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Tissue-nonspecific alkaline phosphatase is an anti-inflammatory nucleotidase

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Short running title: TNAP is an anti-inflammatory nucleotidase

Declaration of interests: none

List of nonstandard abbreviations:

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4 ATP: adenosine triphosphate; BGLAP: bone Gla protein (osteocalcin); CASP: cryopyrin-
5 associated periodic syndrome; CNO: chronic non-bacterial osteomyelitis; CRMO: chronic
6 recurrent multifocal osteomyelitis; DIRA: deficiency of interleukin-1 receptor antagonist;
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8 DMEM: Dulbecco's Modified Eagle Medium; GABA; Gamma-aminobutyric acid; GAPDH:
9 glyceraldehyde-3-phosphate dehydrogenase; HPP: hypophosphatasia; IL: interleukin; MRI:
10 magnetic resonance imaging; MSC: mesenchymal stem cell; MV: matrix vesicle; NLRP3:
11 NOD-like receptor family, pyrin domain containing 3; NPP1: ectonucleotide pyrophosphatase
12 phosphodiesterase 1; PBMC: peripheral blood mononuclear cell; pNPP:
13 paranitrophenylphosphate; PP_i : inorganic pyrophosphate; RPLP0: ribosomal protein lateral
14 stalk subunit P0; RT-qPCR: reverse transcription-quantitative polymerase chain reaction;
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16 TNAP: tissue-nonspecific alkaline phosphatase.
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Abstract

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4 Tissue-nonspecific alkaline phosphatase (TNAP) is necessary for skeletal mineralization by
5 its ability to hydrolyze the mineralization inhibitor inorganic pyrophosphate (PP_i), which is
6 mainly generated from extracellular ATP by ectonucleotide pyrophosphatase
7 phosphodiesterase 1 (NPP1). Since children with TNAP deficiency develop bone metaphyseal
8 auto-inflammations in addition to rickets, we hypothesized that TNAP also exerts anti-
9 inflammatory effects relying on the hydrolysis of pro-inflammatory adenosine nucleotides
10 into the anti-inflammatory adenosine. We explored this hypothesis in bone metaphyses of
11 TNAP-deficient mice, in mineralizing hypertrophic chondrocytes and osteoblasts, and non-
12 mineralizing mesenchymal stem cells (MSCs) and neutrophils, which express TNAP and are
13 present, or can be recruited in the metaphysis. Bones metaphyses of 7-day-old *Alpl*^{+/-} mice
14 (encoding TNAP) had significantly increased levels of *Il-1β* and *Il-6* and decreased levels of
15 the anti-inflammatory *Il-10* cytokine as compared with *Alpl*^{+/+} mice. In bone metaphyses,
16 murine hypertrophic chondrocytes and osteoblasts, *Alpl* mRNA levels were much higher than
17 those of the adenosine nucleotidases *Npp1*, *Cd39* and *Cd73*. In hypertrophic chondrocytes,
18 inhibition of TNAP with 25 μM of MLS-0038949 decreased the hydrolysis of AMP and ATP.
19 However, TNAP inhibition did not significantly modulate ATP- and adenosine-associated
20 effects in these cells. We observed that TNAP protein in hypertrophic chondrocytes was not
21 kept at the cell membrane but sent to the extracellular matrix in matrix vesicles, explaining
22 why TNAP participated in the hydrolysis of ATP but did not modulate its autocrine pro-
23 inflammatory effects. In MSCs, TNAP did not participate in ATP hydrolysis nor in secretion
24 of inflammatory mediators. In contrast, in neutrophils, TNAP inhibition with MLS-0038949
25 significantly exacerbated ATP-associated activation and secretion of IL-1β, and extended cell
26 survival. Collectively, these results demonstrate that TNAP is a nucleotidase in both
27 hypertrophic chondrocytes and neutrophils, and that this nucleotidase function is associated
28 with autocrine effects on inflammation only in neutrophils.

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51 **Keywords:** tissue-nonspecific alkaline phosphatase; ATP; inflammation; nucleotidase;
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1 Introduction

