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The human ectoenzyme CD73 (5' Ectonucleotidase):

analysis of its role in physiology and pathology by means of specific

monoclonal antibodies.

Candidate: Valentina MARIANI   **Tutor:** Fabio MALAVASI

Coordinator of the PhD Program:

Emilio HIRSCH

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Abstract

CD73, also known as ecto-5'-nucleotidase (E.C.3.1.3.5), is a dimeric protein anchored to the plasma membrane through a glycosyl-phosphatidylinositol (GPI)-anchor. Through its enzymatic activity, CD73 catalyzes the dephosphorylation of the extracellular nucleotide adenosine monophosphate (AMP) to generate the nucleoside adenosine (ADO), which is a potent immunosuppressor. Until recently, this CD73-mediated step was always envisaged as part of the classical pathway of ADO production, brought about by ATP degradation through the sequential activities of the ectoenzymes CD39 (NTPDase1) and CD73. However, in 2013, our Laboratory identified an alternative pathway for ADO production which does not involve CD39, but instead requires another two ectoenzymes: CD38, a NAD glycohydrolase, and CD203a (PC-1, Ectonucleotide Pyrophosphatase/Phosphodiesterase 1). As both adenosinergic pathways converge on CD73, we focused our attention on this molecule.

In addition to its enzymatic activity, CD73 also possesses a receptor function. This functional role has been mainly studied in T lymphocytes, where CD73 supplies a costimulatory signal for the T cell receptor (TCR), inducing proliferation in activated T cells. This costimulatory activity predicts the existence of a natural ligand of CD73, which is as yet unidentified. For experimental purposes, the problem of the unknown ligand can be overcome using agonistic antibodies which mimick the binding of the natural ligand and trigger downstream signaling. The anti-CD73 monoclonal antibody (mAb) 1E9 is such an agonistic antibody. In the first part of this Ph.D. project, a novel anti-CD73 mAb, called CB73, was identified and its agonistic properties characterized. We observed that CB73 binding to activated CD73$^+$ T cells led to increased total intracellular phosphorylation and increased surface expression of CD25 (Interleukin 2 Receptor Subunit Alpha).

CD73 is also expressed in B lymphocytes, where its receptor function is much less studied. In the second part of this doctoral project, the role of CD73 in B lymphocyte function was investigated. The results indicate that CD73 can also act as costimulatory receptor for the B cell receptor (BCR): in CD73^{+} B cells activated through BCR engagement, binding of CB73 mAb further enhanced signaling triggered by BCR stimulation alone.

CD73, as many others GPI-anchored receptor, possesses a Src binding domain. The Src family tyrosine kinase Fyn, which is involved in BCR-induced signaling, was found to be essential for intracellular signal transduction of CD73 in mouse. Our hypothesis is that CD73 acts as a costimulatory receptor for the BCR interacting with Fyn, which is active at the beginning of the BCR-signaling, leading to overactivation of BCR pathways.

CD73 is expressed in many normal human tissues but is also upregulated in malignancies, where cancer cells can take advantage of the high ADO levels present in the tumor microenvironment to escape from immune surveillance. CD73 enzymatic blockade has been shown to have antitumor effects, especially in combination with anti-PD-1 and anti-CTLA-4 antibodies. In our experimental model, we observed that CB73 mAb could reduce ADO production by 80%. Enzymatic blockade takes place in a non-competitive manner, suggesting allosteric modulation of CD73 by the CB73 mAb.

The final part of this work was focused on the link between CD73 and Osteopontin/secreted phosphoprotein 1 (OPN/SPP1), an important cytokine involved in the regulation of bone mineralization. Our studies centered on a family with mutated *NT5E* gene (a homozygous missense mutation), resulting into a non-functional molecule. The phenotype shows extensive calcification of the lower limb arteries and small joint capsules. Given the correlation between absence of CD73 and calcification, we analyzed the role of CD73 in OPN expression in an EBV-established cell line model. In the presence of BCR engagement and IL-

4 costimulation, CD73$^-$ cells did not produce OPN: in contrast, after CD73 restoration, CD73$^+$ cells expressed OPN, suggesting a link between CD73 and OPN production. Activation of the kinases Akt and Erk, involved in OPN production, were analyzed after BCR activation and IL-4 incubation; however, the presence or absence of CD73 did not appear influential. Thus, we hypothesized that the role of CD73 in OPN production is more related to its enzymatic activity rather than to its receptor role. As previously hypothesized by others, the link between CD73 and calcification may be based on the activity of tissue non-specific alkaline phosphatase (TNAP). However, our results suggest that CD73 deficiency may lead to increased extracellular AMP, which in turn activates the AMP-activated protein kinase (AMPK), leading to greater OPN production. The absence of ADO causes higher activation of TNAP, that can therefore dephosphorylate OPN increasing vascular mineralization.

Abbreviations

ACN	Acetonitrile
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADO	Adenosine
ADOR	Adenosine receptor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APCP	Adenosine 5'-(α,β-methylene) diphosphate
BCR	B cell receptor
DYP	Dypiridamole
EBV	Epstein-Barr Virus
E5NT	Ecto-5'-nucleotidase
EGF	Epidermal growth factor
EHNA	Erythro-9-(2-hydroxy-3-nonyl)-adenine
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase-1
ERK	Extracellular signal-related kinase
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
Gfi 1	Growth factor independence-1
GPI	Glycosylphosphatidyl inositol
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus

HPLC	High Performance Liquid Chromatography
INF	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinases
MFI	Mean Fluorescence Intensity
NAD^+	β-Nicotinamide Adenine Dinucleotide
OPN	Osteopontin
pAb	Polyclonal antibody
PBMC	Peripheral blood mononuclear cells
Pi	Inorganic phosphate
PMA	Phorbol 12-myristate 13-acetate
PPi	Pyrophosphate
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
RNAi	RNA interference
R_t	Retention time
TCR	T cell receptor
TGF	Transforming growth factor
TNAP	Tissue-nonspecific alkaline phosphatase
TNF	Tumor necrosis factor

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Premise

The aim of this PhD thesis is to highlight some of the characteristics of CD73, a human protein endowed with dual ectoenzymatic and receptor functions, which have not yet been characterized in full. The relevance of CD73 is two-fold: at the conceptual level, CD73 illustrates the crucial role played by enzymes and receptors in modulating significant aspects of the life of cells. At the translational level, CD73 is now being used in clinical trials aimed at modulating the immune response, either by manipulating the tumor cells (*i.e.,* immune evasion) or the effector cells (*i.e.,* immunosuppression).

Chapter 1. Introduction

1.1 - CD73

1.1.1 - CD73 protein structure

Human CD73 is encoded by the *NT5E* gene located at 6q14.3.[1] Structurally, the CD73 protein is a dimer composed of two identical 70 kDa subunits, each consisting of 523 amino acids, and linked together by non-covalent bonds. CD73 is bound to the plasma membrane through a GPI anchor attached via a C-terminal serine residue. The N-terminal domain contains a 26-amino acid signal peptide which coordinates the binding of two catalytic divalent metal ions (Co^{2+} and Zn^{2+}); the C-terminal domain contains the substrate-binding pocket (Figure 1). Thus, the substrate binds at the interface of the two domains.[2] CD73 also exist in soluble form (sCD73), resulting from the hydrolysis of membrane-bound forms by phosphatidylinositol-specific phospholipase C or by proteolytic cleavage.[3] Soluble forms have been described in several body fluids, including synovial fluid[4] and serum.[5]

1.1.2 - CD73 distribution

In humans CD73 is expressed by leukocytes from peripheral blood, lymph nodes, thymus (prevalently by reticular fibroblast and epithelial cells), spleen and bone marrow as well as colon, kidney, brain, liver, heart and lung.[6,7] CD73 is also upregulated in several types of cancer, including prostate cancer, ovarian carcinoma, breast cancer, melanoma, colon cancer, leukemia, head and neck cancer, glioblastoma and papillary thyroid carcinoma.[8] CD73 expression in cancer is often associated with resistance to chemotherapy, increased risk of metastasis and poor prognosis.[9,10]

1.1.3 - CD73 function

1.1.3.1 - CD73 as enzyme

CD73 is an ecto-5'-nucleotidase that catalyze the dephosphorylation of extracellular nucleoside monophosphates into nucleosides, with AMP as the preferred substrate and adenosine (ADO) as final product.[11] (Figure 1). Inhibitors of CD73 enzymatic activity are known, including ATP, ADP and its stable analogue adenosine 5'-(α,β-methylene) diphosphate (APCP). Although these inhibitors all bind to the catalytic site in CD73, they are not hydrolysed.[12] Extracellular ADO can then activate four types of widely distributed adenosine receptors (ADOR), named A_1, A_2A, A_2B and A_3. All four are G-protein coupled receptors with seven transmembrane domains. ADO can also be internalized through dipyridamole (DYP)-sensitive carriers.[13]
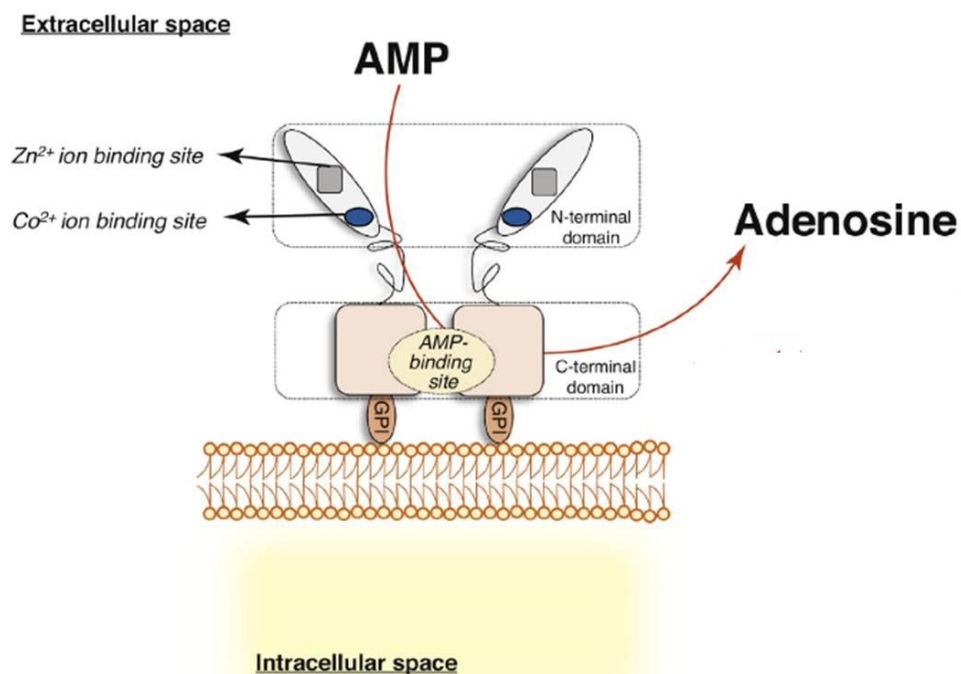
Figure 1. Structure and enzymatic function of CD73.

(Adapted from Antonioli L. *et al.*, in "Drug Discovery Today", 2017)

In addition to the AMP metabolic pathway, CD73 is also involved in NAD^+ metabolism. Although by far best known for its role as a coenzyme in redox reactions, NAD^+ can be released into the extracellular milieu and can be degraded by several enzymes such as NAD^+ glycohydrolases (*e.g.,* CD38, CD157/Bst1) and ectonucleotide pyrophosphatase phosphodiesterase 1 (*e.g.,* CD203a/ENPP1), which are involved in the pathways leading to ADO production.[14]

Through the production of ADO, CD73 affects many different physiological functions, such as: (i) ion transport in intact tissues; (ii) tissue barrier function (*e.g.,* closing inter-endothelial gaps during neutrophil transmigration); (iii) the adaptive response to hypoxia (the *NT5E* promoter contains a binding site for hypoxia-inducing-factor, HIF-1α); (iv) in ischemia by generating a more ischemia-tolerant phenotype[11] and (v) a crucial role in the downregulation of inflammation.[15]

Extracellular ADO, produced by CD73, is a critical regulatory autocrine and paracrine factor that accumulates in the tumor microenvironment. The concentration of this nucleoside, physiologically present at low levels in the interstitial fluids, can rapidly increase in response to pathological conditions, such as inflammation, hypoxia, ischemia or trauma.[16] Extracellular accumulation of ADO present during acute injury has protective effects by defending cells and tissues from excessive inflammatory responses and immune-mediated damage, as well as supporting a self-limiting immune response to promote healing processes.[17] On the other hand, the persistence of high levels of ADO during the acute-injury phase can become deleterious by activating pathways leading to immune suppression or reduction of wound-healing processes, resulting in a fibrotic remodeling.[18]

ADO may chronically accumulate in the tumor environment and stroma, a process associated with the generation of an immunosuppressed niche that favors the onset of

neoplasia.[19] Tumor microenvironments contain factors that drive CD73 expression: the *NT5E* promoter contains an HIF responsive element which favors the expression of CD73 in the hypoxic tumor microenvironment.[20] Consequently, ADO levels can be upregulated up to 20-fold compared to healthy tissues and reach micromolar concentration, enough to activate all ADORs. This connection between hypoxia and the adenosinergic system creates a pro-tumorigenic loop where the hypoxic tumor microenvironment drives the accumulation of extracellular ADO leading to ADOR-mediated tumor immune evasion.[21]

CD73 promotes spontaneous metastasis by cancer cells by enhancing migration in an adenosinergic manner by the A_2B ADOR and downstream pro-migratory mechanism.[22] Treatment of cancer cells with the CD73 enzymatic inhibitor APCP or by RNA interference (RNAi)-mediated silencing of CD73 leads to decreased adhesion of tumor cells to extracellular matrix proteins, fibronectin and laminin.[23] CD73 silencing by RNAi was shown to inhibit adhesion and invasion of cancer cells more potently than APCP, suggesting that the ability of CD73 to promote adhesion and invasion may relate to other mechanisms besides to its catalytic activity.

1.1.3.2 - CD73 as receptor

In addition to its enzymatic role, CD73 has also a receptor function. This functional activity of CD73 contributes to cell adhesion, in particular to the binding of lymphocytes to endothelial cells[24,25] and the adhesion of B cells to follicular dendritic cells.[26]

CD73 is also a costimulatory molecule for T cells. In addition to specific antigen receptors, other molecules contribute to T cell activation by acting as costimulators. Anti-CD73 mAbs in combination with suboptimal engagement of the T cell receptor (TCR) or in the presence of phorbol 12-myristate 13-acetate (PMA), stimulate human peripheral blood T cells to become

active, proliferate, express the IL-2R and secrete IL-2.[27,28] Moreover, recent studies in HIV-infected patients show that CD73 expression in CD8^{+} T cells is associated with increased antigen-specific responses, leading a potential impact of disease outcome. Purified CD73^{+} memory CD8^{+} T cells display a stronger ability to produce IL-2 and TNF-α upon HIV-1 specific stimulation compared to CD73^{-} counterparts.[29]

1.1.4 - CD73 as therapeutic target

The important role of CD73 in regulating tumor growth and metastasis through ADO production has opened new routes to the development of anti-CD73 therapies for different human cancers. Small molecular inhibitors (*e.g.,* APCP) or monoclonal antibodies (mAbs) that target CD73 suggest that anti-CD73 therapy may be an important therapeutic strategy for effective control of tumor growth and metastasis.[30,31]

One of the reasons behind the development of mAbs in oncology is their historically higher approval rates compared to small molecules. The success of mAbs comes partly from their higher specificity and longer half-life, and because they can exert both direct on-target and indirect immune-dependent antitumoral effects (*e.g.,* ADCC and ADCP).[31] The purpose of developing anti-CD73 mAbs is to induce antitumor immune responses. In the last two years, a novel human, non-competitive, high-affinity antagonistic antibody was developed against CD73, namely, MEDI9447. This antibody hinders the conversion of CD73 from a catalytically inactive to an active conformation. CD73 blockade by MEDI9447 reverses ADO-mediated suppression of CD4^{+} T cells and is associated with increased antigen presentation and enhanced lymphocyte activation, resulting in a greater release of proinflammatory Th_1 cytokines (TNFα, IL-1β and IFNγ). Moreover, *in vivo* MEDI9447 induces inhibition of syngenic tumor growth. At odds with other cancer immunotherapies, MEDI9447 was found to shape

the composition of both myeloid and lymphoid infiltrating leukocyte populations within the tumor microenvironment. MEDI9447 also increased the infiltration of several immune cell populations such as CD8^{+} effector cells and activated macrophages.[32-34] MEDI9447 also exhibits synergic activity upon combined administration with anti-PD-1 antibodies.[34] This confirms previous observations on the synergies between anti-CD73 mAb treatment and other T cell-directed immunotherapies, such as anti-PD-1 and anti-CTLA-4 mAbs.[35]

Treatment with anti-CD73 mAbs also has the potential to enhance the response to chemotherapy, resulting in immunogenic cell death. Indeed, CD73 expression correlates with the resistance of cancer cell lines to TRAIL-induced death, UV radiation and chemotherapeutics.[36] Targeting CD73 represents a novel approach that may enhance the efficacy of conventional and emerging therapies in the treatment of a wide spectrum of cancer types.[37]

1.2 - Monoclonal antibodies (mAbs)

Antibodies are composed of three functional units: two antigen-binding fragments (Fabs) that contain the variable region and confer antigen specificity and one constant fragment (Fc), linking antibodies to immune effector functions.[38] The concept of using antibodies to selectively target tumors was proposed by Paul Ehrlich over a century ago, while the advent of hybridoma technology in 1975 by Köhler and Milstein enabled the production of mAbs. These are antibodies that contain uniform variable regions and are thus specific for a single epitope. Antibodies are important therapeutic agents for cancer. In recent years, it has become clear that antibodies possess several clinically relevant mechanisms of action; for example, they can modulate tumor-related signaling. Moreover, antibodies exhibit different

immunomodulatory properties and, by directly activating or inhibiting molecules of the immune system, they can promote the induction of antitumor immune responses.[39]

1.2.1 - Antibody mimicry

Some mAbs operate as molecular agonists, substituting for the natural ligand of the receptor and inducing downstream signals. Although it is a common notion that an antibody can block the function of a receptor, evidence derived from studies in autoimmunity suggested that mAbs could also elicit a function. Antibody-mediated agonistic activity induces the receptor to dimerize or to crosslink, mimicking the activity of the specific natural ligand. The mAbs that efficiently trigger cell functions are defined as true agonistic antibodies. Antibodies may also bind epitopes distant from the orthogonal active site and they are defined as allosteric modulators. There are different mechanisms of allosteric binding of an agonistic antibody: (i) they may cause conformational changes leading to a functional modulation by sterically hindering ligand binding or (ii) they may bind selectively to stabilize different conformations of the receptor, thereby either enhancing (positive cooperativity) or decreasing (negative cooperativity) levels of signaling (Figure 2). The allosteric agonistic modulators represent an appealing alternative to the natural or endogenous ligand because they can modulate the response to the orthosteric agonists. The development of allosteric modulators represents a practicable research area for the design of therapeutic drugs. Such allosteric regulators provide advantages over recombinant ligands or traditional drugs, which are usually competitive inhibitors of the natural ligand. Among other advantages, binding of an allosteric regulator at a distant site provides reduced side-effects, saturability, and modulation in the presence of the true agonist.[38,40,41]
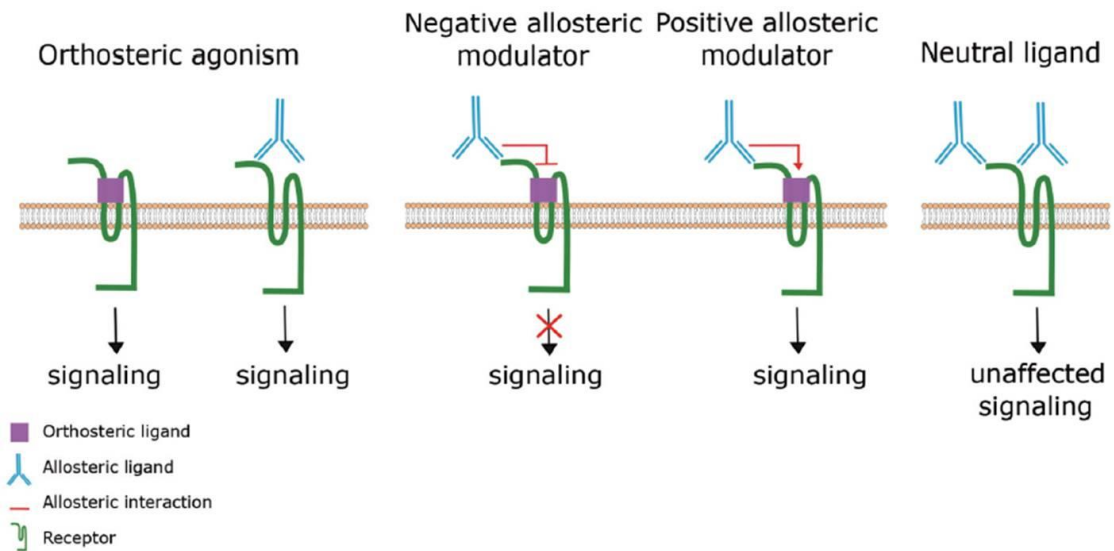
Figure 2. Orthosteric and allosteric agonism of mAbs on natural ligand function.

