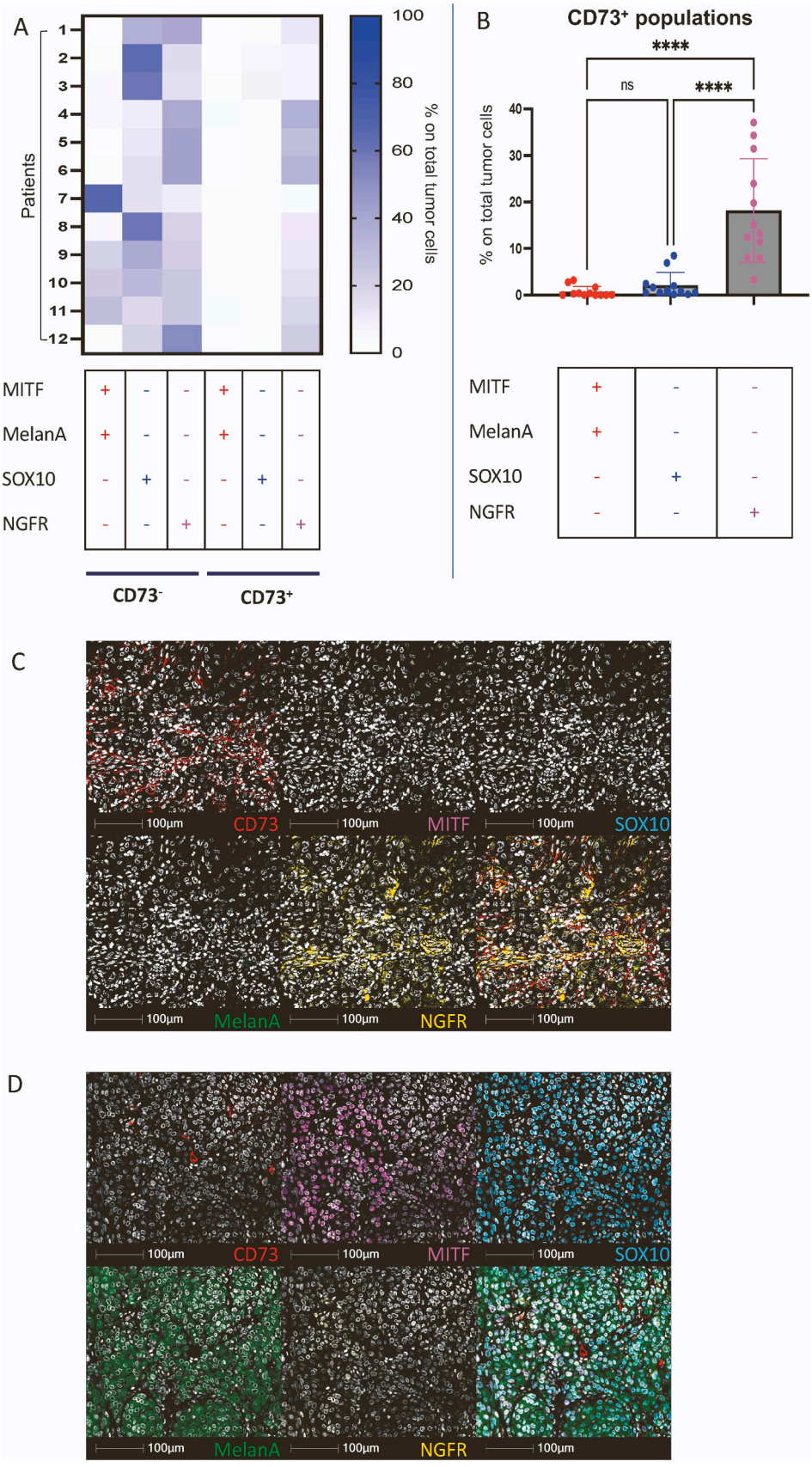

Fig. 6. Analysis of CD73 expression in human melanoma. A) Heat map showing the frequency of CD73- or CD73 + melanoma populations in each patient. The values plotted correspond to the mean of the percentage calculated considering the biological replicates for each patient (for the following patients we analyzed three cores: 3, 7, 8, 11, 12; while 2 cores were analyzed for the patients 1, 4, 6; and one core for 2, 5, 9, 10). Cores were excluded when the biological material was not sufficient for the analysis or when the tissue resulted damaged. The percentage for each group is calculated on the total of tumor cells. B) The expression of CD73 is significantly more frequent in NGFR^{+} cells, compared to MITF^{+} Melan-A^{+} or SOX10^{+} cells. In the graph, each dot corresponds to the mean of the percentage calculated considering the biological replicates for each patient, as in A. Mean ± s.d. is shown. P values are from two-way ANOVA. Followed by Turkey's multiple comparison test. **** = $p < 0.0001$. C) Representative image showing the concomitant expression of CD73 and NGFR, a marker of melanoma dedifferentiation. Notably, differentiation markers are not detected. D) Representative image showing the low expression of CD73 in tumor cells on the contrary positive for melanoma differentiation proteins. C, D) DAPI was used to detect nuclei, showed in white.

enzymatic function of CD73 inhibits the invasion of A375 melanoma cells, in line with previous published data [5,64].

Many agents targeting TGF-β have been developed over the years and many of them are being examined in clinical trials in different types of cancer in monotherapy or in combination with other chemotherapy or immune-therapeutics, showing good tolerability and encouraging results of efficacy [65]. Since TGF-β triggered by nutrient deprivation may contribute to inducing the protein expression of CD73, which is enzymatically active in producing adenosine, it is reasonable to suppose that targeting both TGF-β and adenosine signaling pathways would be effective. A very recent published study proves the therapeutic efficacy of CD73/TGF-β dual-blockade using a bifunctional anti CD73/TGF-β construct in a mouse model of triple negative breast cancer [66]. In this setting, given the critical role of both CD73/adenosine and TGF-β signaling pathways in mediating tumor immune escape and tumor cells proliferation and invasion, it will be interesting in future studies to investigate the efficacy of CD73/TGF-β dual blockade in melanoma.