Ossification proceeds by two different processes [1]. Long bones are formed by endochondral ossification, during which growth plate cartilage is first mineralized and progressively replaced by new bone. Alternatively, flat bones grow by intramembranous ossification where there is no need for a cartilage template. In both processes, extracellular matrix mineralization is a relatively simple biological process that merely requires the coexpression of two broadly expressed proteins, a fibrillar collagen (type II collagen in growth plate cartilage and type I collagen in bone) and tissue-nonspecific alkaline phosphatase (TNAP) [2]. Mutations in the genes encoding type II collagen are associated with different forms of abnormal growth known as type II collagenopathies [3], and those that occur in type I collagen genes with *osteogenesis imperfecta* [4]. On the other hand, mutations in *ALPL*, the gene encoding human TNAP, lead to hypophosphatasia (HPP). Based on disease severity, HPP has been divided into 5 major subtypes: the perinatal form, the infantile form, the childhood form, the adult form and odontohypophosphatasia [5, 6]. The perinatal form is lethal at birth or soon after. In stillborns, bones can appear nearly devoid of minerals. In the infantile form, symptoms usually appear during the first six months, in particular with radiographic evidence of widespread demineralization and rachitic changes in the metaphyses, often leading to lethality. The childhood form is associated with dental problems, delayed walking and bone fractures. The adult forms manifest by osteomalacia and premature tooth loss [6]. The pro-mineralizing function of TNAP relies on its ability to hydrolyze extracellularly the mineralization inhibitor inorganic pyrophosphate (PP_i) [2, 7]. Intracellularly formed PP_i can be exported by the transporter progressive protein ankylosis (ANK), but the major fraction of extracellular PP_i is likely generated extracellularly from adenosine triphosphate (ATP) by the transmembrane enzyme ectonucleotide pyrophosphatase phosphodiesterase -1 (NPP1) [8]. TNAP, attached by a GPI-anchor to the membrane of mineralizing cells, is transferred with its GPI anchor to the extracellular collagen matrix in the membrane of so-called matrix vesicles (MVs) to initiate collagen mineralization [9].

TNAP has at least one other substrate and function. TNAP dephosphorylates pyridoxal phosphate into pyridoxal to participate in vitamin B6-dependent reactions, in particular GABA synthesis [6]. This function explains in part why both HPP patients and *Alpl*^{-/-} mice experience epileptic seizures [6, 10]. PP_i and pyridoxal phosphate are probably not the only TNAP substrates *in vivo* since *in vitro*, TNAP has the ability to hydrolyze many different

1 phosphorylated compounds [11]. Moreover, the expression of TNAP in many different
2 locations such as liver, kidney, blood or brain suggests that TNAP exerts other functions,
3 possibly by hydrolyzing other substrates. Thorough examination of the symptoms of the
4 childhood form of HPP suggests a role for TNAP in inflammation. Indeed, cases of chronic
5 recurrent multifocal osteomyelitis (CRMO), a sterile auto-inflammatory disease [12], have
6 been reported independently by the groups of MP Whyte and H Girschick in unrelated HPP
7 children who suffered chronic, multifocal, periarticular pain and soft tissue swelling [13, 14].
8 Magnetic resonance imaging (MRI) evidenced bone marrow edema in these children, and
9 non-steroidal anti-inflammatory drugs diminished their pain [13-17]. More recently, whole-
10 body MRI of four children with the childhood form of HPP revealed bone metaphysis
11 inflammation in all of them [15]. It was therefore proposed that chronic bone inflammation
12 should be considered an additional feature of HPP [14].
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23 CRMO inflammation in childhood HPP develops at the metaphysis [15]. This is where TNAP
24 is the most active during growth, being expressed by mineralizing hypertrophic chondrocytes
25 and mature osteoblasts. We hypothesized that metaphysis inflammation in HPP is due to the
26 pathological persistence of high extracellular ATP, normally hydrolyzed by TNAP by these
27 cells. ATP can indeed be secreted by virtually all cells, through transporters such as connexins
28 and pannexins, in response to inflammatory molecules, mechanical stress, or danger signals
29 [18]. ATP then binds to P2 purinergic receptors to trigger intracellular signaling pathways
30 leading to pro-inflammatory responses. ATP for instance binds to P2X₇ receptor to activate
31 the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, which
32 cleaves pro-interleukin-1 β (pro-IL-1 β) into mature IL-1 β and triggers its secretion through the
33 cleavage and activation of the pore-forming protein gasdermin D [19]. ATP also binds to P2Y
34 receptors to delay the apoptosis of neutrophils [20] and activate their migration [21]. Two
35 ubiquitous ectonucleotidases, CD39 and CD73, are involved in adenosine nucleotide
36 dephosphorylation to control inflammation in a time- and location-dependent manner [18].
37 CD39 removes two P_i from ATP to generate adenosine monophosphate (AMP), which is
38 further hydrolyzed into adenosine by CD73. Adenosine then binds to P1 receptors to activate
39 anti-inflammatory responses. Several recent articles suggested that TNAP may be able to
40 hydrolyze adenosine nucleotides *in vivo*. In the blood, TNAP is suspected to limit
41 inflammation by its ability to dephosphorylate AMP into adenosine [22]. TNAP also appears
42 to generate adenosine in the brain and the spinal cord [10, 23]. In our study, we explored the
43 putative anti-inflammatory functions of TNAP using mouse and human mesenchymal and
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hematopoietic cells, wild-type mice, and TNAP-deficient mice. To our knowledge, bone inflammation has never been explored in these mice, but it was reported that they suffer from allodynia and hyperalgesia [24].