(Adapted from Horenstein A.L. *et al.*, in "Human Antibodies", 2017)

One example of specific mAbs used as ligand surrogate are the mAbs employed in CD38 functional analysis. CD38 display multiple functions, primarily attributed to its role as a receptor and lately as an ectoenzyme (NAD$^+$ glycohydrolase that leads to the generation of ADPR and nicotinamide as products). The effects induced by mAb ligation led to the hypothesis that CD38 might have a counterreceptor working as an adhesion molecule, subsequently identified as the surface molecule CD31. Indeed, the interaction between CD38 and CD31 reproduced what was seen with the agonistic mAb, inducing activation, cytokine release and proliferation in selected lymphocyte populations.[38,42-44] Another observation obtained using agonistic mAbs specific for CD38 concerns the selective induction of CD73 in human lymphocytes after CD38 ligation. CD38 cross-linking induced rapid export to the cell surface of pre-formed CD73, derived from an intracellular pool. Translocation lasted for 8 h, after which CD73 was removed from the cell surface by enzymatic cleavage. The CD73 protein induced on the cell surface upon CD38 stimulation was functionally active, acting as

a potent agonistic transducer that lowered the activation threshold of cord blood T cells by the CD3/TCR complex via a monocyte-independent mechanism.[45,46]

The analysis of the non-enzymatic functions of CD73 was yet another example of antibody mimicry. The natural ligand of CD73 is not presently known, but by using a specific anti-CD73 mAb the following functions of CD73 were uncovered: (i) the capacity to act as costimulatory molecule for CD2 and CD3 and (ii) the capacity to induce proliferation in T lymphocytes.[27,28]

Chapter 2. Materials and Methods

2.1 - Cells and cell lines

Cells and cell lines were cultured in RPMI-1640 media with 5-10% fetal calf serum (HyClone, Logan, UT, U.S.A), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, U.S.A) (hereafter referred to as complete medium). NIH/3T3-CD73 and Jurkat CD73$^+$ cells, lines stably transfected with the human *NT5E* gene (kindly provided by Dr. L. Thompson)[33] were grown in complete medium supplemented with 400 µg/ml G418 (Sigma-Aldrich, St. Louis, MO, U.S.A). CD73$^+$ EBV cells, line stably transfected with the human *NT5E* gene, was grown in the same medium as the other transfectants. PBMCs were isolated from peripheral blood after centrifugation on a Ficoll-Paque density gradient (GE Healthcare, Chicago, Illinois, U.S.A).

2.2 - Reagents

ADO, AMP, potassium dihydrogen phosphate (KH_2PO_4), acetonitrile (ACN, HPLC-grade reagent) and phorbol 12-myristate 13-acetate (PMA) were all purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) as were the inhibitors erytro-9-(2-hydroxy-3-nonyl)-adenine (EHNA, adenosine deaminase inhibitor) and dypiridamole (DYP, nucleoside transporter inhibitor).

2.3 - Antibodies

Monoclonal antibodies (mAbs) used for flow cytometry in this study include anti-CD73 (clone CB73), anti-CD69 (clone MLR-3), anti-CD3 (clone CBT3G), anti-CD19 (clone CB19), anti-CD16 (clone CB16), and anti-CD14 (clone S39), locally produced and in-house purified by affinity chromatography.[47] The PE-conjugated mouse anti-human CD25 mAb was from Immunostep (Salamanca, Spain). The fluorescein (FITC)-conjugated F(ab')$_2$ fragment goat anti-mouse

IgG+IgM [GαM Ig (G+M) FITC] was from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A).

The mAbs used for Western blot in this study include: anti-CD73 (Sigma-Aldrich Prestige Antibodies, St. Louis, MO, U.S.A), anti-p4EBP1 (Cell Signaling Technology, Danvers, MA, U.S.A), anti-Erk, anti-pErk, anti-Akt, anti-pAkt, anti-mTor, anti-pmTor and anti-cSrc (all from Santa Cruz Biotechnology, Dallas, Texas, U.S.A). The anti-pTyrosine HRP-linked antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas, U.S.A), the anti-rabbit IgG HRP-linked antibody was purchased from Cell Signaling Technology (Danvers, MA, U.S.A) and the goat anti-mouse IgG+IgM H&L HRP-linked antibody was purchased from Abcam (Cambridge, UK).

Isotype-matched irrelevant mAb used was X63.Ag8, an IgG1κ secreted by a parental mouse myeloma. The anti-human IgM/IgA/IgG (anti-BCR) was purchased from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A).

2.4 - Immunophenotypic analysis

Cells (2.5×10^5) were washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) + NaN_3 followed by incubation with the indicated mAb (1 h, 4°C). Cells were then washed twice and incubated with FITC-GαMIg (30 min, 4°C). When directly-labelled antibodies were used, cells were incubated with the mAb (30 min, 4°C) without further incubation with a secondary antibody. The samples were washed and resuspended in PBS and acquired on a FACSCanto (Becton-Dickinson, San Jose, CA, U.S.A), using FACSDiva Software (Becton-Dickinson). Data were analyzed using a FlowJo v10.0.8 software.

2.5 - Western blotting

Cells were starved overnight before treatment. Cell lysates were prepared with Triton X lysis buffer (50 mM Tris-HCl pH 7,4, 150 mM sodium chloride, 1% Triton X, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1% pervanadate and 1% protease inhibitor cocktail). Samples containing 15-30 µg proteins were analyzed by 8-10% SDS-polyacrylamide gel (polyacrylamide from Bio-Rad, CA, U.S.A) and electrophoresed (Bio-Rad electrophoresis system, Bio-Rad, Hercules, CA, U.S.A). The resolved protein bands were then transferred onto pre-activated (ethanol and after transfer buffer) polyvinylidene fluoride (PVDF) (EMD Millipore, Temecula, CA, U.S.A) membranes using a transfer buffer (10 mM Tris base + 100 mM glycine + 0.1% SDS and 20% ethanol). The blots were incubated 1 h at 4°C with blocking buffer (tris-buffered saline, 5% non-fat milk or 2% bovine serum albumin + 1% Tween-20) and overnight (or 1 h if using labelled antibodies) at 4°C with the primary antibody (diluted in tris-buffered saline, 2.5% non-fat milk or 1% bovine serum albumin + 1% Tween-20). Blots were extensively washed (tris-buffered saline + 1% Tween-20), incubated with an HRP-conjugated secondary antibody (diluted in tris-buffered saline + 1% Tween-20), washed (tris-buffered saline + 1% Tween-20) and proteins were visualized using the enhanced chemiluminescent substrate Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, U.S.A). Blot images were obtained with the Molecular Imager ChemiDoc™ XRS+ (Bio-Rad, Hercules, CA, U.S.A). Protein band intensity was evaluated using ImageJ Image Analysis Software.

2.6 - High-performance liquid chromatography (HPLC) measurement of enzymatic activity

Chromatographic analysis was performed with an HPLC System (Beckman Gold 126/166NM, Beckman Coulter, Milan, Italy) equipped with a reverse-phase column (Hamilton C18, 5 μm; 250 x 4.5 mm). Separation of nucleotides and nucleosides was carried out using a mobile-phase buffer (0.125 M citric acid and 0.025 M KH_2PO_4), pH 5.1 with 8% acetonitrile (ACN) in 10 min at a flow rate of 0.8 ml/min. UV absorption spectra were measured at 254 nm.

HPLC-grade standards compounds used to calibrate the signals were dissolved in AIM V serum-free medium (Invitrogen, Paisley, UK), pH 7.4, 0.2 μm filtered and injected at a volume of 20 μL. The retention times (R_t, in min) of ADO standard was 5.56. Peak integration was performed using a Karat software (Beckman Coulter).

Ectonucleotidase activities were measured using cells (1×10^6/ml) pre-treated with the inhibitors EHNA in combination with dipyridamole (15 min, 37°C), and incubated (37°C) in AIM V medium with AMP. Supernatants were successively collected and transferred in tubes containing ACN (1:2) to stabilize ADO, vortexed for 1 min and then centrifuged (13,000 g, 5 min at 4°C). Samples were evaporated to dryness by speed-vac, reconstituted in mobile-phase buffer, and assayed by HPLC. Qualitative identities of the peaks were confirmed by co-migration and absorbance spectra of known reference standards using a R_t window of ±5%. The presence of ADO was also confirmed by spiking standard ADO (100 μM), followed by chromatography. Quantitative measurements were inferred by comparing the peak area of samples with calibration curves for peak areas of each standard compound.

2.7 - EBV immortalization

Continuous lymphoblastoid cell lines were developed by Epstein-Barr virus (EBV) infection of B lymphocytes[48] obtained from a CD73-negative patient (homozygous mutation in *EN5T*).[49]

Control (CD73-positive) EBV cell lines were obtained from both sex- and age-matched individuals; one cell line was derived from a healthy young (25y) female.

2.8 - CD73 expression vector generation and EBV transfection

The full-length cDNA encoding CD73 was obtained by PCR amplification using total RNA from the MSTO-211H human mesothelioma cell line as template. The primers were: 5'-AACATTGGATCCTTTCGCACCCAGTTCACGCGCCA-3' (forward) and 5'-ACATGAATTCGGCAAGGAGAATTTTTGGCTATT-3' (reverse) primers (restriction sites for BamHI in forward primer, and EcoRI in reverse primer are underlined). The pCD73N-puro expression vector was constructed by inserting the BamHI/EcoRI CD73 cDNA fragment into BamHI/EcoRI digested pcDNA3-Puro vector [derived from pcDNA3 (Invitrogen)], in which the neomycin cassette was substitute by a puromycin cassette by Exponential Mega Priming PCR[50]). Recombinant plasmids were transformed into competent *E. coli* bacteria (DH5α, prepped in lab), and the insert of individual clones were validated by enzymatic digestion and direct sequencing (ABI Prism 3130, Applied biosystem, Sanger method). EBV line were transfected with the vector using the Amaxa Cell Line V Optimization Nucleofector Kit (Lonza, Cologne).

2.9 - Cytokine secretion analysis

Cells (1×10^6 cells/ml) were treated with 15 µg/ml of anti-human IgM/IgA/IgG (anti-BCR) and 15 ng/ml of IL-4 for the times indicated. Cytokine levels in culture supernatants or plasma were measured by ELISA kits purchased from R&D Systems for Osteopontin.

2.10 - Statistical analysis

All statistical analysis was performed by Prism 6 software (GraphPad, U.S.A.). Differences were considered significant at $p < 0.05$.

Chapter 3. Results

3.1 - Selection of a monoclonal antibody specific for CD73

The starting point of the research was to characterize a panel of mAbs specific for human CD73 available in our Laboratory in order to identify those with unique functional characteristics.

The first step was to test mAb reactivity with murine NIH/3T3 cells expressing human CD73 (Figure 3). Subsequently, candidate antibodies were analyzed for reactivity with human T and B cell line models. The selection process led to the identification of clone CB73, which fulfilled the desired specificity and binding characteristics. Clone CB73 was expanded and purified to obtain a functional grade antibody.[47]
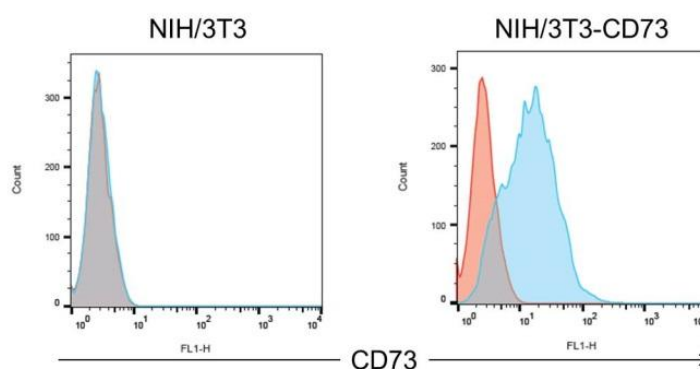
Figure 3. Flow cytometric analysis of CB73 mAb reactivity with NIH/3T3 cells expressing human CD73.

Red histograms shows staining with isotype-matched control while blue histograms show expression of CD73. Left panel, control NIH/3T3; right panel, NIH/3T3-CD73. In each sample, 1 x 10^4 cells were analysed; x-axis = fluorescence intensity, y-axis = number of cells (events).

3.2 - Analysis of the agonistic activity of the CB73 mAb in T cell lines

The ability of CD73 to induce proliferation[28] and to act as costimulatory molecule for CD3 and CD2 molecules[27] in T lymphocytes have been well characterized. As the natural ligand of CD73 is currently unknown, one approach involves testing the capacity of antibodies to deliver signals upon binding, in effect indicating the engagement of a domain normally bound by the natural ligand. We also tested mAbs different from those used in the early periods of CD73 analysis in the 1990s.[27,28,33,51-54] The first part of my Ph.D. project was the analysis of the binding and the agonistic activities exhibited by the CB73 mAb.

3.2.1 - Analysis of CD73 expression in T cell lines

The Jurkat cell line, derived from a human T acute lymphoblastic leukemia, was adopted as a model for T lymphocyte/TCR studies.[55] The Jurkat cell line does not express CD73. To have a positive counterpart for the study, Jurkat cells were transfected with an expression vector containing full-length human CD73 cDNA, generating a Jurkat CD73^{+} line (Figure 4).
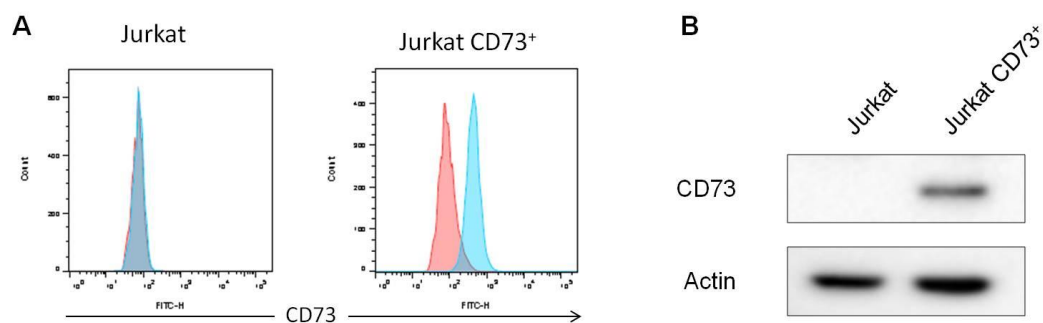
Figure 4. Expression of CD73 on Jurkat cell lines.

A) Cytofluorymetric analysis of CD73 expression on Jurkat and Jurkat CD73^{+} cell lines. Red histograms represent isotype-matched control staining; blue histograms represent expression of CD73. In each sample, 1×10^4 cells were analysed; x-axis = fluorescence intensity, y-axis = number of cells (events). B) Western blot analysis of CD73 in Jurkat and Jurkat CD73^{+} cells probed with anti-CD73 mAb.