In further support of the high expression of CD73 in tumor cells, we extended and deepened the analysis of CD73 protein expression in melanoma cells based on their phenotype, in tissue from patients. We observed that, in melanoma metastases, CD73 is highly expressed in tumor cells that are negative for MITF, Melan-A or SOX10, but positive for NGFR. This observation is in line with published data by Reinhardt and colleagues, who indicate that *CD73* gene expression is associated to a nascent and fully established invasive melanoma phenotype [22]. Specifically, the authors propose CD73 as a marker of activation of the EMT-like invasive program, being CD73 highly expressed by $MITF^{low}$ melanoma cells, and also expressed by a subset of $MITF^{high}$ melanoma cells where it marks the transition to the invasive phenotype [22]. Our results represent the first in situ proof of what observed by gene analyses in the before mentioned paper. The loss of melanoma-associated antigens is one of the mechanism behind resistance to immune checkpoint inhibitors in melanoma patients [67–69]. The effectiveness of these drugs depends on the reinvigoration of the antitumor immune response, that is not sufficient when the tumor cells lose their differentiation antigens: in such case, the T-cells cannot recognize the tumor cells, even if reactivated by the immune checkpoint inhibitors. In this context, the enhanced expression of CD73 by tumor cells may be an additional mechanism promoting the immune escape and the progression of disease.

5. Conclusions

In summary, our study demonstrates that in a condition of lower nutrient availability melanoma cells by releasing TGF-β up-regulate CD73 that produces adenosine. Within human melanoma metastases CD73 is highly expressed in invasive-like tumor cells. Understanding the mechanism regulating the CD73 expression and function in the tumor microenvironment may be informative for the development of effective combinatorial therapeutic regimens for cancer.

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CRediT authorship contribution statement

SM conceived the project. CG, RT, MH and SM designed experiments, analyzed and interpreted the data; CG, RT, LO, RB, SL performed experiments. JL provided clinical specimens. GR and CEM provided reagents. CG, RT and SM drafted the original manuscript. SL, CEM and MH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

MH received honoria from BMS, Novartis and research support from TME (NOXXON), all unrelated to this work. The other authors declare no potential competing interests.

Data Availability

Data will be made available on request.

Acknowledgements

The graphical abstract was created with BioRender.com.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115225.

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