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2 Materials and methods

2.1 Animals and sample collection

Alpl mice [25] were bred in the CERCO animal facilities in accordance with the Guide for the Care and Use of Laboratory Animals and the guidelines of the local institutional animal care and use committee. Study was approved by the Regional (Midi Pyrénées) Ethics Committee (MP/06/79/11/12). Femurs and tibias were extracted from 7-day-old male animals (8 *Alpl*^{+/+} and 3 *Alpl*^{+/-}) in the morning. Immediately after mouse dissection, femurs and tibias were frozen in liquid nitrogen. Metaphysis-containing bone samples were grinded in a liquid nitrogen cooled mortar, and RNA was extracted as described below. Mice genotypes were specified *a posteriori* using tail samples as previously published [10].

2.2 Cell cultures

Primary osteoblasts and chondrocytes were isolated from newborn (4-6 days) SWISS mice by successive enzymatic digestions of calvaria, and articular cartilage from femoral head and knee, respectively [26]. Animal experimentations were conducted according to French and European laws and approved by our local ethic committee (approval numbers A 69266 0501 and BH2012-63). The animal procedures were performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Cells were routinely cultured at 37°C in a humidified atmosphere with 5% of CO₂ in Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L glucose) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), 20 mmol/L HEPES, and 2 mmol/L L-glutamine. Culture media were changed every three days. To stimulate cell differentiation, ascorbic acid (50 µg/mL) was added in both cell culture at confluence; to induce mineralization, β-glycerophosphate (10 mM) was added at confluence in chondrocytes and 6 days after confluence in osteoblasts [26].

MSCs from 4 donors were used [a 34-year old female and 22-, 23- and 36-year-old males (Lonza, Walkersville, USA; certified positive for CD29, CD44, CD105 and CD166, and negative for CD14, CD34 and CD45)]. MSCs were seeded at a density of 5,000 cells per cm² and routinely cultured in DMEM containing 10% (v/v) FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), 20 mmol/L HEPES, and 2 mmol/L L-glutamine. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in air. Cells were subcultured at approximately 80-90% confluence with trypsin/EDTA. To induce osteoblast differentiation,

1 medium was replaced at confluence by an osteogenic medium, consisting of DMEM with
2 10% FCS, containing 10 nM of 1,25(OH)₂D₃, 50 µg/mL of ascorbate and 10 mM of β-GP
3 [27].
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7 Neutrophils were obtained from peripheral blood from 4 healthy adult donors (one 45 year-
8 old male and 3 females aged 36, 54 and 55). Blood cells were separated using a density
9 gradient centrifugation (Pancoll human, P04-60500, PAN Biotech) as described by the
10 manufacturer. Red blood cells and neutrophils were then separated in the presence of 3%
11 dextran and red blood cells were finally lysed (BD Pharm lyse, BD Bioscience). Neutrophils
12 were cultured in DMEM containing 2% of FCS and treated with 0.5 µg/mL of
13 lipopolysaccharide (LPS) O111:B4 from *Escherichia coli* (from Sigma) for 3 hours and then
14 with 2 mM of ATP for 45 min.
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23 In all cells, TNAP inhibition was achieved with 25 µM MLS-0038949 (from Merck) [28],
24 since we determined in preliminary experiments that this dose was efficient to fully inhibit the
25 hydrolysis of *para*-nitrophenylphosphate (*p*NPP) by bone cells. ARL-67156 was added at 100
26 µM to inhibit CD39 and NPP1 [29] and AOPCP [adenosine-5'-O-[(phosphonomethyl)
27 phosphonic acid]] at 100 µM to inhibit CD73 [30]. ARL-67156 and AOPCP were from Santa-
28 Cruz.
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36 2.3 RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT- 37 qPCR) 38

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40 Total RNAs were extracted from grinded bones using the TRI reagent (Sigma), and from cells
41 using the NucleoSpin RNA II kit (Macherey-Nagel) following the manufacturers' protocols.
42 DNA was eliminated from bone RNA by DNase from Roche. 1 µg of RNA was retro-
43 transcribed into cDNA with Superscript II reverse transcriptase (Life Technologies) and
44 quantitative PCR was performed using a CFX-96 system (Biorad). Primers and PCR
45 conditions are given in Table 1. The obtained products were checked by sequencing. Relative
46 quantification was performed using the 2^{-ΔCq} method by the CFX Manager software (Biorad).
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54 2.4 Measurement of ATP and AMP hydrolysis 55