3.2.2 - Analysis of the intracellular tyrosine phosphorylation after CD73 ligation with CB73 mAb

The ability of CD73 ligation to increase tyrosine phosphorylation of several intracellular proteins was first shown using the anti-CD73 mAb 1E9.[51] This result was the starting point for our analysis of total intracellular tyrosine phosphorylation levels using the CB73 clone selected. Jurkat and Jurkat CD73$^+$ cells were incubated with CB73 mAb (125 µg/ml)[28] for either 30 min or 4 h at 37°C. Jurkat cell lysates were prepared and tyrosine phosphorylation was analyzed by Western blot (Figure 5).
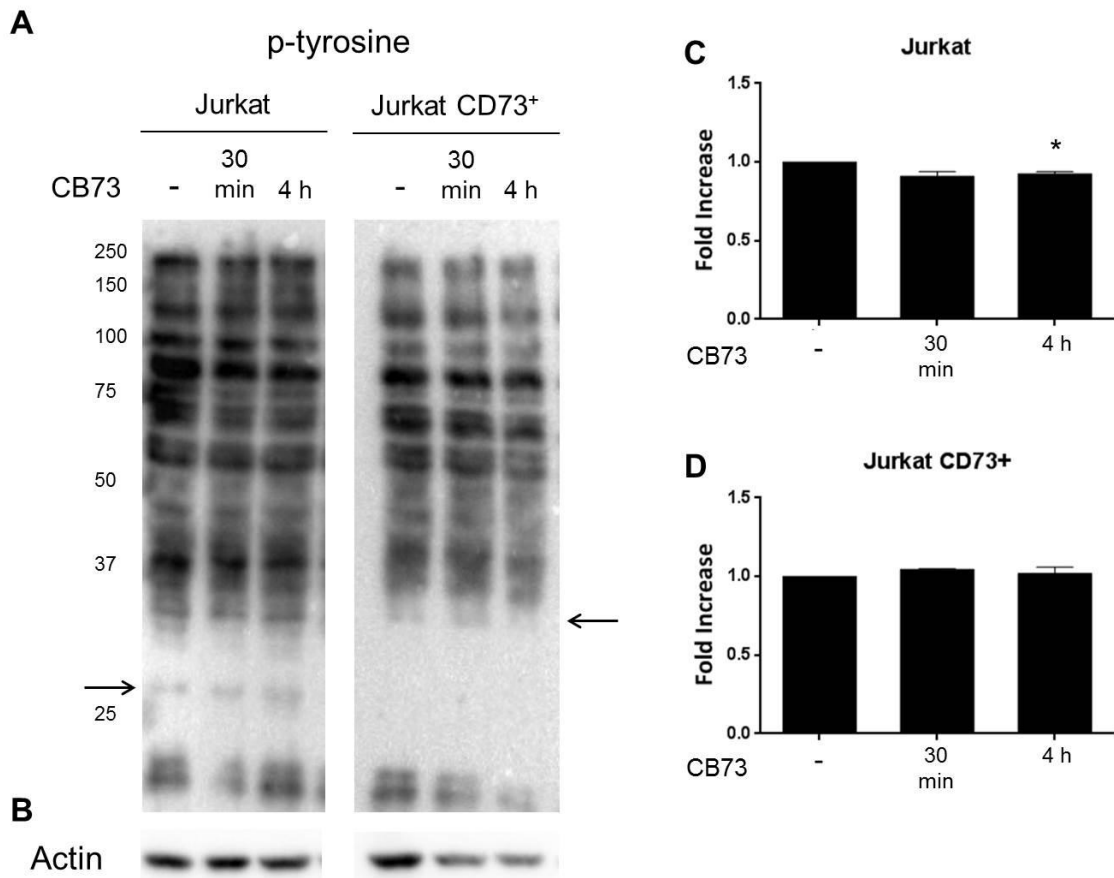
Figure 5. Western blot analysis of total tyrosine phosphorylation induced by CD73 binding in Jurkat cells.

Jurkat and Jurkat CD73$^+$ were incubated with CB73 mAb for 30 min or 4 h. A) Representative Western blot probed with anti-pTyrosine mAb detecting phosphorylated tyrosine residues in whole cells lysates. B) Blot obtained with anti-actin mAb. C-D) Histograms show modification levels of total tyrosine phosphorylation following Western blotting of Jurkat and Jurkat CD73$^+$ cells treated with CB73. Error bars are ± SEM.

At any experimental conditions, in the Jurkat CD73$^+$ cell line, we observed decreased phosphorylation of two proteins of 27 kDa and 32 kDa, compared to Jurkat cells. In contrast, there were no significant differences in total tyrosine phosphorylation levels between control Jurkat and Jurkat CD73$^+$ following incubation with CB73 mAb. Prior knowledge that CD73 has the characteristics of an accessory molecule suggested the possibility that it was necessary to pre-activate cells. Therefore, the experiment was repeated after Jurkat cell

activation. Cells were activated by treatment with PMA (Phorbol 12-myristate 13-acetate, a protein kinase C activator)[56] (100 ng/ml) for 24h before incubation with CB73 mAb. To confirm cell activation, expression of the early activation marker CD69 was analyzed[57] (Figure 6).
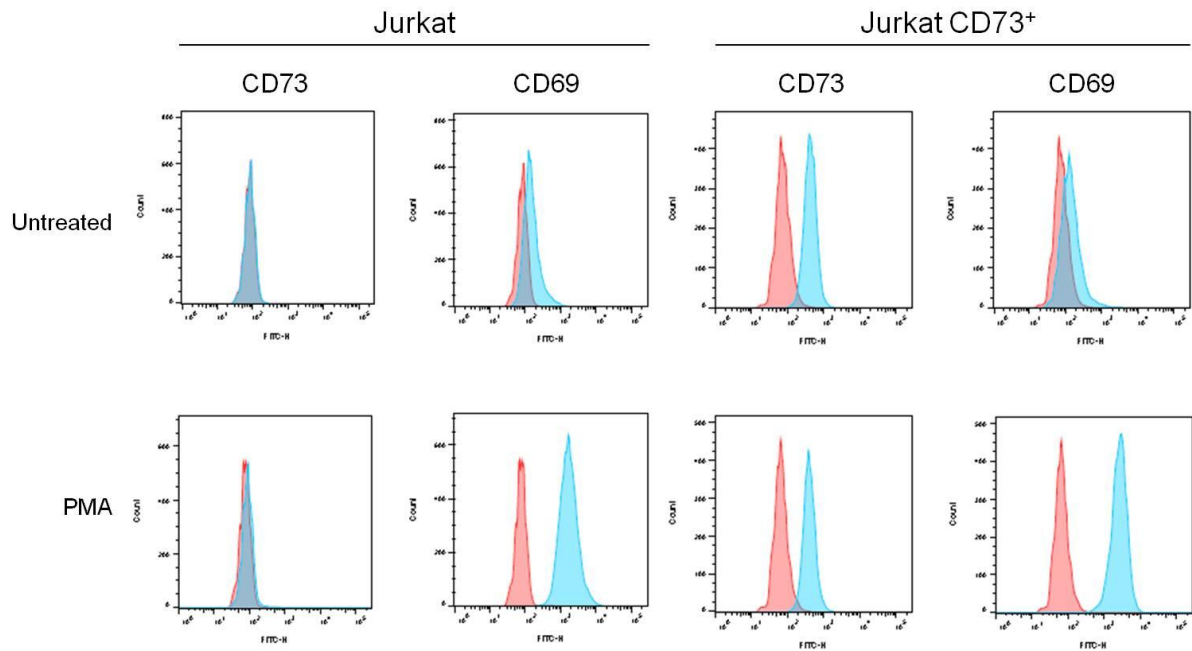
Figure 6. Comparative cytofluorymetric analysis of CD73 and CD69 expression in resting and PMA-activated Jurkat cell lines.

Control and CD73-positive Jurkat cells were stained with primary antibodies against CD73 and CD69. Red histograms represent isotype-matched control staining and blue histograms depict the expression of the indicated antigen. In each sample, 1×10^4 cells were analysed; x-axis = fluorescence intensity, y-axis = number of cells (events).

Control Jurkat and Jurkat CD73$^+$ were incubated at 37°C with CB73 mAb (125 µg/ml) for 1 h, 30 min, 15 min, 5 min or 3 min. Whole cell lysates were analyzed by Western blotting for total tyrosine phosphorylation (Figure 7).
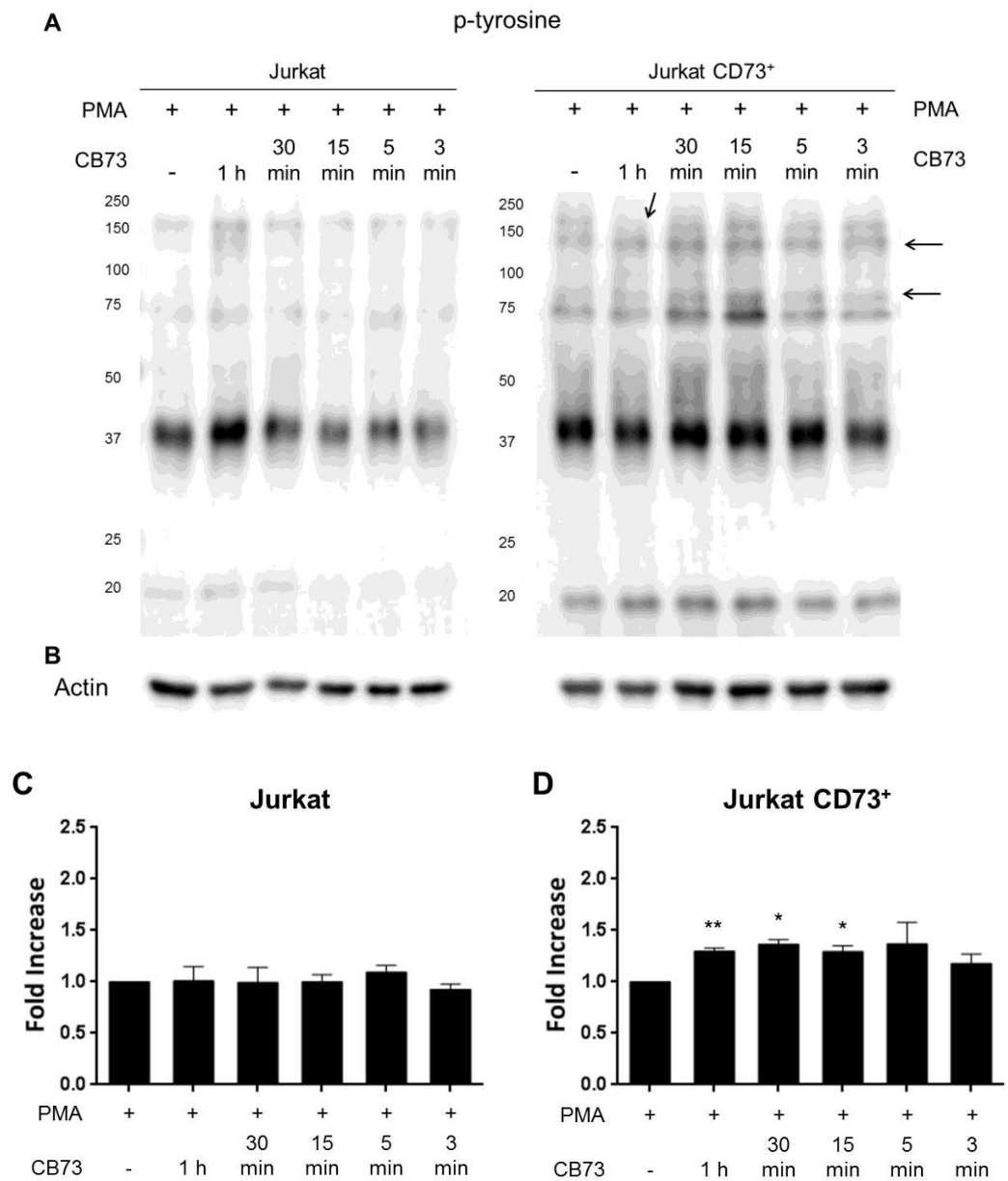
Figure 7. Analysis of total tyrosine phosphorylation in Jurkat cells pre-activated with PMA and incubated with CB73 mAb.

Control Jurkat and Jurkat CD73$^+$ were activated with PMA prior to incubation with anti-CD73 CB73 mAb for 1 h, 30 min, 15 min, 5 min and 3 min. A) Representative Western blot probed with anti-pTyrosine mAb detecting phosphorylated tyrosine residues from whole cells lysates. B) Blot obtained with anti-actin mAb. C-D) Histograms show modification levels of total tyrosine phosphorylation of Jurkat CD73$^+$ and Jurkat cells pre-activated and treated with CB73 mAb compared to pre-activated cells. Error bars are ± SEM.

As shown in Figure 7, PMA activation modified the pattern of total intracellular phosphorylation in Jurkat cells. CD73-mediated signals triggered by CB73 mAb binding induced increased levels of total tyrosine phosphorylation in Jurkat CD73^{+} with respect to CD73^{-} Jurkat controls. The process of phosphorylation started quickly (3 min) and was maintained along the time course of the experiment, suggesting that the CB73 mAb has agonistic properties. In addition, in Jurkat CD73^{+} cells two phosphorylated proteins at 132 kDa and 83 kDa were evident, which were absent in Jurkat controls. Further, a dephosphorylated protein at 143 kDa was apparent in Jurkat CD73^{+} cells after 1 h of incubation with CB73 mAb.

3.2.3 - Analysis of CD25 expression after CD73 ligation

T lymphocyte costimulation by CD3/CD73 has been reported to induce expression of CD25 (or IL-2 receptor α).[27] We tested CB73 mAb for the capacity to trigger expression of CD25. Jurkat CD73^{+} and Jurkat control cells were pretreated with PMA followed by incubation with CB73 mAb (50 μg/ml or 125 μg/ml) for 24 h or 48 h. Cells were then analyzed by flow cytometry for CD25 expression. Pretreatment with PMA and incubation with CB73 mAb induced an increase in the number of CD25-positive cells as compared to untreated cells. According to the MFI (mean fluorescence intensity), CB73 binding to Jurkat CD73^{+} cell line was followed by increased MFI levels when compared with PMA alone. This functional effect increased with time, with greatest increase observed after 48 h incubation with the CB73 mAb. These effects were dose-independent, being maintained also at the lower concentration used here of 50 μg/ml (Figure 8).
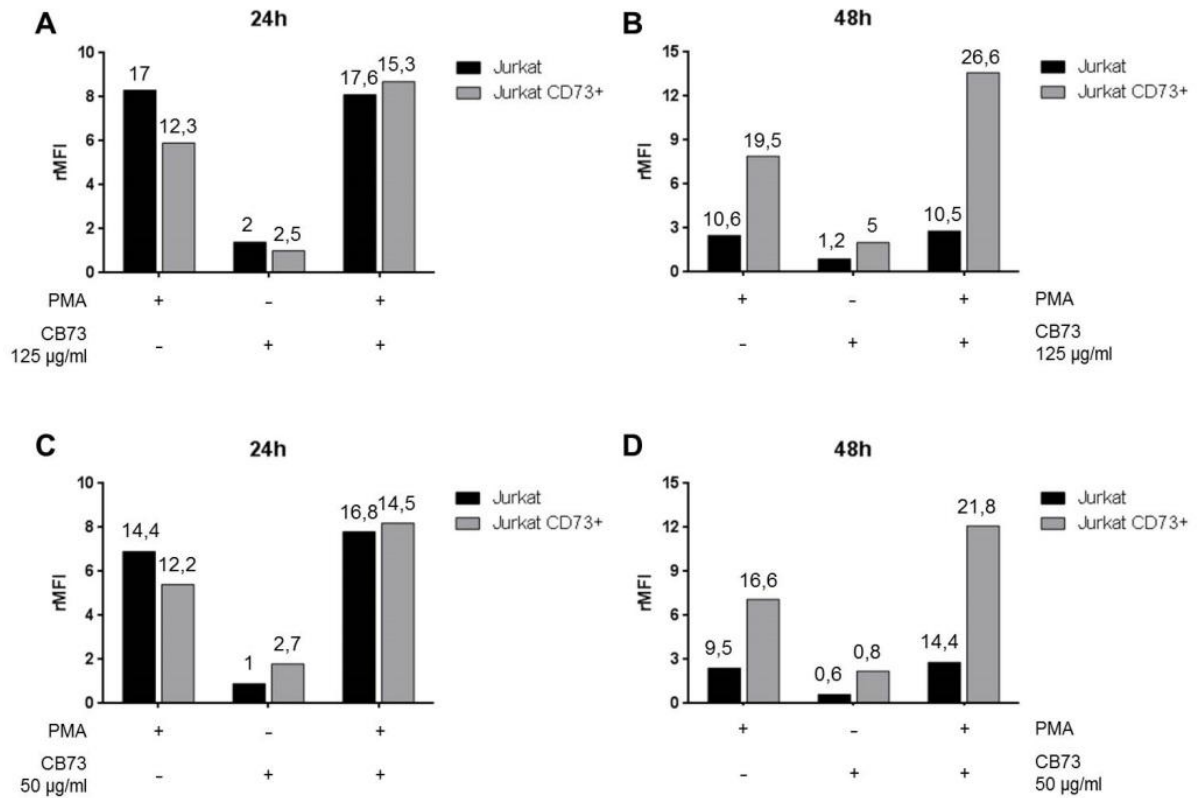
Figure 8. Relative MFI of Jurkat and Jurkat CD73^{+} incubated with PMA and/or CB73 mAb and stained for CD25.

Histograms represent relative MFI (rMFI) of CD25 staining obtained from the ratio of MFI value of each sample with respect to the untreated sample. The numbers on the top of each column represent the ratio between the percentage of positive cells of each sample with the untreated. A,B) Control Jurkat and Jurkat CD73^{+} cells were incubated with PMA and/or CB73 (125 µg/ml) for times indicated. C,D) Jurkat CD73^{+} and Jurkat cell lines incubated with PMA and/or CB73 (50 µg/ml) for times indicated.

3.2.4 - Analysis of Erk phosphorylation after CD73 ligation

The MAPK pathway is one of the major signaling pathways involved in regulating CD25 expression.[58,59] Since extracellular signal-related kinase Erk is one of the most representative key points of this pathway, Erk phosphorylation levels were analyzed by Western blotting in our Jurkat model. Control Jurkat and Jurkat CD73^{+} cells were pre-activated with PMA (24 h) and incubated at 37°C with the CB73 mAb (50 μg/ml or 125 μg/ml) for 1 h, 30 min, 15 min, 5 min and 3 min (Figure 9).
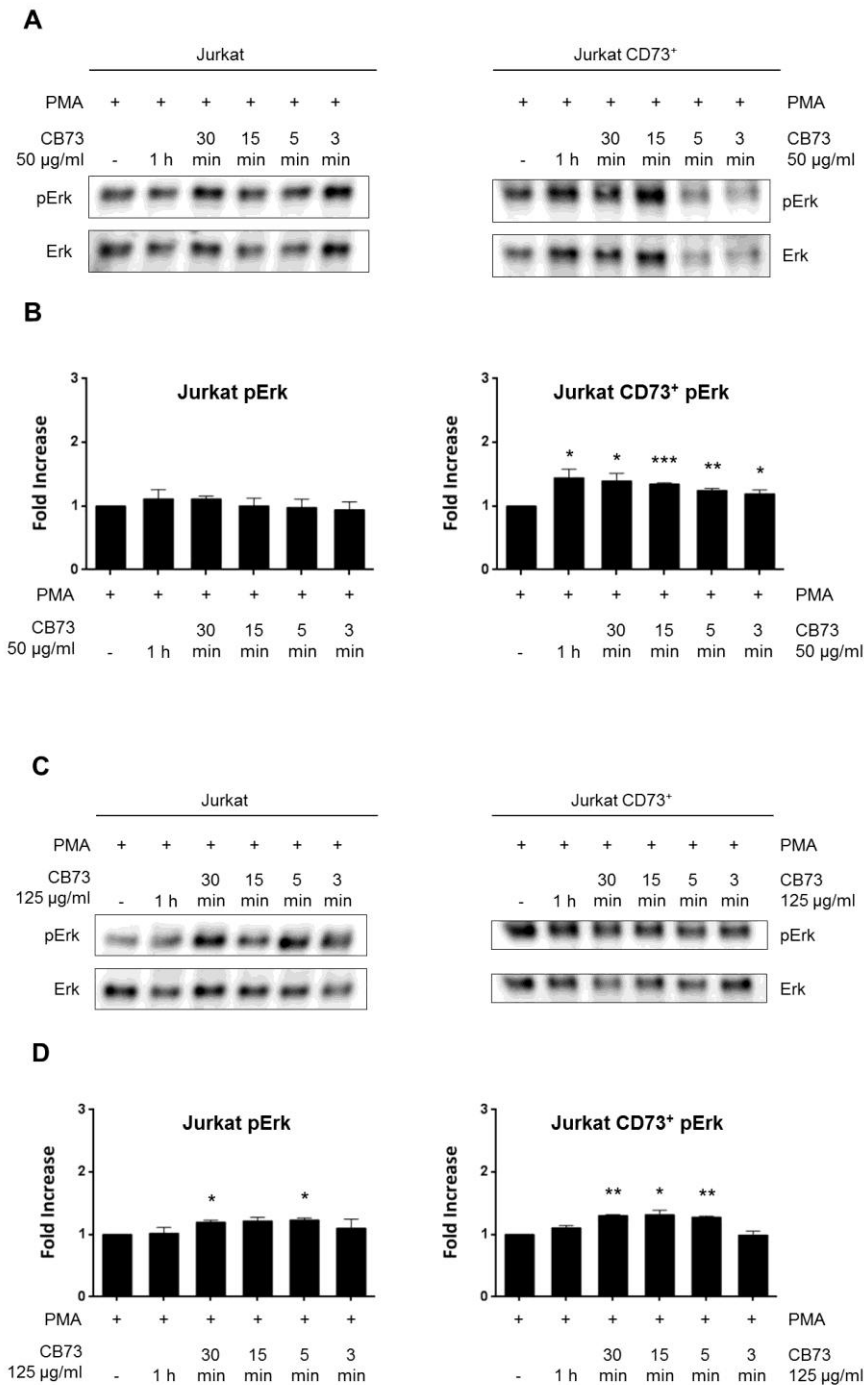
Figure 9. Erk phosphorylation levels in Jurkat and Jurkat CD73$^+$ cells after PMA activation and incubation with CB73 mAb.

A,C) Representative Western blots of total cell lysates from Control and CD73-positive Jurkat cells activated by PMA, treated with CB73 mAb (either 50 µg/ml or 125 µg/ml) for 1 h, 30 min, 15 min, 5 min and 3 min, and probed with anti-Erk or anti-phosphoErk (pErk) mAb. B,D) Histograms summarize the modification levels of phosphorylated Erk in Jurkat and Jurkat CD73$^+$ cells pre-activated and treated with mAb CB73 compared to pre-activated cells without CB73 mAb incubation. Error bars are ± SEM.

Ligation of CD73 by CB73 mAb in CD73$^+$ Jurkat cells led to increased phosphorylation levels of Erk as compared to the Jurkat controls. This effect is maintained also at low concentration of the mAb and is maintained along the time course of the experiment. The observed increase in Erk phosphorylation confirms that CB73-mediated triggering of CD73 activates the MAPK pathway and subsequent CD25 expression. The bulk of these findings suggest that the CB73 mAb is endowed with agonistic capacity in T lymphocytes.

3.3 - Analysis of the agonistic activity of CB73 mAb in non-pathological B cells

We then addressed our attention to human B lymphocytes, where the costimulatory abilities of CD73 have not, to our knowledge, yet been investigated.

3.3.1 - Immortalization of normal B lymphocytes

The experience gathered using the Jurkat cell line model of T lymphocytes was adapted for similar studies in the B cell compartment. However, in this case, the model adopted was EBV-immortalized B lymphocytes, an extremely useful *in vitro* model with which to study the B cell compartment. An advantage of EBV-derived B cell lines is that they can be easily maintained in culture without need of special growth requirements, and they grow continuously for months if necessary, all the time maintaining the functional and genetic characteristics of the donor cell.

B lymphocytes were obtained from a healthy donor, the cells were exposed to EBV virus, stabilized in vitro, analyzed for phenotypic characteristics and then used for the experiments. This cell line was designated Donor EBV line (Figure 14).

3.3.2 - BCR pathway analysis after CD73 ligation in the Donor EBV line

Since CD73 acts as a costimulatory molecule for the human TCR[27], the next question was does CD73 play a similar role in signaling mediated by the BCR, *i.e.*, the antigen receptor system of the B cell lineage? To answer this question, we performed experiments that paralleled those performed in T cells. The Donor EBV cells, which express CD73 (Figure 14), were incubated with CB73 mAb and the effects on phosphorylation were analyzed. We focused on the activation of two kinases, Erk and Akt, involved in the MAPK pathway and in the PI3K/Akt pathway, respectively. Both MAPK and PI3K are among the major pathways activated by the BCR.

Donor EBV cells were incubated at 37°C with CB73 mAb for 1 h, 30 min, 15 min, 5 min and 3 min. Cells were then lysed and analyzed by Western blotting for phosphorylation of the kinases Erk and Akt (Figure 10).
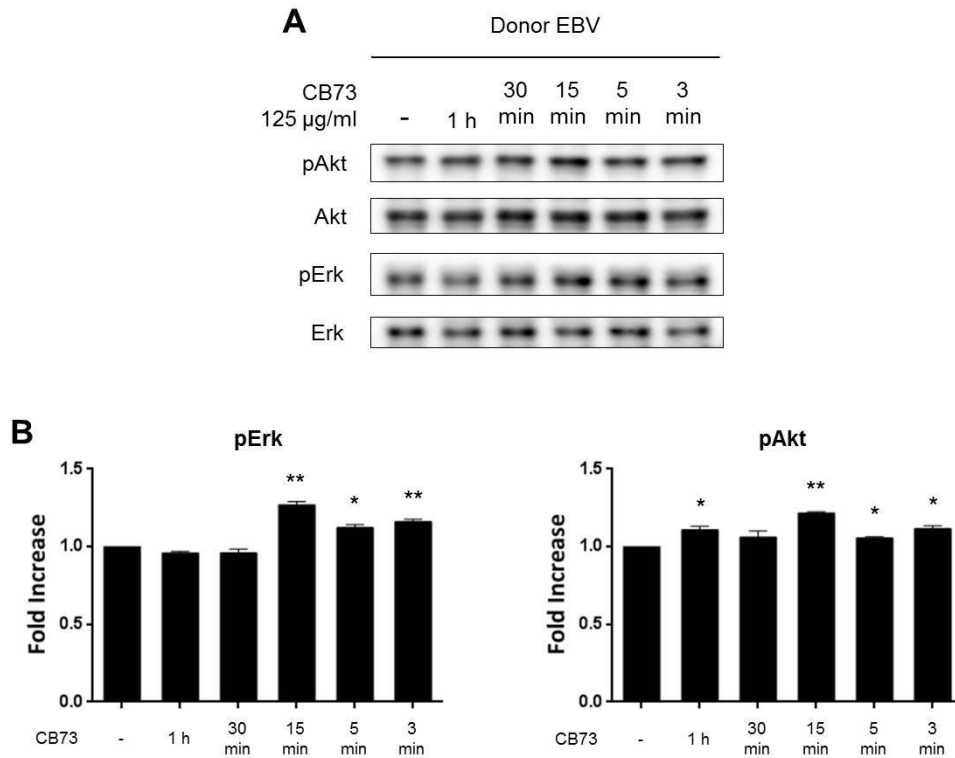
Figure 10. Analysis of Erk and Akt phosphorylation in Donor EBV cells after incubation with CB73 mAb.

A) Donor EBV cells were incubated with CB73 mAb for the times indicated. Representative Western blot detecting total and phosphorylated Erk and Akt in whole cell lysates of Donor EBV cells incubated with CB73 mAb the times indicated. B) Histograms summarizing the modification levels of phosphorylated Erk and Akt in the Donor EBV cell line incubated with CB73 mAb compared to untreated cells. Error bars are ± SEM.

As shown in Figure 10, Akt and Erk phosphorylation increased after CB73 mAb incubation, especially after the shorter incubation times. These first results supported the hypothesis that CD73 may mediate intracellular signals also in B cells.

Having observed that the agonistic activity of CB73 mAb was more pronounced after cell activation and using CB73 mAb at a concentration of 50 µg/ml, a similar approach was adopted for Donor EBV cells. Activation was obtained by engaging the BCR by incubating Donor EBV cells for 24 h with a polyclonal anti-BCR Ab (30 µg/ml). Donor EBV cells were then

incubated at 37°C with the CB73 mAb (50 µg/ml) for 30 min, 5 min and 3 min, respectively. Total cell lysates were analyzed for whole-cell tyrosine phosphorylation by Western blot (Figure 11).
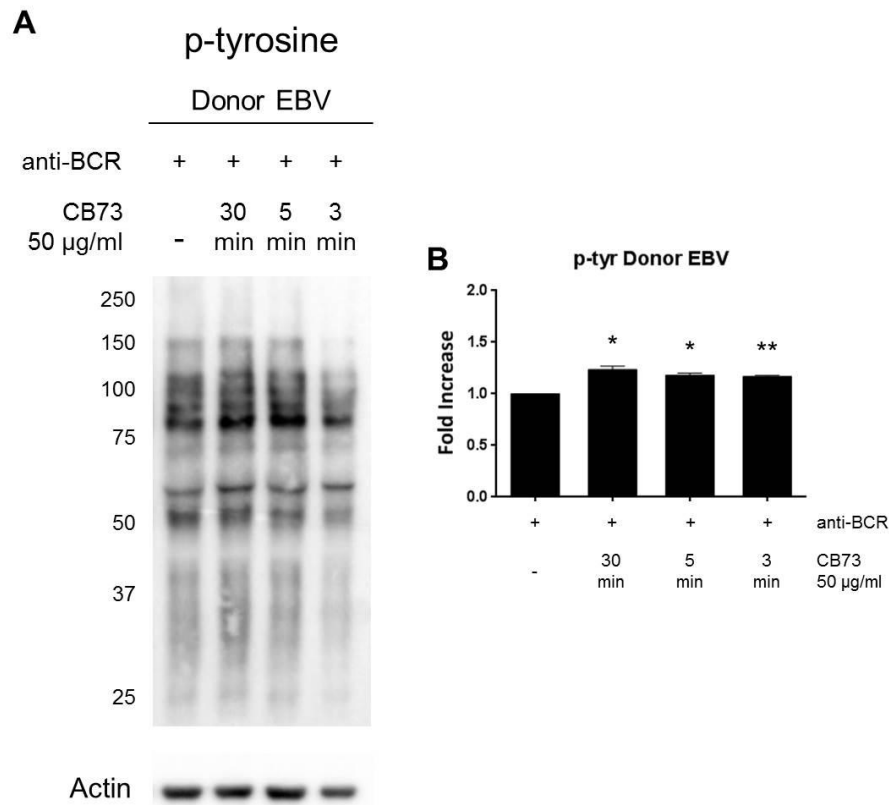
Figure 11. Analysis of total tyrosine phosphorylation in Donor EBV cells line after pre-activation and incubation with CB73 mAb.

A) Donor EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot probed with anti-pTyrosine mAb detecting phosphorylated tyrosine residues and actin from whole cells lysates. B) Histograms summarize the modification levels of total tyrosine phosphorylation compared to pre-activated cells. Error bars are ± SEM.

Following this approach, Donor EBV cells were found to display increased levels of tyrosine phosphorylation. The evaluation of total protein phosphorylation in tyrosine provides a general overview about cytoplasmic signals. The limit is represented by the fact that the

analysis does not provide information about the activity of kinases phosphorylated in serine and/or threonine or which pathways are activated. To answer these questions, cell lysates were analyzed for the activation of the major BCR-induced pathways, namely MAPK (Erk), PI3K/Akt (Akt, the translation initiation factor 4EBP1 and the kinase mTor) and the kinase cSrc (a member of Src family)[60] (Figure 12).
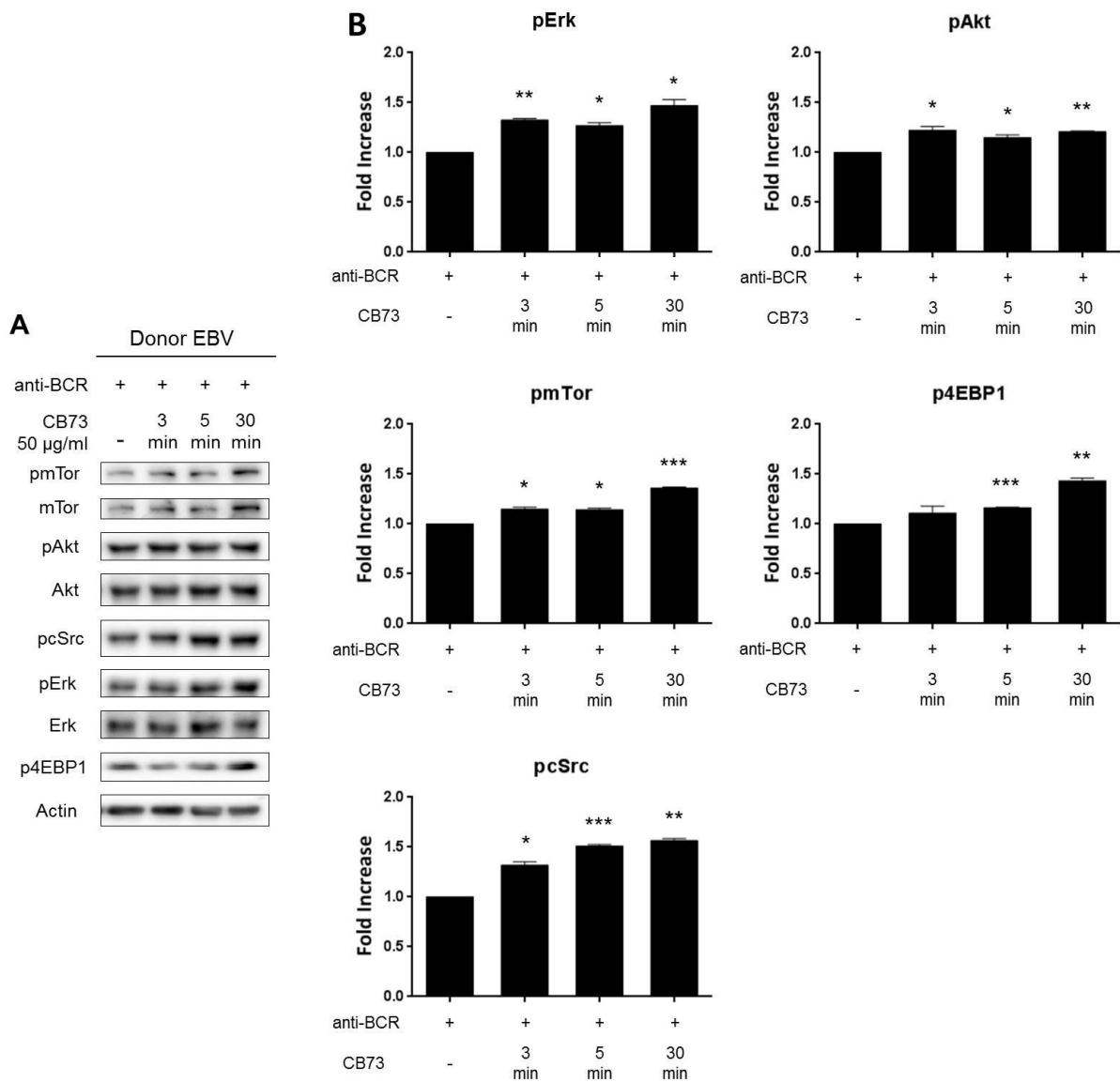
Figure 12. Erk, cSrc and Akt pathway phosphorylation levels of Donor EBV cell line after pre-activation and incubation with CB73 mAb for 30 min, 5 min and 3 min.

A) Donor EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot detecting phosphorylated aforementioned kinases and total protein from whole cells lysates. B) Histograms summarize the modification levels of protein phosphorylation compared to pre-activated cells. Error bars are ± SEM.

The results obtained indicated that all the proteins analyzed display increased phosphorylation in the presence of CB73 mAb, prevalently after approx. 30 min of incubation. These findings confirm that CB73 mAb is: (i) endowed with agonistic properties not only in T cells but also in B lymphocytes and (ii) the signaling events triggered by CB73 include BCR-controlled pathways.

3.4 - Analysis of the agonistic activity of CB73 mAb in pathological B cell lines

Three families with autosomal recessive CD73 deficiency were identified in our Department of Medical Genetics in Torino, and then studied as part of a small group of patients (three families) with similar disease.[49] The Torino family that came to our attention has three affected sisters who show the same homozygous missense mutation (c.1073G → A, p.C358Y) in the *NT5E* gene, resulting in significantly reduced trafficking of a CD73 devoid of AMPase activity to the cell surface.[61] These siblings represent a unique human model, constituting a natural human CD73 knock-out model. For our investigations into the functional role of the CD73/CB73 antigen/antibody pair, these patients represent a most important resource. We obtained B lymphocytes from these patients in order to generate CD73-negative EBV cells lines, which were characterized during the course of this doctoral work.

3.4.1 - *NT5E*-mutated B cells

3.4.1.1 - Blood populations and immortalization of B cells from patients with *NT5E* mutation

One of the siblings of the Torino family with the homozygous *NT5E* mutation was first analyzed to establish the composition of the leukocyte populations present in peripheral blood. At issue was the fact that a patient could live and thrive in the absence of CD73, and hence without the ability to produce extracellular ADO. Notwithstanding the extremely limited number of patients described with mutations in *NT5E* (nine in the world), their clinical characteristics apparently suggest no history of autoimmune diseases nor any impairment of the immune or inflammatory responses. However, these patients do share one clinical characteristic: severe arterial calcification, specially in the lower limbs. Cytofluorimetric analysis of PBMC from the *NT5E* patient showed that the main cell subpopulations were within normal ranges (Figure 13).
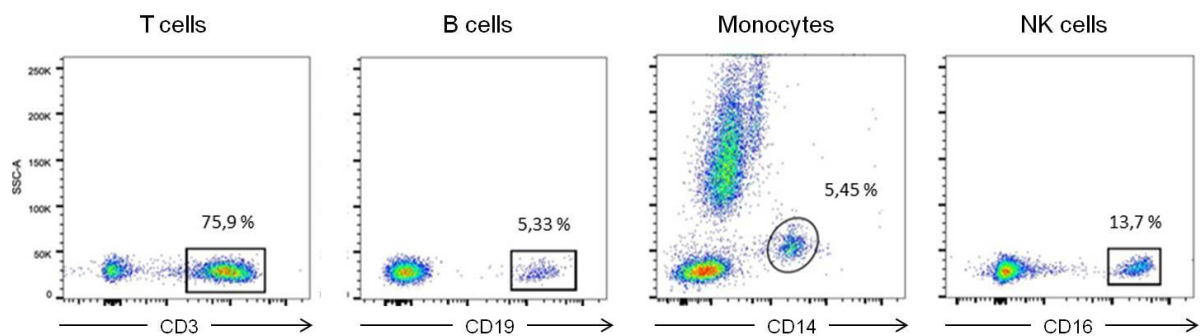
Figure 13. Cytofluorimetric analysis of PBMC isolated from CD73⁻ patient.
PBMC isolated from the *NT5E* patient were stained with primary antibodies against CD3, CD19, CD14 and CD16.