56 Osteoblasts and chondrocytes were cultured for 16 days as described above. Then, cells were
57 incubated in Tris-HCl buffer (pH = 7.4) containing MgCl₂ (1 mM), CaCl₂ (1 mM) and the
58 substrate (10 µM ATP or 25 µM AMP) at room temperature. Aliquots were collected at
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different time points and P_i release was determined by Malachite Green assay as described elsewhere [31]. The results were expressed as μM of P_i by comparing to a standard curve of P_i , and initial reaction rates were determined from the curves representing P_i concentrations *versus* time.

2.5 Quantification of extracellular ATP and intracellular cAMP

Osteoblasts and chondrocytes were differentiated as indicated above. Culture media were removed and replaced with serum-free DMEM with or without TNAP inhibitor (25 μM MLS-0038949). Aliquots of media were collected every 5 minutes and extracellular ATP levels were measured using the Promega ATP assay kit (ENLITEN Luciferase/Luciferin reagent) and read in the luminometer Fluoroskan Ascent® 1506450 (ThermoLabsystems). After differentiation, chondrocytes were cultured for 24 h in DMEM containing 0.1% BSA instead of FCS. AMP and Ro 20-1724, a phosphodiesterase inhibitor, were then added to the medium. At different time points, intracellular cAMP was measured by ELISA (Enzo Life Sciences), according to the manufacturer's instructions.

2.6 Measurement of TNAP activity

For the determination of TNAP activity using *p*NPP as substrate [31] cells were harvested in 0.2% (V:V) Nonidet P-40 and disrupted by sonication. TNAP specific activity was expressed as nmol of *p*-nitrophenolate formed/min/mg of protein.

2.7 MV purification and characterization

MVs were purified as described in details [32]. Hypertrophic chondrocytes were incubated with collagenase from *Clostridium histolyticum* (200 U/mL, type IA; Sigma) in a synthetic cartilage lymph (SCL) buffer at pH 7.4, at 37°C for 3h. The digests were centrifuged at 800 g and 30,000 g during 30 min at 4°C to remove cell debris and microsomes, respectively. The supernatants were centrifuged at 250,000 g during 30 min to pellet the MV, followed by re-suspension in SCL buffer (pH 7.4). In addition to these collagen-associated MVs, extracellular collagen-free vesicles and exosomes were isolated according to the protocol by Wuthier and collaborators [33]. After culture the medium was collected and centrifuged at 1,000 g during 30 min to remove apoptotic bodies. Collagen-free vesicles were harvested from the supernatant by centrifugation at 100,000 g for 30 min at 4°C. Western blots were performed by incubation with antibody against TNAP or CD73 (Abcam) overnight at 4°C followed by incubating with peroxidase-conjugated secondary antibody (1:5,000 dilution).

1 Immunostained bands were detected with the Enhanced Chemiluminescence method (ECL
2 select, GE Healthcare).

3 4 5 6 7 8 2.8 Western-blot and ELISA for IL-1 β

9 Neutrophils were cultured and treated as described above. Cell culture supernatants were
10 collected, centrifuged at 2,000 g for 5 min and analyzed for IL-1 β secretion by ELISA
11 [Immunotools GmbH (Friesoythe, Germany)]. Cell lysates (10 μ g protein) were separated by
12 SDS-PAGE, transferred onto Hybond-P membranes (GE Healthcare Life Sciences), and
13 subjected to Western blot analysis. Briefly, for the analysis of IL-1 β , the primary antibody
14 incubation was carried out for 4 h at room temperature with a 1:1,000 dilution of the mouse
15 monoclonal antibodies (Mab201, RD Systems). Second antibody incubation was carried out
16 with a 1:5,000 dilution of anti-mouse immunoglobulin G antibody conjugated to HRP
17 (Sigma). Immunostained bands were detected by the ECL method.
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28 2.9 Determination of neutrophil viability and death

29 Neutrophil viability after treatment with 25 μ M MLS-0038949 for 24 h was determined using
30 the MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay. MLS-
31 0038949 cytotoxicity in the same conditions was estimated with the Cytotoxicity Detection
32 kit from Roche based on the measurement of lactate dehydrogenase (LDH) activity released
33 from damaged cells.
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41 2.10 Statistical analysis

42 All cell experiments were repeated independently at least three times and up to 9 times
43 (indicated in the legends). Results are expressed as mean \pm the standard error of the mean
44 (SEM). Statistical analysis was performed with Past 3.22. The Shapiro-Wilk test was used to
45 determine data distribution and the F test to determine whether variances were equal or not.
46 Depending on the results of these tests, paired or unpaired Student's *t*-test, or Welch's *t*-test or
47 Wilcoxon test were used. The differences between groups were considered significant with *
48 at $p < 0.05$, ** at $p < 0.01$, and *** at $p < 0.001$.
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3 Results

3.1 Metaphysis-containing bone fragments from *Alpl*-deficient mice have increased levels of inflammatory cytokines

In order to explore the possible anti-inflammatory function of TNAP, we first were interested to determine whether metaphysis inflammation in childhood HPP [15] is associated with alterations in specific inflammatory cytokines. Childhood HPP is however a rare disease, which makes virtually impossible to obtain bone biopsies from children with HPP and the corresponding control biopsies. We therefore used *Alpl*-deficient mice, and more precisely *Alpl*^{+/-} mice, since *Alpl*^{-/-} mice die at birth or soon after. We measured a 50% decrease in *Alpl* mRNA levels in *Alpl*^{+/-} as compared with *Alpl*^{+/+} mice (Fig. 1), confirming a recent study [34]. Moreover, we confirmed that *Alpl*^{+/-} metaphyses have decreased levels of *Bglap* encoding osteocalcin and of *Col10a1* encoding the $\alpha 1$ chain of type X collagen, as also recently reported in the same mice [35] and in cultured hypertrophic chondrocytes [36]. These results are in agreement with reports indicating that TNAP-deficient mice have disorganized growth plates, characterized by an arrest in chondrogenesis [37, 38]. We then measured the levels of inflammatory markers, first focusing on the pro-inflammatory *Il-1 β* and *Il-6* and the anti-inflammatory *Il-10*, which are thought to be dysregulated in CRMO [39]. We measured significantly increased levels of *Il-1 β* and *Il-6* in *Alpl*^{+/-} bones as compared to *Alpl*^{+/+} bones. Levels of *Il-1 β* and *Il-6* were positively associated ($r=0.7$, $p=0.015$), which was coherent with IL-1 β being a major inducer of IL-6. We also measured reduced levels of the anti-inflammatory cytokine *Il-10*. Finally, we didn't observe differences in the levels of several other inflammatory cytokines and chemokines, including *Il-1Ra* encoding IL-1 receptor antagonist, *Tnf- α* (Fig. 1) or *Il-8* (data not shown). Collectively, these data confirm that the metaphyses of *Alpl*^{+/-} bones are characterized by disturbed chondrocyte maturation, and further indicate that they may also be characterized by dysregulated inflammatory cytokine expression.

3.2 TNAP is a nucleotidase in hypertrophic chondrocytes

1 We then investigated whether TNAP is an ectonucleotidase in osteoblasts and hypertrophic
2 chondrocytes. We first compared the levels of transcripts encoding TNAP with those of
3 *CD39*, *NPP1* and *CD73* (Fig. 2A). We found that TNAP was the most highly expressed
4 ectonucleotidase in metaphysis-containing bone fragments of 7-day-old wild-type mice (Fig.
5 2B). Moreover, in hypertrophic chondrocytes and osteoblasts differentiated for 16 days [26],
6 the levels of *Alpl* were much higher than those of *Npp1*, *Cd39* and *Cd73* (Figures 2C and 2D
7 respectively). These results suggested that TNAP might be involved in ATP and AMP
8 hydrolysis in hypertrophic chondrocytes and osteoblasts. To address this question, we
9 measured the hydrolysis of exogenously added AMP and ATP in hypertrophic chondrocytes
10 or osteoblasts treated or not with the TNAP inhibitor MLS-0038949 [28], the CD73 inhibitor
11 AOPCP [30], and the inhibitor of both NPP1 and CD39 ARL-67156 [29] (Fig. 2A). We first
12 checked that 100 μ M of AOPCP or 100 μ M of ARL-67156 did not inhibit the hydrolysis of
13 *p*NPP, whereas 25 μ M of MLS-0038949 totally prevented this hydrolysis (data not shown).
14 This result indicated that AOPCP and ARL-67156 have no inhibitory effect on TNAP.
15 Inhibition of TNAP or CD39/NPP1 in chondrocytes slightly reduced the hydrolysis of
16 exogenously added ATP, and this reduction was more pronounced when the three enzymes
17 were inhibited together (Fig. 3A). To ascertain that TNAP participates in ATP hydrolysis in
18 hypertrophic chondrocytes, we measured the extracellular levels of ATP that was exported
19 from the cytoplasm to the extracellular compartment after a cellular stress. After a change to
20 new medium without FCS, extracellular ATP levels peaked at 5 minutes and returned to basal
21 levels after 20 minutes (Fig. 3C). TNAP inhibition with MLS-0038949 significantly increased
22 extracellular ATP concentrations. Finally, TNAP inhibition with MLS-0038949 and CD73
23 inhibition with AOPCP both significantly reduced the hydrolysis of exogenously added AMP
24 and again this reduction was strengthened by the concomitant use of both inhibitors (Fig. 3E).
25 These data indicate that in hypertrophic chondrocyte cultures, TNAP participates in both ATP
26 and AMP hydrolysis. In contrary, TNAP inhibition with MLS-0038949 in osteoblasts did not
27 reduce the hydrolysis of exogenously added ATP (Fig. 3B), nor did it increase the levels of
28 extracellular ATP in osteoblasts cultures (Fig. 3D). Moreover, TNAP inhibition neither
29 reduced the hydrolysis of exogenously added AMP (Fig. 3F), suggesting that in osteoblasts,
30 TNAP is not a strong ectonucleotidase.