Also to avoid frequent blood withdrawal from these rare patients, EBV was used to immortalize cells from the B lymphocyte compartment. The CD73⁻ EBV line obtained was

confirmed to lack detectable surface CD73 by indirect immunofluorescence (IF). Donor EBV was used as CD73-positive control (Figure 14).
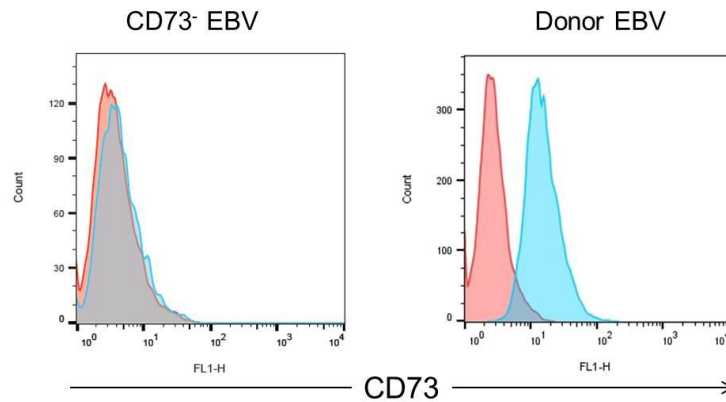
Figure 14. Comparative cytofluorymetric analysis of normal (Donor) and CD73⁻ EBV cell lines. EBV cell lines with and without CD73 were stained with primary antibodies against CD73. Red histograms represent isotype-matched control staining while blue histograms represent CD73 expression. In each sample, 10^4 cells were analysed; x-axis = fluorescence intensity, y-axis = number of cells (events).

3.4.1.2 - BCR pathway analysis after CD73 ligation on CD73⁻ EBV cell line

The costimulatory role of CD73 in association with BCR-induced pathways was tested in the EBV CD73⁻ model. Akt and Erk phosphorylation levels were analyzed by Western blotting from whole cell lysates of CD73⁻ EBV incubated at 37°C with the mAb CB73 for 1 h, 30 min, 15 min, 5 min and 3 min (Figure 15).
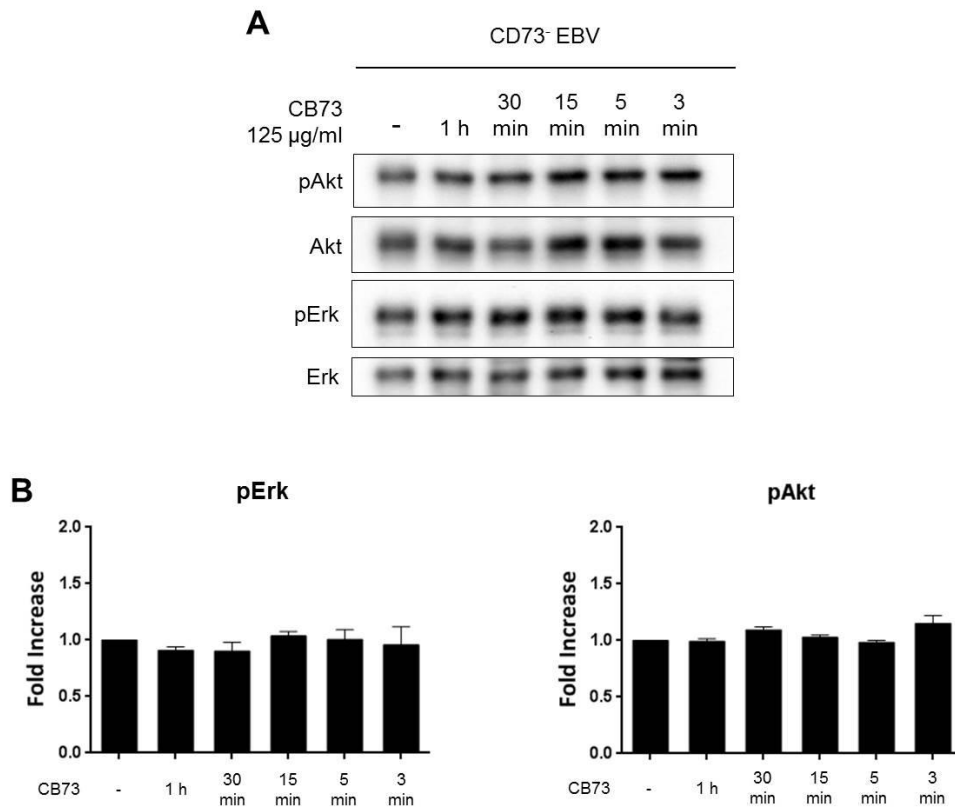
Figure 15. Analysis of Erk and Akt phosphorylation in CD73⁻ EBV cells after incubation with CB73 mAb.

A) CD73⁻ EBV cells were incubated with CB73 mAb for the times indicated. Representative Western blot detecting total and phosphorylated Erk and Akt in whole cell lysates of CD73⁻ EBV cells incubated with CB73 mAb the times indicated. B) Histograms summarizing the modification levels of phosphorylated Erk and Akt in the CD73⁻ EBV cell line incubated with CB73 mAb compared to untreated cells. Error bars are ± SEM.

The analysis of CD73⁻ mediated signaling in resting condition did not show significant differences in Erk and Akt phosphorylation levels.

Considering the experience accumulated by adopting a protocol based on activation and incubation at low amounts of CB73 mAb, CD73⁻ EBV cell line was pre-activated and CB73 mAb concentration was decreased to 50 µg/ml. After CD73⁻ EBV cells incubation with CB73 mAb for 30 min, 5 min and 3 min, total tyrosine phosphorylation was analyzed by Western blot (Figure 16).
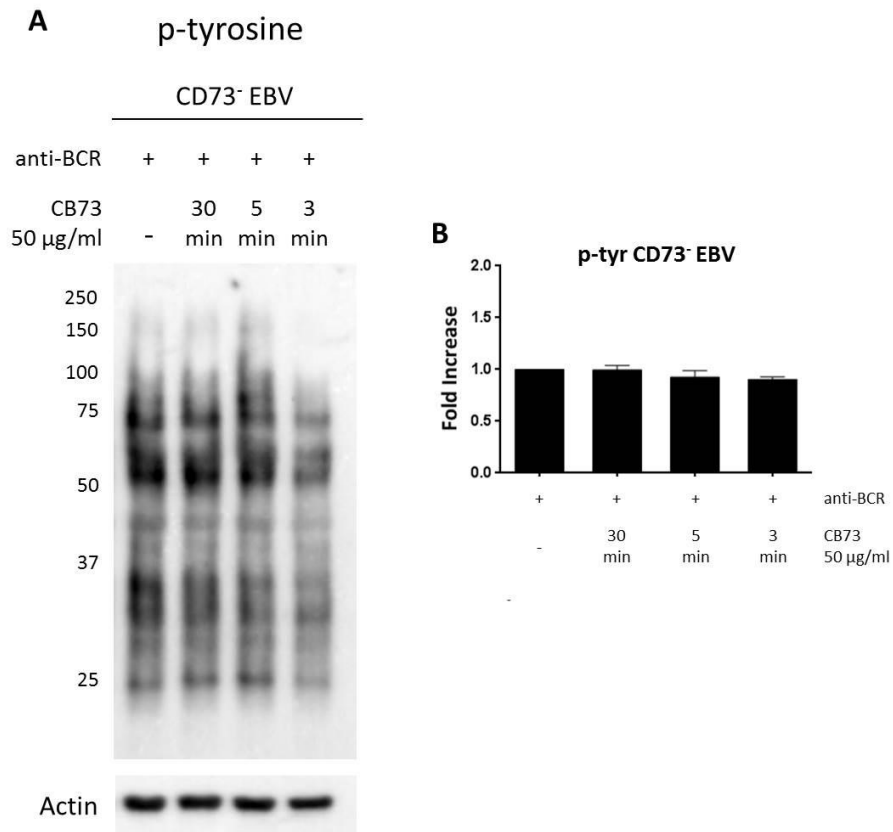
Figure 16. Analysis of total tyrosine phosphorylation in CD73⁻ EBV cells line after pre-activation and incubation with CB73 mAb.

A) CD73⁻ EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot probed with anti-pTyrosine mAb detecting phosphorylated tyrosine residues and actin from whole cells lysates. B) Histograms summarize the modification levels of total tyrosine phosphorylation compared to pre-activated cells. Error bars are ± SEM.

There are no significant differences in total tyrosine phosphorylation levels at any incubation times.

After total tyrosine phosphorylation analysis, BCR-induced pathways were analyzed more specifically and there are no significant differences in phosphorylation levels of all the kinases analyzed (Figure 17).
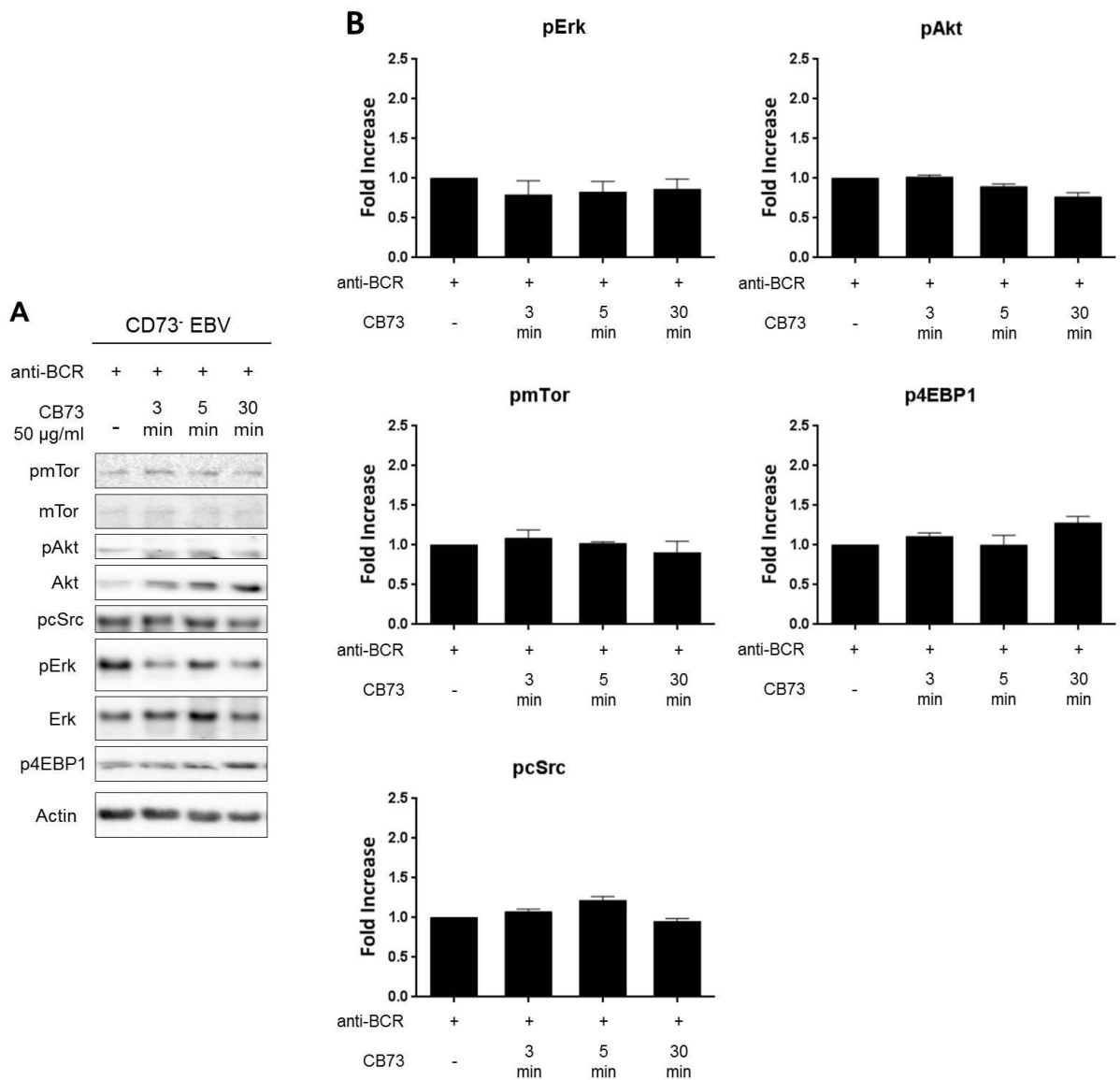
Figure 17. Erk, cSrc and Akt pathway phosphorylation levels of CD73⁻ EBV cell line after pre-activation and incubation with CB73 mAb for 30 min, 5 min and 3 min.

A) CD73⁻ EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot detecting phosphorylated aforementioned kinases and total protein from whole cells lysates. B) Histograms summarize the modification levels of protein phosphorylation compared to pre-activated cells. Error bars are ± SEM.

Results obtained on CD73⁻ EBV cell line give more consistency to the conclusions derived from Donor EBV, confirming the involvement of CD73 in the BCR-controlled pathways.

3.4.2 - CD73-restored EBV cell line

3.4.2.1 - Transfection of CD73⁻ EBV cell line with human CD73 cDNA

To have a "positive" control for the CD73⁻ EBV cell line (*i.e.*, a counterpart cell line expressing CD73) in the analysis of the role of CD73 in intracellular signaling in B cells, the CD73⁻ EBV cell line was transfected with the human CD73 cDNA, producing a CD73⁺ EBV cell line. Transfection was validated through flow cytometry and immunoblotting (Figure 18).
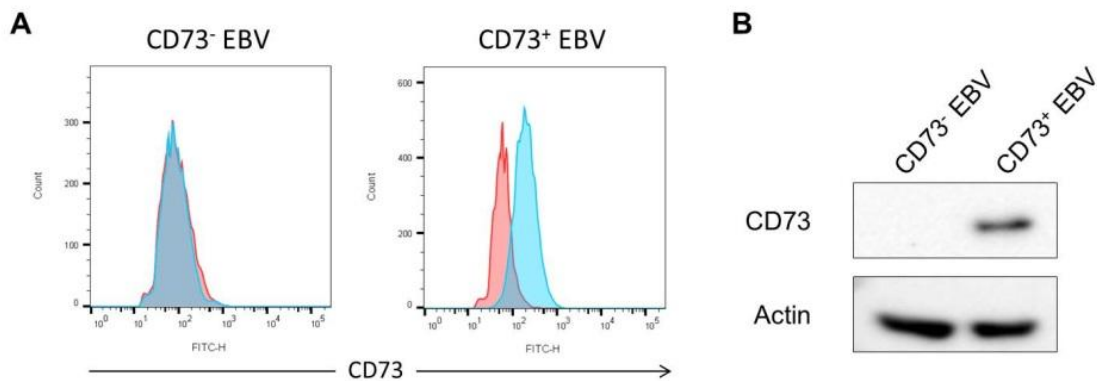
Figure 18. Expression of CD73 on CD73⁺ EBV and CD73⁻ EBV cell lines.

A) Cytofluorymetric analysis of CD73 expression on CD73⁺ EBV and CD73⁻ EBV cell lines. Red histograms demarcate isotype-matched control staining and blue histograms depict the expression of CD73. B) Western blot of whole cell lysates from CD73⁺ EBV and CD73⁻ EBV cell lines probed with anti-CD73 mAb.

Next, we compared the enzymatic activity (AMPase) of EBV CD73$^-$ and CD73$^+$ EBV cell lines.

As predicted, EBV CD73$^+$ was capable of producing ADO using AMP as substrate (Figure 19)
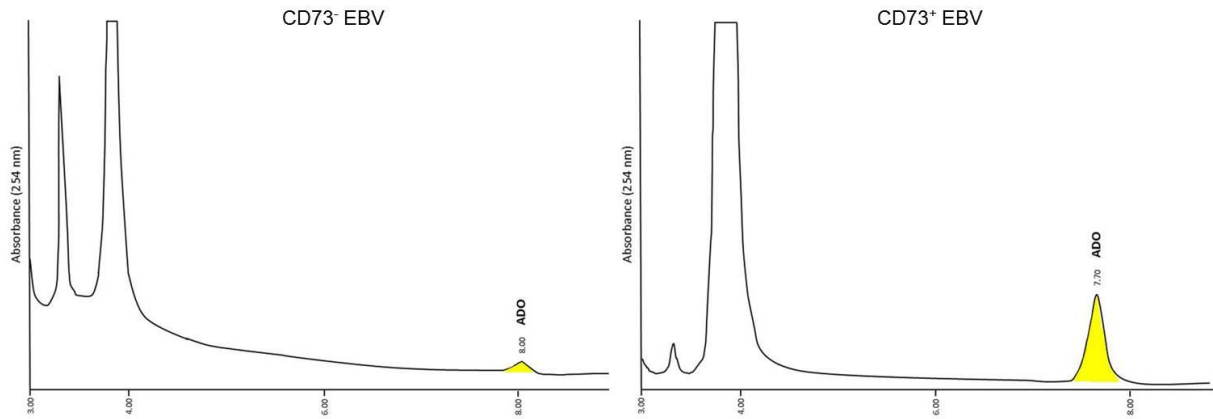
Figure 19. Comparative production of ADO in CD73$^+$ EBV and CD73$^-$ EBV cell lines.
EBV cell lines were incubated with 100 µM AMP and the production of ADO was determined by HPLC.

3.4.2.2 - BCR pathway analysis after CD73 ligation in CD73-restored EBV cell line

We next evaluated the effects of having restored CD73 expression in the EBV cell line in terms of intracellular signaling, gauged by modification of the phosphorylation status of Akt and Erk. Whole cell lysates were obtained from CD73$^+$ EBV incubated at 37°C with CB73 mAb for 1 h, 30 min, 15 min, 5 min and 3 min (Figure 20), and examined by Western blotting.
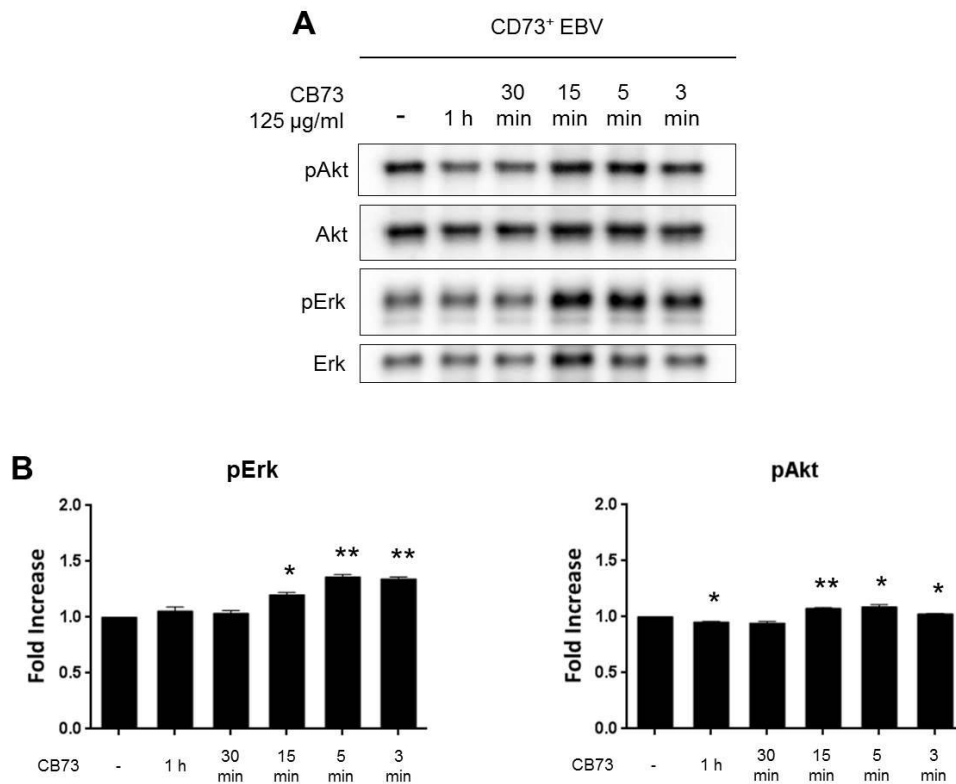
Figure 20. Analysis of Erk and Akt phosphorylation in CD73⁺ EBV cells after incubation with CB73 mAb.

A) CD73$^+$ EBV cells were incubated with CB73 mAb for the times indicated. Representative Western blot detecting total and phosphorylated Erk and Akt in whole cell lysates of CD73$^+$ EBV cells incubated with CB73 mAb the times indicated. B) Histograms summarizing the modification levels of phosphorylated Erk and Akt in the CD73$^+$ EBV cell line incubated with CB73 mAb compared to untreated cells. Error bars are ± SEM.

Modification levels of phosphorylated Erk and Akt are similar to those obtained from Donor EBV (Figure 13), with an increase of phosphorylation of both kinases, especially at brief incubation times (from 3 min to 15 min of incubation with CB73 mAb).

To confirm the result of the work done with Donor EBV and CD73$^-$ EBV cell lines, CD73$^+$ EBV cells were pre-activated with the anti-BCR pAb for 24 h and incubated with the mAb CB73 for 30 min, 5 min and 3 min. Total cell lysates were then analyzed for tyrosine phosphorylation (Figure 21).
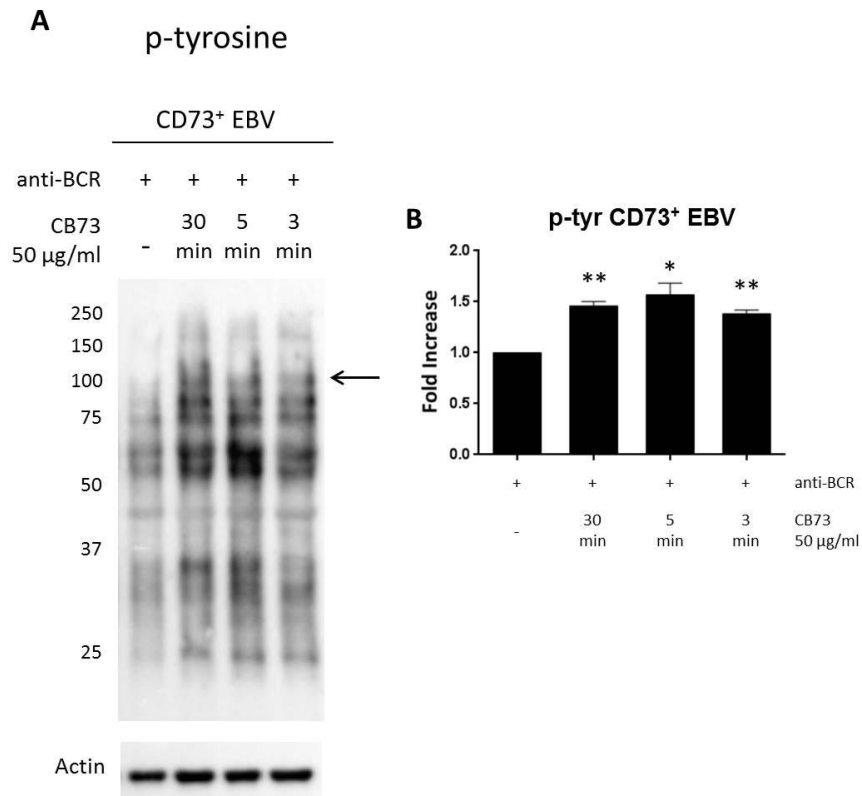
Figure 21. Analysis of total tyrosine phosphorylation in CD73$^+$ EBV cells line after pre-activation and incubation with CB73 mAb.

A) CD73$^+$ EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot probed with anti-pTyrosine mAb detecting phosphorylated tyrosine residues and actin from whole cells lysates. B) Histograms summarize the modification levels of total tyrosine phosphorylation compared to pre-activated cells. Error bars are ± SEM.

In the presence of the CB73 mAb, CD73$^+$ EBV presents a higher and maintained along the time total tyrosine phosphorylation, at levels greater than Donor EBV cells. Furthermore, CD73$^+$ EBV cells incubated with CB73 mAb present a phosphorylated protein at 99 kDa absent in CD73$^-$ EBV cell line in the same experimental conditions.

After analyzing total tyrosine phosphorylation, the attention was focused on the BCR-controlled pathways. Pre-activated CD73$^+$ EBV cells were incubated with the CB73 mAb and the phosphorylation levels of the proteins involved in BCR pathways were analyzed by Western blot (Figure 22).
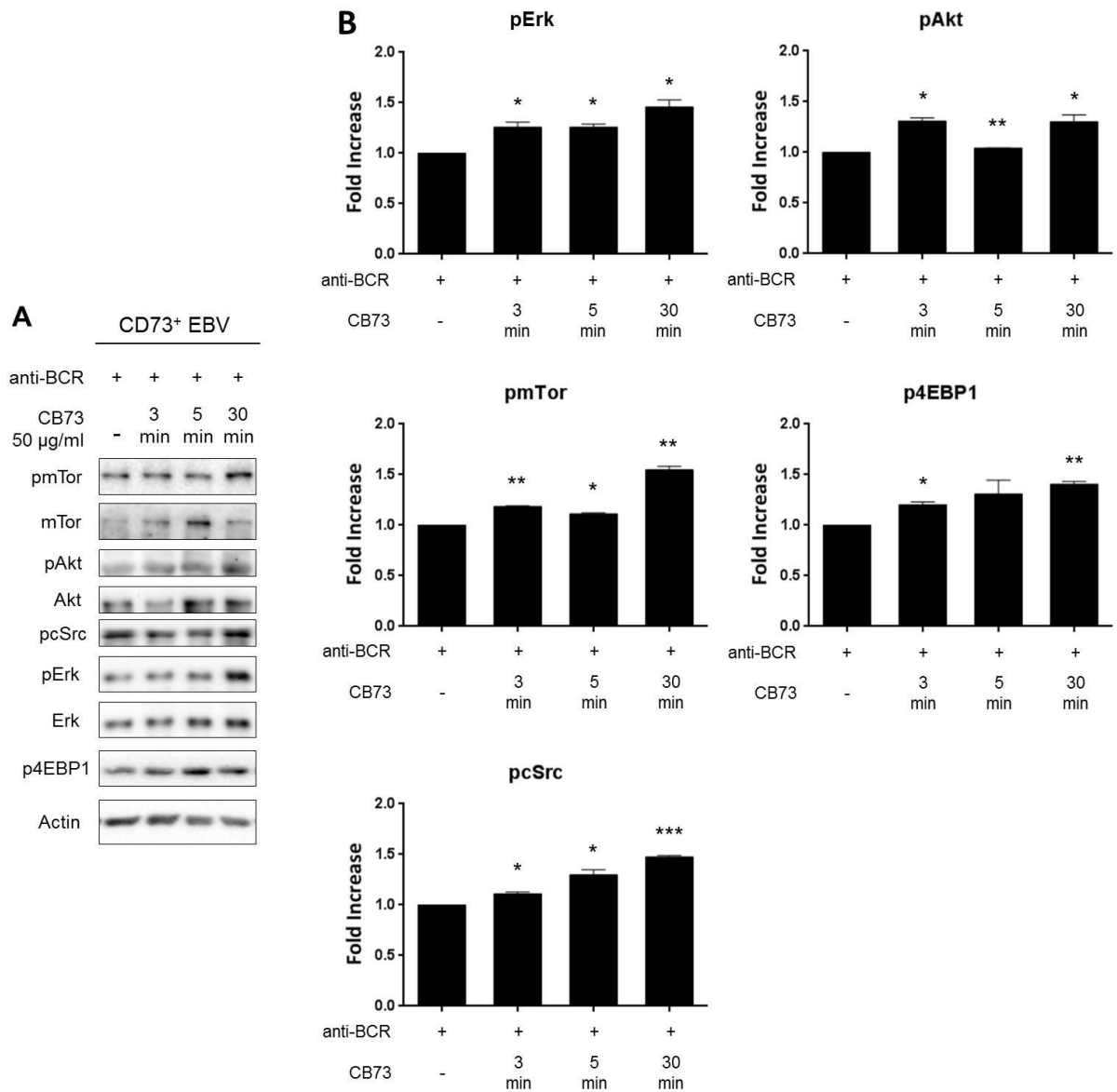
Figure 22. Erk, cSrc and Akt pathway phosphorylation levels of CD73+ EBV cell line after pre-activation and incubation with CB73 mAb for 30 min, 5 min and 3 min.

A) CD73+ EBV cells were activated via BCR followed by incubation with CB73 mAb for the times indicated. Representative Western blot detecting phosphorylated aforementioned kinases and total protein from whole cells lysates. B) Histograms summarize the modification levels of protein phosphorylation compared to pre-activated cells. Error bars are ± SEM.

CD73$^+$ cells pre-treated with anti-BCR and exposed to CB73 mAb, increased phosphorylation levels of all kinases, with a significant improvement of the levels in comparison with that obtained with CD73$^-$ cells. The activation kinetic is similar to that observed on Donor EBV, confirming the results obtained on normal B lymphocytes.

3.5 - Selection of a CD73-specific mAb that inhibits enzymatic activity

CD73 plays a critical role in the conversion of AMP into ADO, and is considered the rate-limiting enzyme in the generation of extracellular ADO. ADO is a key immunosuppressive factor involved in tumor immune escape. Therefore, inhibition of CD73 catalytic activity by means of specific mAbs could be predicted to reduce the growth of primary tumors and metastasis *in vivo*, and support for this hypothesis has been obtained showing that blocking the catalytic activity of CD73 led to increased activity of cytotoxic T lymphocytes, reducing growth of primary tumors and metastasis in a syngeneic murine model of breast cancer.[62] These results are considered to be secondary to the inhibition of ADO production. For this reason, we tested the ability of CB73 mAb, selected for its agonistic properties, to inhibit the enzymatic activity of CD73.

3.5.1 - Selection and analysis of an anti-CD73 mAb able to inhibit the enzymatic activity

To evaluate the capacity of CB73 mAb to inhibit CD73 enzymatic activity, EBV-derived cell lines were pre-treated with the mAb at different concentrations (from 1 μg/ml to 100

µg/ml) for 15 min at 37°C, incubated for 30 min with AMP (100 µM) and the consumption of the substrate was measured with HPLC-based assays. As shown by the chromatographic profiles (Figure 23), addition of CB73 mAb inhibited ADO production, an effect which is concentration-dependent (from 1 µg/ml to 100 µg/ml). The identities of the peaks were confirmed by comparing the Retention time (R_t) of spiked standard ADO (Figure 23).
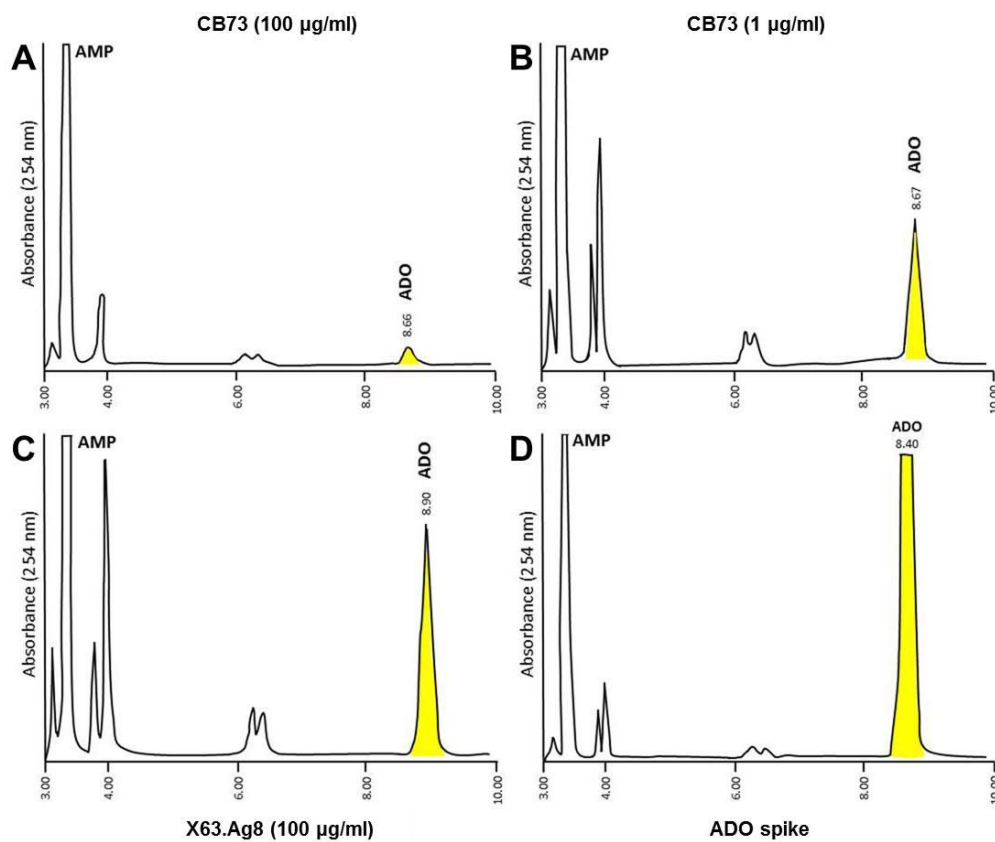
Figure 23. Effect of CB73 mAb on CD73 enzymatic activity of CD73^{+} EBV cell line incubated with AMP (100 µM) for 30 min.

A) ADO production in cell medium from CD73^{+} EBV cell line pre-treated for 15 min with 100 µg/ml of CB73 mAb. The consumption of the substrate was measured with HPLC-based assay.

B) ADO production in cell medium from CD73^{+} EBV cell line pre-treated for 15 min with 1 µg/ml of CB73 mAb. The consumption of the substrate was measured with HPLC-based assay.

C) ADO production in cell medium from CD73^{+} EBV cell line pre-treated for 15 min with 100 µg/ml of the irrelevant mouse IgG$_1$ mAb X63.Ag8. The consumption of the substrate was measured with HPLC-based assay. D) Spike of the standard ADO measured with HPLC-based assay.

AMP consumption by CD73^{+} EBV cells decreased in 30 min by 80% in the presence of CB73 mAb (Figure 24).
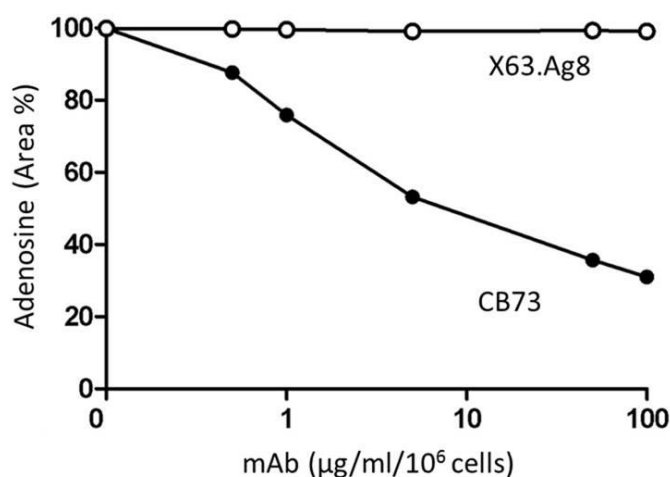
Figure 24. Effect of CB73 mAb concentration/number of cells on ADO production.
CD73^{+} EBV cells were incubated with CB73 mAb (from 1 µg/ml to 100 µg/ml) in presence of AMP (100 µM). The consumption of the substrate was subsequently measured with HPLC-based assay.