3.3 TNAP nucleotidase activity in hypertrophic chondrocytes is not associated with an autocrine anti-inflammatory function

1 We next investigated whether TNAP in hypertrophic chondrocytes is able to reduce the pro-
2 inflammatory effects of ATP. Treatment of hypertrophic chondrocytes with 100 μ M of ATP
3 triggered a peak in *Il-6* expression that returned to basal levels after about 2 hours (Fig. 4A).
4 Pretreating cells with TNAP and/or CD39/NPP1 inhibitors slightly increased *Il-6* expression
5 in response to ATP, but their effects were not significant. We next hypothesized that TNAP
6 may exert anti-inflammatory effects via the production of adenosine rather than the lowering
7 of ATP concentration. Adenosine is known to mediate its effects by binding to G-protein
8 associated receptors acting on intracellular cAMP production by adenylate cyclase [40]. We
9 observed that treatment of hypertrophic chondrocytes with adenosine significantly increased
10 the content of intracellular cAMP (data not shown). We therefore investigated whether TNAP
11 inhibition might block AMP dephosphorylation into adenosine and alter the production of
12 cAMP. Figure 4B shows that AMP dose-dependently increased cAMP production but that
13 TNAP inhibition had modest and non-significant inhibitory effects. Collectively, these data
14 indicated that despite being much more expressed than other nucleotidases, TNAP inhibition
15 modestly decreased ATP and AMP hydrolysis and had no convincing anti-inflammatory
16 effects in hypertrophic chondrocytes. We speculated that these weak effects may be due to the
17 fact that TNAP is not kept at the cell membrane in hypertrophic chondrocytes but sent in MVs
18 to induce collagen mineralization [9]. We addressed this question by performing western-blot
19 experiments to detect TNAP in cell lysates and in MVs. Figure 4C clearly shows that TNAP
20 protein is massively transferred from the cell membrane to MVs. This massive transfer into
21 MVs is specific since exosomes and apoptotic bodies released by hypertrophic chondrocytes
22 were not enriched in TNAP. In contrast to TNAP, CD73 was not sent to MVs, but remained at
23 the cell membrane. CD39, which was very weakly expressed in hypertrophic chondrocytes
24 (Fig. 2C), could not be detected by western-blots in these preparations. Finally, it was
25 tempting to speculate that TNAP might exert anti-inflammatory effects only when
26 hypertrophic chondrocytes are stimulated by inflammation, but this is probably not the case
27 since inflammatory stimulation of these cells with IL-1 β strongly decreased TNAP
28 expression, while increasing that of CD73 (Fig. 4D).
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51 3.4 TNAP is an anti-inflammatory nucleotidase in hematopoietic neutrophils, but not in 52 mesenchymal cells

53 Since in hypertrophic chondrocytes, TNAP is involved in mineralization but not in
54 inflammation, we hypothesized that TNAP might participate in inflammation in two non-
55 mineralizing cells in the bone metaphysis: mesenchymal stem cells (MSCs) and/or
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neutrophils. Indeed, MSCs express TNAP before their differentiation into osteoblasts or chondrocytes [41], and it was recently reported that they may use TNAP to dephosphorylate ATP [35]. We checked and confirmed that MSCs indeed have a significant but weak TNAP activity before their differentiation into osteoblasts (Fig. 5A). TNAP activity in MSCs from 4 adult donors was 23.24 ± 8.82 mU/mg, as compared to 2456.32 ± 226.9 mU/mg in mouse hypertrophic chondrocytes, and 2208.83 ± 157.3 in mouse osteoblasts. Interestingly, TNAP activity in undifferentiated MSCs was increased upon treatment with IL-1 β , while it was decreased in MSCs that had been previously differentiated into osteoblasts (Fig. 5A), confirming the inhibitory effect of IL-1 β on TNAP in osteoblasts (Fig. 5B). Whereas TNAP was active in undifferentiated MSCs, its expression was nonetheless very weak as compared to that of *NPP1* and *CD73* (Fig. 5C), making unlikely that it significantly contributed to ATP and AMP hydrolysis in these cells. We indeed observed that TNAP inhibition with MLS-0038949 weakly reduced ATP hydrolysis, and only when MSCs had been pretreated for 4 days with IL-1 β (Fig. 5D). However, inhibition of NPP1 with ARL-67156 was much more efficient to reduce ATP hydrolysis, indicating that TNAP contribution was quite modest as compared to that of NPP1. Finally, we failed to detect any anti-inflammatory function of TNAP in MSCs.