3.5.2 - Inhibitory kinetics of CB73 mAb

The better understand the mechanism(s) employed by CB73 mAb in blocking the enzymatic function performed by CD73, kinetic studies were undertaken. The CD73^{+} EBV cell line was incubated with the CB73 mAb (100 µg/ml), or with X63.Ag8 mAb (IgG$_1$) used as isotype-matched control. Cells were then incubated with increasing concentrations of AMP. CB73 mAb decreased the maximum velocity of the enzymatic consumption of AMP (Vmax), without influencing the affinity constant (Km) (Figure 25). These results led us to the conclusion that CB73 mAb inhibits AMP hydrolysis in a non-competitive fashion, probably through a conformational change of the CD73 enzymatic protein.
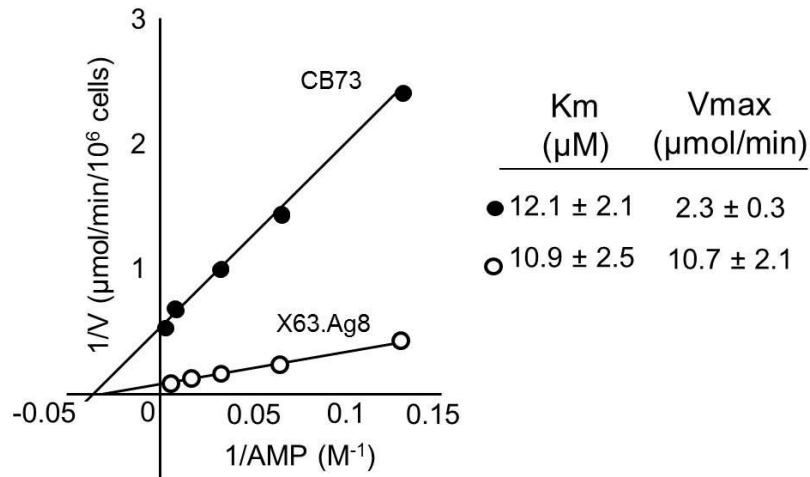
Figure 25. CB73 mAb and kinetic parameters of the CD73 enzymatic activity.

CD73^{+} EBV cell line was incubated with the CB73 mAb (100 µg/ml) or with the isotype-matched control X63.Ag8 mAb. Cells were then incubated with increasing concentrations of AMP.

3.6 - The role of other molecules in vascular calcification in CD73-null patients

The clinical manifestations shared by the patients with CD73-null mutations are due to the accumulation of calcium phosphate crystals within the arterial walls, prevalently in the lower limbs, but also in the joint capsules of both hands and feet. In trying to identify a link between the impairment of CD73 function and vascular calcification, we analyzed the production of several soluble factors, of which Osteopontin appears to be the most promising.

3.6.1 - Osteopontin

Osteopontin (OPN), a member of the small integrin-binding N-linked glycoprotein family,[63] has a molecular weight that varies from 44-75 kDa, depending of post-translational

modifications.[64] The OPN glycoprotein has multiple functions and names, among which secreted phosphoprotein 1 (SPP1), bone sialoprotein 1 (BSP1) and early T-lymphocyte activation 1 (ETA1). Furthermore, it is expressed by different cells and tissues, including kidney, brain, bone, cartilage, gut, activated macrophages and lymphocytes. It is also present in biological fluids such as plasma, milk, blood, seminal fluid and urine.[65-67] OPN is also expressed in numerous forms of cancer, including mesothelioma and ovarian, prostate, colon, breast and lung carcinomas.[64] OPN is attributed the physiological role of regulating bone remodeling and inhibiting ectopic calcification, as well as influencing inflammation, cell adhesion, migration and survival. In pathology, OPN is involved in calcification of soft tissues, kidney disease and cardiovascular disease.[64,68,69] OPN expression is upregulated by various factors, among which cytokines involved in inflammation, EGF, TGF-β and hormones (*e.g.,* steroids and retinoic acid).[64]

3.6.1.1 - Analysis of Osteopontin production

The first step towards attempting to establish a correlation between CD73 function and OPN was to analyze plasma levels of OPN in CD73-null individuals, using as control an age- and sex-matched CD73-expressing healthy individual (Figure 26).
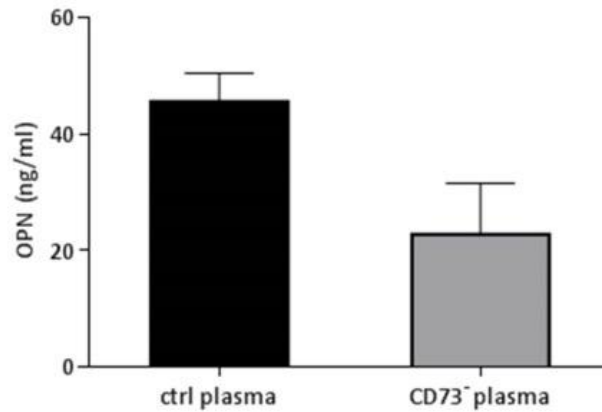
Figure 26. Analysis of OPN levels in plasma fluids of a CD73⁻ patient and an age-matched control by ELISA.

Using ELISA to analyze OPN levels in plasma, the results obtained indicate that the CD73-null patient had lower plasma OPN than control plasma.

As OPN has important effects on B lymphocytes, where it promotes proliferation and Ig production.[70] Therefore, B cells were selected for their capacity to produce OPN. However, B lymphocytes do not produce detectable levels of OPN, even after activation of the classical pathway through the BCR. A second signal is necessary, mediated by an alternative pathway regulated by IL-4.[70,71] OPN production was first analyzed comparing its production in: (i) CD73⁻ PMBC and PBMC from an healthy donor (CD73⁺) after activation with anti-BCR antibody (15 μg/ml) and IL-4 (15 ng/ml) (Figure 27 A) and (ii) in CD73⁻ EBV and CD73⁺ EBV cell lines, also after activation with anti-BCR antibody (15 μg/ml) and IL-4 (15 ng/ml) (Figure 27 B).
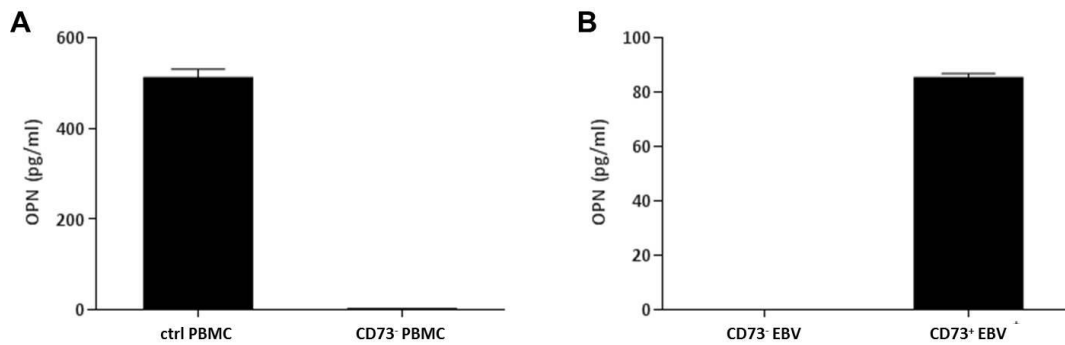
Figure 27. Analysis of OPN production on CD73⁻ PBMC, CD73⁻ EBV and CD73⁺ EBV cell lines by ELISA.

A) PBMC isolated from the patient and an age-matched control were stimulated with an anti-BCR antibody and IL-4 and supernatants were analyzed after 24 h by ELISA. B) EBV cell lines were stimulated with an anti-BCR antibody and IL-4 and the supernatants were analyzed after 24 h by ELISA.

As observed, neither the CD73⁻ PBMC nor the CD73⁻ EBV line produced OPN, whereas control PBMC (CD73⁺) produce high levels of OPN. CD73⁺ EBV cells produced OPN, although less than control PBMC, indicating a possible role for CD73 in OPN production in B cells.

3.6.1.2 - Intracellular phosphorylation induced by anti-BCR antibody and IL-4 in CD73-null EBV cells

As described above, B lymphocytes activated through the BCR do not produce detectable levels of OPN, requiring the presence of IL-4 to give a second signal through an alternative pathway (in which by the need for PI3K in ERK activation is eliminated). To evaluate signaling events induced by BCR and IL-4 in our EBV model, the first step was to expose CD73⁻ EBV and CD73⁺ EBV cell lines to anti-BCR antibody (15 µg/ml) and/or IL-4 (15 ng/ml) for 24 h at 37°C. Cell lysates were prepared and analyzed for Akt and Erk phosphorylation by Western blotting. As no differences were observed between the two cell lines, experiments were repeated but reducing the incubation time to 5 min (Figure 28).
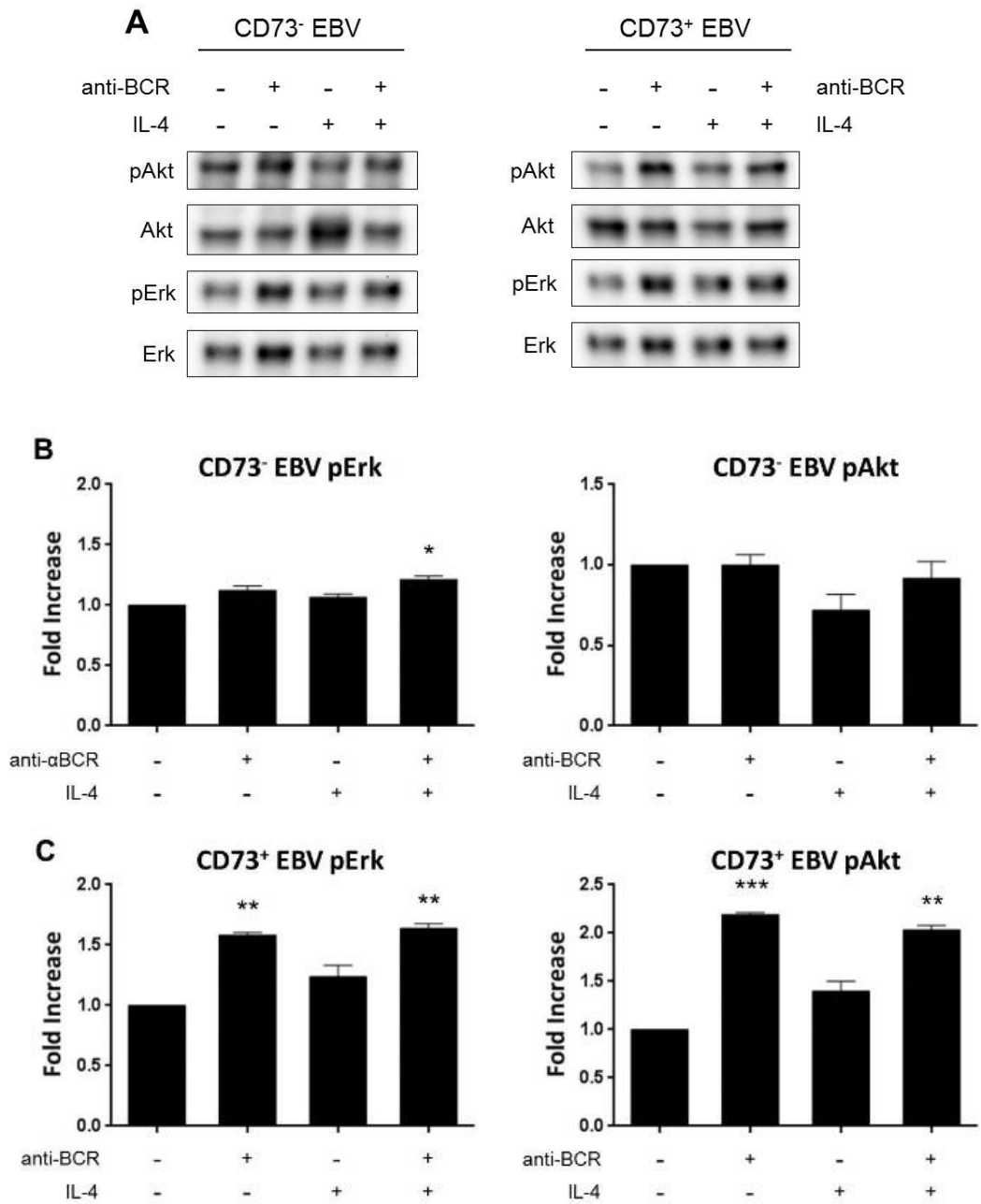
Figure 28. Erk and Akt phosphorylation levels of CD73⁻ EBV and CD73⁺ EBV cell lines after incubation with anti-BCR pAb and/or IL-4 for 5 min.

A) CD73⁻ and CD73⁺ EBV cells were incubated with anti-BCR pAb and/or IL-4. Representative Western blot detecting phosphorylated aforementioned kinases and total protein from whole cells lysates. B) Histograms summarize the modification levels of phosphorylated Erk and Akt of CD73⁻ EBV cell line compared to untreated cells. C) Histograms summarize the modification levels of phosphorylated Erk and Akt of CD73⁺ EBV cell line compared to untreated cells. Error bars are ± SEM.

The modified experimental conditions showed that phosphorylation of both the Erk and Akt was higher in CD73$^+$ cells with respect to CD73$^-$ cells. Incubation of CD73$^+$ EBV cells with IL-4 was apparently able to increase phosphorylation of Akt and Erk. Simultaneous exposure to anti-BCR antibody and IL-4 in CD73$^+$ cells was followed by increased phosphorylation of both kinases, although at levels similar to those obtained in the presence of anti-BCR antibody alone.

3.6.1.3 - Intracellular phosphorylation induced by anti-BCR antibody and IL-4 in normal EBV cells (CD73$^+$)

To verify that the results obtained previously with CD73$^-$ EBV and CD73$^+$ EBV cell lines were not an experimental artifact, similar experiments were repeated using Donor EBV cell line. Donor EBV cells were exposed to anti-BCR antibody (15 µg/ml) and/or IL-4 (15 ng/ml) for 24 h and cell lysates were analyzed for Erk and Akt phosphorylation by Western blot. As no differences were observed, experiment was repeated but reducing the incubation time to 5 min (Figure 29).
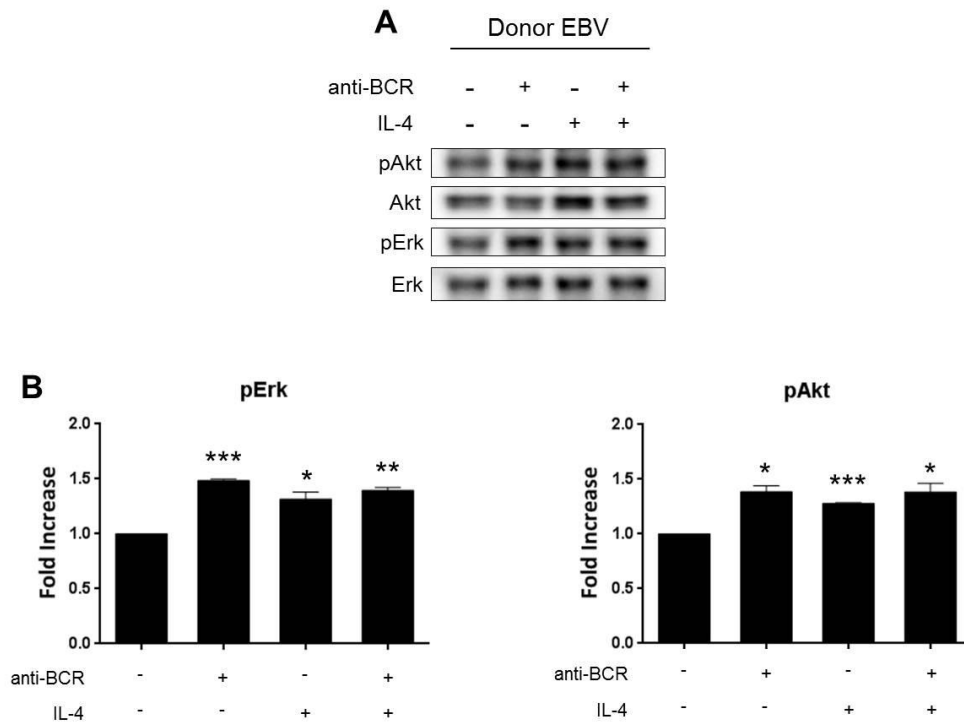
Figure 29. Erk and Akt phosphorylation of Donor EBV cell line after incubation with anti-BCR pAb and/or IL-4 for 5 min.

A) Donor EBV cells were incubated with anti-BCR pAb and/or IL-4. Representative Western blot detecting phosphorylated aforementioned kinases and total protein from whole cells lysates.

B) Histograms summarize the modification levels of phosphorylated Erk and Akt of Donor EBV cell line compared to untreated cells. Error bars are ± SEM.

Decreasing the incubation time to 5 min led to increased phosphorylation in Erk and Akt similar to what was observed in CD73$^+$ EBV cells. The differences on Donor EBV compared to CD73$^+$ EBV cells concerns a general lower Akt phosphorylation level.

Chapter 4. Discussion

In 2013, our Laboratory identified an alternative ectoenzymatic pathway that led to ADO production and that was independent of CD39.[14] This novel pathway involved a discrete set of ectoenzymes: (i) CD38 (NAD glycohydrolase/ADP ribosyl cyclase); (ii) CD203a (ectonucleotide pyrophosphatase/phosphodiesterase 1) and (iii) CD73 (ecto-5-nucleotidase. While the role in adaptive immunity of the ectoenzymatic activities of CD38 and CD203a have received considerable attention, studies on the functional role of CD73 in adaptive immunity have lagged behind. This prompted us to focus our attention on the analysis of human CD73.

Alongside its enzymatic activity, CD73 also presents receptor functions, and this aspect has been mainly studied in human lymphocytes. Intracellular signaling via CD73 in T cells indicate that CD73 plays a role in cell adhesion between lymphocytes and endothelial cells.[24] In addition, CD73 can act as a costimulatory molecule for the TCR[27] and induce proliferation in T lymphocytes.[28] Furthermore, T cell activation through CD73 does not require neither the GPI anchor[52] nor its ecto-5'-nucleotidase activity.[33]

The receptor activities of CD73 presume the existence of a natural ligand for CD73. However, as yet, such a natural ligand has not yet been identified. In the absence of a natural ligand, ligand mimicks can substitute for the lack of the real thing, and anti-CD73 mAbs successfully mimick the actions of its supposed natural ligand, as has been shown for some T-cell co-stimulatory molecules and their ligands (*e.g.*, CD28 and B7-1/B7-2).[72] In most of these studies, the problem of the missing ligand was overcome by using the anti-CD73 mAb, 1E9 , produced by Thompson and coll.[54]

In the first part of this work, a new anti-CD73 mAb, CB73, was identified and found to have agonistic properties. Triggering CD73$^+$ T cells with CB73 mAb caused reduced phosphorylation of two proteins of 27 kDa and 32 kDa, a result that differs from that

obtained with the 1E9 mAb.[51] After cell activation with PMA (a mitogenic DAG surrogate that leads to protein kinase C activation and subsequent T cell activation, enhancing also proliferation of T cells involving GPI-anchored molecules)[73-75], CB73 ligation led to yet another pattern of phosphorylation, with increased phosphorylation of two proteins of 83 kDa and 132 kDa, and decreased phosphorylation of 143 kDa protein in CD73^{+} cells. Compared to CD73^{-} cells, total tyrosine phosphorylation was increased and maintained in time in CD73^{+} cells, pre-activated and incubated with CB73 mAb. These results confirm the ability of CD73 to induce intracellular signals and suggest an agonistic role for CB73 mAb.