In summary, hypertrophic chondrocytes, and maybe also MSCs, may use TNAP to hydrolyze ATP into adenosine, but the changes in ATP and adenosine levels do not seem to impact these mesenchymal cells in an autocrine action. We hypothesized that changes in ATP levels in the metaphysis of TNAP-deficient bones induce inflammation through the involvement of neutrophils. Indeed, neutrophils are often the first cells to be recruited in inflamed or damaged tissues, and ATP release in tissues is a strong signal that initiates neutrophil recruitment [42]. Moreover, neutrophils are the only hematopoietic cells that express TNAP [43]. We first confirmed that neutrophils from healthy donors had a much higher TNAP activity than peripheral blood mononuclear cells (PBMCs) from the same donors (Fig. 6A), and observed that *ALPL* expression was increased by a pro-inflammatory stimulation with LPS (Fig. 6B). Interestingly, TNAP was, together with CD39, the most highly expressed nucleotidase in neutrophils, which expressed no detectable *NPP1* and very weak *CD73* levels (Fig. 6C) [44]. We then explored whether TNAP participates in the control of IL-1 β secretion, which was reported to be at least in part ATP- and NLRP3-dependent in neutrophils [45]. Figure 6D indicates that IL-1 β secretion induced by a 3-hrs treatment with LPS was significantly increased by a short 15-min co-treatment with ATP, confirming that NLRP3 was likely

1 involved. Interestingly, TNAP inhibition with MLS-0038949 significantly increased LPS and
2 LPS/ATP stimulated IL-1 β secretion (Fig. 6D). This stimulation of the mature 17 kDa IL-1 β
3 secretion by MLS-0038949 was accompanied by a reduction in the intracellular levels of the
4 31 kDa precursor form (Fig. 6D), strongly suggesting that TNAP inhibition increased IL-1 β
5 secretion by exacerbating ATP-associated NLRP3 activation [45]. TNAP-deficient
6 neutrophils not only secreted more IL-1 β , they also lived longer since TNAP inhibition by
7 MLS-0038949 significantly increased cell viability and reduced cell death, as shown with
8 MTT and LDH activity respectively (Fig. 6E). This seems in agreement with previous work
9 reporting that ATP delays neutrophil apoptosis [20].
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4 Discussion