One of the consequences of T cell activation through the TCR[76,77] or via PMA[78] is the production of the proliferative cytokine IL-2 and its specific receptor CD25. Costimulation of CD3 and CD73 (with the 1E9 mAb) induces CD25 expression.[27] After cell pre-activation, also CB73 mAb was found to increase expression surface CD25 in a CD73^{+} T cell line. CD25 expression is normally induced by several pathways, among these the MAPK pathway, with Erk being one of the most studied kinases.[58,59] CB73 mAb, most notable at the lower concentrations used here (50 μg/ml), induced Erk to increase phosphorylation levels in pre-activated CD73^{+} T cells. This is a further confirm of the agonistic activity of the CB73.

The non-enzymatic functions of CD73 in T lymphocytes are well-known, but the role of CD73 in B lymphocytes has been less studied. To date, CD73 has been described as mediating adhesion of B cells both to follicular dendritic cells[26] and to endothelial cells.[24] However, the ability of these interactions of CD73 to induce intracellular signals is less known. Our results, using the functional CB73 mAb, indicate that CD73 may function as be a costimulatory molecule also in B lymphocytes. The main BCR-mediated signal cascade is mediated by the PLC-γ2 and PI3K pathways, which in turn trigger the activation of various other enzymes. Of these, PKC, Erk and Ras lead to the activation of a host of transcriptional factors.[79] Our

results demonstrate that CD73 ligation is followed by activation of BCR-induced pathways. Indeed, CB73 mAb binding to CD73 in activated cells is followed by increased phosphorylation of Erk, cSrc, Akt and of its downstream effectors mTor and 4EBP1. This effect is rapid (observed within 3 min) and is maintained in time (at least up to 30 min). The increased substrate phosphorylation was observed present also without prior cell activation, although in this case the effect was reduced and lasted less (15 min). These results were obtained in an EBV cell line derived from a patient naturally lacking CD73 and corrected by transfection with the human CD73 cDNA. The control was an EBV cell line obtained from a healthy donor. The control ruled out that the observed events were due to an artifact of transfection.

The absence of selected phosphorylated proteins detectable only after CB73 incubation lead us to hypothesize that CD73 may play a physiological role by reinforcing BCR-induced pathways or by decreasing the threshold for their activation. This has also been proposed to occur in naïve T cells.[51] Most GPI-anchored proteins co-isolate with the Src family non-receptor protein tyrosine kinases (PTKs). A reasonable inference is that signaling via GPI-anchored proteins could proceed through the above pathway.[80,81] The CD73 protein bears an epitope recognized by antibodies against Src homology domain 3 (SH-3)[82]: thus a working hypothesis is that CD73's signal transduction capacities may be secondary to the an association between CD73 and Src kinases. Strengthening this hypothesis is the fact that in murine models, the kinase Fyn is required for CD73-mediated signal transduction.[83] Fyn is a PTK member of the Src family essential for BCR signaling. Indeed, Fyn causes the phosphorylation of Syk and Btk and consequently the activation of PLC-γ2.[84-91] CD73 signals could strengthen the BCR cascade though its interactions with Fyn, which is active in the

early stages of the BCR pathway (Figure 30). Indirectly, these observations also confirm the agonistic activity of the CB73 mAb.
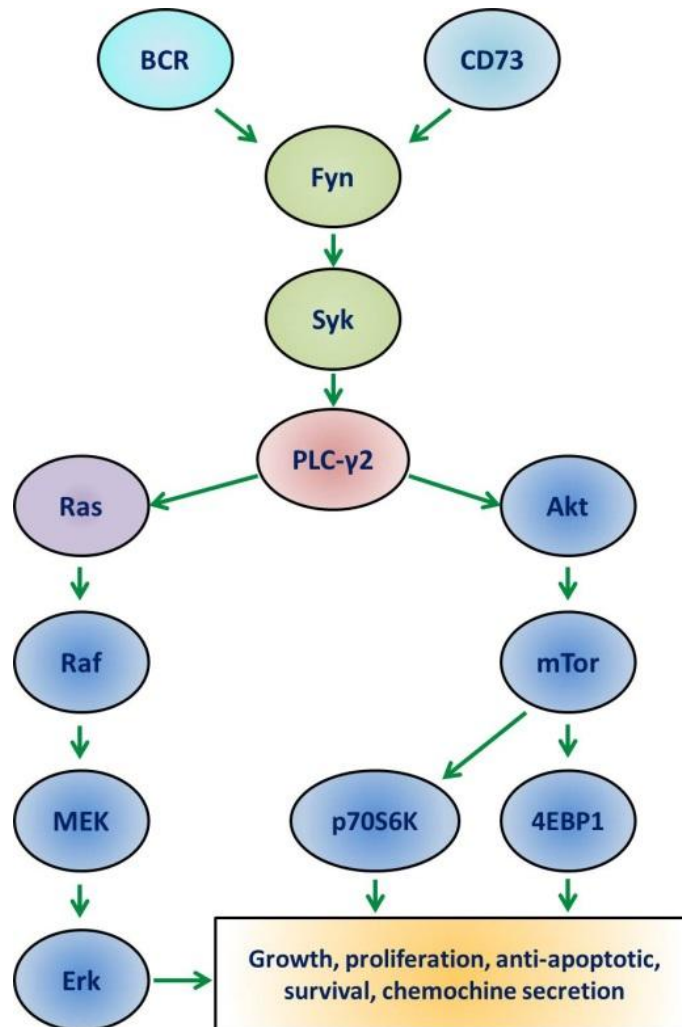
Figure 30. Proposed connection between CD73 and BCR pathways.

CD73 interacts with the kinase Fyn, active at in the early steps of the BCR pathways, strengthening the BCR cascade. Fyn activate the kinase Syk, which in turn leads to the activation of PLC-γ2 causing the activation of Ras/MAPK and Akt pathways.

By controlling the production of extracellular ADO, CD73 can exert different functions in different cell types, such as protecting from ischemia and hypoxia, controlling tissue barrier functions, influencing ion and fluid transport, among others.[11] However, CD73 also plays an important role in the control of tumor progression. The tumor microenvironment is usually

an inhospitable place, unsuitable for the survival of normal cells, being characterized by low levels of oxygen, glucose and other nutrients, and a low pH.[92] As an escape strategy, tumor cells reprogram their metabolic machinery, for instance by altering purine metabolism and increasing expression of CD73. Interacting with its four receptors A_1, A_2A, A_2B and A_3, extracellular ADO conditions the activity of different cell types present in the tumor environment. In this manner, ADO induces the tumor cells to migrate, invade and proliferate *in vitro*. The results *in vivo* are that tumor cells evade immune surveillance and tumors grow thanks to angiogenesis, all hallmarks of poor disease outcome.[19,30,93]

CD73 expression in tumors can be regulated by several mechanisms: (i) hormones – for example, the estrogen receptor negatively regulates CD73 expression[94], while thyroid hormones can increase its expression[95-97]; (ii) a hypoxic microenvironment - the CD73 gene promoter includes an HIF-1α binding site[98,99]; (iii) inflammatory factors – for example, IFN-γ, LPS and glutamate all decrease CD73 expression[100], while IFN-α and IFN-β upregulate CD73 expression[101,102]; (iv) epigenetic modifications[103,104]. CD73 overexpression is also associated with resistance to antitumor agents: indeed CD73 overexpression mediates vincristine resistance in glioblastoma multiforme cells[105] and conferred chemoresistance to doxorubicin in murine model of breast cancer[9]. In virtue of its involvement in immune escape, CD73 is also involved in resistance to immunotherapy.[106] Targeting CD73 with small-molecule inhibitors or mAbs is followed by antitumoral effects.[22,23,105,107-109] The link observed between CD73 and resistance to anticancer treatments suggests the usefulness of combining anti-CD73 treatment with chemotherapy or immunotherapy. Indeed, CD73 blockade synergize with anthracycline[9] and immunotherapeutic drugs like anti-PD1 mAb and anti-CTLA-4 mAb.[35]

In the second part of the work we investigated the ability of our CB73 mAb to inhibit the 5'-NT activity of CD73. CB73 mAb has the ability to decrease by 80% the production of ADO, blocking AMP hydrolysis noncompetitively. The three-dimensional structure of the CD73 protein, both substrate-free and substrate-bound, indicate that catalysis requires a large conformational switch[2], suggesting that CB73 mAb could act as an allosteric modulator, stabilizing the enzyme conformation in an inactive form or could change the conformation and perhaps hide the ligand binding site.[38]

Immunotherapy is a promising field, mainly because antibodies have superior specificity and longer half-life when compared to small-molecule inhibitors. Encouraging results have been obtained in this study with CB73 mAb: this model may pave the way to transfer our conclusions to clinical diagnostics and eventually for *in vivo* treatment, in the appropriate antibody setting.

We were fortunate to have one member of the family affected by the CD73-null mutation participate in part of the present studies. These individuals show extensive calcifications of the lower-extremity arteries and small joint capsule.[49] This correlation between the absence of CD73 and presence of vascular calcification was subsequently observed also in murine models[110], and CD73 loss also plays a role in atherogenesis[111], a pathological condition that is often followed by calcification.[112]

One of the most important cytokines involved in the physiology of calcification is Osteopontin (OPN)[113], but OPN also has pro-inflammatory effects and is secreted by several immune cells such as macrophages, dendritic cells, T and B lymphocytes.[114] B lymphocytes are not reported as producing detectable levels of OPN, even after activation of the classical pathway through BCR, necessitating a second signal mediated by an alternative pathway regulated by IL-4. The two pathways co-exist in BCR- and IL-4-treated B lymphocytes. The

classical BCR-initiated signaling cascade requires a numbers of mediators and the absence (or inhibition) of any of these elements terminates BCR signaling, blocking downstream steps such as Erk phosphorylation. The situation is different for B lymphocytes that have been exposed to IL-4, because IL-4 induce an alternate signaling pathway that specifically affects Erk activation. Thus, IL-4, but not other cytokines, induces a new pathway for BCR signaling that is not part of the repertoire of naïve B cells, creating a new pathway in which the need for PI3K in Erk activation was eliminated.[70,71,114,115] The important role of Erk in OPN production has been demonstrated in head and neck squamous cell carcinoma[116], and in dendritic cells and macrophages after TCR stimulation.[117] The results of the present work indicate that restoration of CD73 function in CD73⁻ B cells enables these cells to produce OPN, suggesting a role for CD73 in OPN production. Simultaneous activation of BCR and IL-4 pathways in B cells expressing CD73 induces major activation of Erk and Akt as compared to CD73⁻ B cells. However, the activation levels are similar to those obtained after BCR activation alone.

Another issue in trying to explain the above discrepancy is that OPN production is more related to the enzymatic activities of CD73 than to its receptor functions. St. Hilaire and coll.[49] proposed a mechanism to explain vascular calcification observed in CD73⁻ patients (Figure 31). The ectoenzyme CD203a converts ATP to AMP and pyrophosphate (PPi), while CD73 metabolizes AMP to ADO and inorganic phosphate. CD73 deficiency would lead to lower levels of ADO, eliminating the inhibition of TNAP (Tissue-Nonspecific Alkaline Phosphatase, which degrades PPi), in turn leading to reduced levels of PPi, which is a potent inhibitor of calcification.
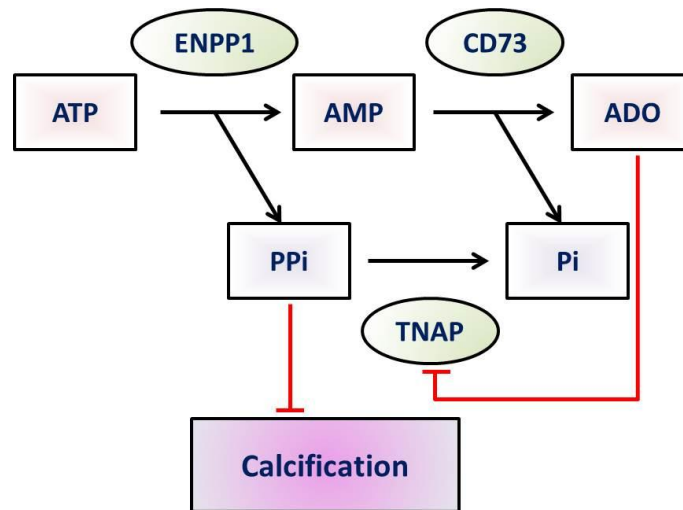
Figure 31. Mechanism proposed by St. Hilaire *et al*. for increased mineralization due to CD73 deficiency.

ENPP1 converts ATP to AMP and PPi. CD73 converts AMP to ADO and Pi. TNAP degrades PPi, an inhibitor of calcification, while ADO inhibits TNAP, inducing higher levels of PPi. Deficiency of CD73 results in decreased ADO levels, eliminating the inhibition of TNAP. Increased TNAP activity results in decreased PPi and increased cell calcification. From St. Hilaire *et al*., in N Engl J Med (2011).

Based on the results presented here, the enzyme-based metabolic mechanism proposed might not be the main cause of the vascular calcifications observed in CD73-null individuals, but OPN could be involved as proto- or deuteragonist. OPN is not a simple cytokine, instead it is a multiform product which includes many peptides, including splice variants and post-translational modifications. For instance, OPN phosphorylations play important roles for its function. Indeed, non-phosphorylated OPN increases mineralization of vascular smooth-muscle cells, while phosphorylated OPN is a potent inhibitor of vascular calcification.[118,119] Often the literature does not accurately report on whether OPN observed in the studies is phosphorylated or in a dephosphorylated form: this might generate contrasting results and conclusions. A possibility that has not yet been considered is that the lack of CD73 may lead to higher AMP levels and, consequently, to increase the AMP:ATP ratio. This in turn could

provoke activation of AMPK (AMP-activated protein kinase)[120]. AMPK may downregulate the transcriptional repressor Gfi 1 (growth factor independence-1) and in turn upregulate OPN levels increasing osteogenesis.[121] Instead, in the presence of CD73, ADO provokes antagonistic effects on AMPK activation.[122] From these axes, it seems that the absence of CD73 increases OPN expression and yields high calcification levels. Indeed, CD73⁻ patients are characterized by greatly increased calcium deposition. Moreover, in the absence of CD73, TNAP is more active and could dephosphorylate OPN, one of its natural ligands.[123] A consequence of this would be vascular calcification. In contrast, presence of CD73 would lead to an inhibition of TNAP activity by ADO, thereby increasing the level of phosphorylated OPN. On the other hand, CD73 expression may provoke an increased levels of PPi inhibiting calcifications. PPi, in turn, could increase OPN levels through the Erk and p38 MAPK signaling pathway.[124] All these mechanisms (Figure 32) can explain more exhaustively the link between CD73 and vascular calcifications, correlating ADO and OPN.
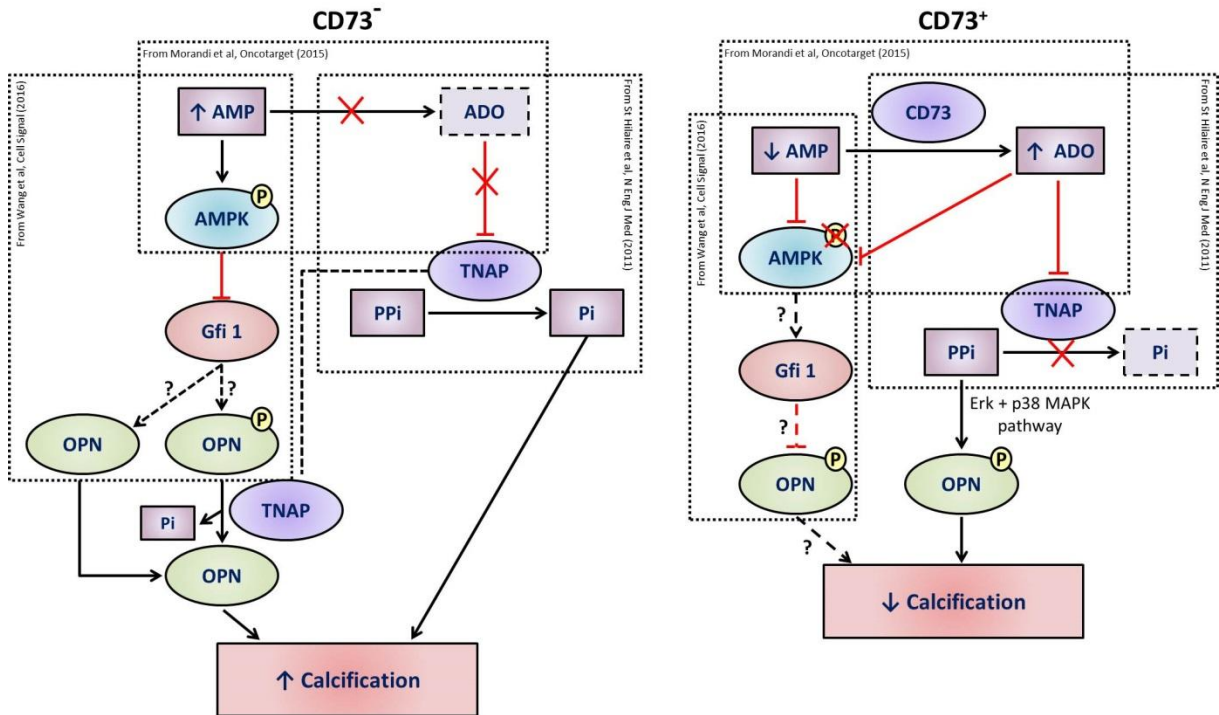
Figure 32. Our proposed mechanism of calcification due to CD73 deficiency.

In the absence of CD73, higher levels of AMP lead to the activation of AMPK. Activated AMPK decreases the levels of the transcriptional repressor Gfi 1, enabling OPN production. With ADO absence, TNAP can dephosphorylate OPN causing increased calcification. Moreover, TNAP activation leads to increased levels of Pi, inducing calcification.

In the presence of CD73, ADO blocks AMPK activation leading to lower levels of OPN. On the other hand, ADO blocks TNAP leading to higher levels of PPi, which in turn induces increased production of OPN (probably the phosphorylated form) and reduction of calcification.

The question marks (?) present in the figure indicate our uncertainty about which form of OPN is produced (CD73⁻ scheme) or the signaling pathway after AMPK inactivation (CD73⁺ scheme).

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PUBLICATIONS

"Antibody mimicry, receptors and clinical applications."

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POSTERS

"Identification of a murine monoclonal antibody specific for human CD73 with potential to inhibit extracellular production of adenosine."

Chillemi A, Quarona V, Zito A, Lo Buono N, Azzolino O, Morandi F, Zaccarello G, **Mariani V**,

Pistoia V, Horenstein AL, Malavasi F.

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Malavasi F.

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Signalling, Drugs and Targets. Bonn, Germany (July 23–27, 2014).

ABSTRACTS

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