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4 TNAP-deficient mice have disturbed hypertrophic chondrocyte maturation and growth plate
5 cartilage development [36-38]. Results of our study further indicate that *Alpl*^{+/-} mice also have
6 increased levels of *Il-1β* and *Il-6* in their bone metaphysis. Interestingly, it was recently
7 reported that experimental pancreatitis induced more *Il-1β* and *Il-6* in the pancreas of *Alpl*^{+/-}
8 mice as compared to that of *Alpl*^{+/+} mice [46]. This suggests that TNAP deficiency up-
9 regulates these cytokines in several tissues. Since there was a significant positive association
10 between *Il-1β* and *Il-6* levels in the metaphyses, and since IL-1β is usually upstream of IL-6
11 in the inflammatory cascade, it is likely that TNAP deficiency led to IL-1β activation, which
12 then induced IL-6. It is almost certain that TNAP deficiency in hypertrophic chondrocytes, or
13 in other mesenchymal cells, does not induce autocrine effects that result in increased IL-1β
14 secretion. Indeed, although *Il-1β* mRNA levels were increased in hypertrophic chondrocytes
15 and MSCs in response to pro-inflammatory stimulation with TNF-α, we always failed to
16 detect any intracellular cleavage of the precursor form of IL-1β, and any secretion of active
17 IL-1β by ELISA or Western-blot (data not shown). It is likely that NLRP3 is not functional in
18 mesenchymal cells and that IL-1β is activated and secreted by other mechanisms. Indeed,
19 NLRP3-activating mutations in myeloid cells but not in chondrocytes or mesenchymal
20 precursors trigger chronic inflammation and growth plate dysplasia, that mimic the
21 cryopyrin-associated periodic syndrome (CASP, due to NLRP3 activating mutations) [47].
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38 Our hypothesis is that neutrophils, rather than cells from the mesenchymal lineage, are
39 activated by supraphysiological ATP levels. As already mentioned above, ATP release in
40 damaged tissues is a strong signal that initiates neutrophil recruitment [42]. Moreover,
41 neutrophils express TNAP, which is more expressed in these cells in response to
42 inflammatory stimulation. When TNAP was chemically inhibited in neutrophils, intracellular
43 levels of the IL-1β precursor form were decreased, and extracellular levels of the cleaved
44 mature IL-1β form were increased. These results are in agreement with the recent report that
45 neutrophils from *Alpl*^{+/-} mice secrete more IL-6 than those from wildtype mice [46].
46 Therefore, we think reasonable to hypothesize that the increased expression of *Il-1β* and *Il-6*
47 in the bone metaphysis of *Alpl*^{+/-} mice occurs in neutrophils or is induced by neutrophils.
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58 Neutrophils are likely responsible for increased cytokine expression in the metaphysis of
59 *Alpl*^{+/-} mice, and we think that the signal that induces their recruitment and activation in the
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1 metaphysis is the abnormally elevated levels of extracellular ATP resulting from TNAP
2 deficiency in hypertrophic chondrocytes. Indeed, TNAP inhibition in hypertrophic
3 chondrocytes resulted in significantly higher extracellular ATP levels. Since the hypertrophic
4 zone is where the growth plate becomes vascularized, these increased ATP levels are likely to
5 induce the recruitment of neutrophils [42].
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10 In the particular context of childhood hypophosphatasia, the fact that IL-1 β inhibits TNAP
11 expression in mineralizing hypertrophic chondrocytes and osteoblasts suggests that a vicious
12 cycle takes place where TNAP deficiency in mineralizing cells induces neutrophil
13 recruitment, which further exacerbates TNAP deficiency in mineralizing cells and accelerates
14 rickets. More generally, considering the importance of neutrophils in blood and tissue
15 inflammation [48], such a function of TNAP as an anti-inflammatory nucleotidase deserves
16 much consideration.
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35 *data and wrote the paper; L. Bessueille, A. Briolay, J. Como, C. Mansouri, S. Mebarek, M.*
36 *Gleizes, A. El Jamal, E. Mornet and C. Fonta performed research and analyzed data; C.*
37 *Dumontet and E.L. Matera developed neutrophil purification and cultures; R. Buchet and J.L.*
38 *Millan analyzed data.*
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Figure legends

Figure 1: Disturbed levels of hypertrophic chondrocyte markers and inflammatory cytokines in the metaphysis-containing bone fragments of *Alpl*^{+/-} mice. RT-qPCR analysis of mRNA levels in the metaphysis-containing femurs and tibias of 7-day-old *Alpl*^{+/+} and *Alpl*^{+/-} mice. * indicates a statistical difference with p<0.05; ** a difference with p<0.01.

Figure 2: expression of TNAP, and ectonucleotidases in metaphysis-containing bone fragments, hypertrophic chondrocytes and osteoblasts. A: enzymes known to be involved in ATP hydrolysis into adenosine, and their inhibitor. B: RT-qPCR analysis of nucleotidase-encoding mRNA levels in the metaphysis-containing femurs and tibias of 7-day-old wildtype mice. Mouse primary chondrocytes (C, n=4 independent experiments) and primary osteoblasts (D, n=3) were cultured as detailed in the Materials and methods section and mRNA levels were quantified by RT-qPCR. * indicates a statistical difference with p<0.05; ** a difference with p<0.01; *** a difference with p<0.001.

Figure 3: ectonucleotidase activity of TNAP in hypertrophic chondrocytes and osteoblasts. Mouse primary chondrocytes (A, C and E) and primary osteoblasts (B, D and F) were cultured as detailed in the Materials and methods section for 16 days. Hydrolysis of the substrate (10 μM ATP or 25 μM AMP) into P_i was measured by the Malachite green assay in the presence or absence of 25 μM of MLS-0038949 and 100 μM of ARL-67156 (A, n=5 and B, n=4), or in the presence or absence of 25 μM of MLS-0038949 and 100 μM of AOPCP (E, n=5 and F, n=4). Endogenously released ATP was quantified with the bioluminescent Luciferin-Luciferase reaction in presence (○) or absence (□) of MLS-0038949 (C, n=5 and D, n=3). * indicates a statistical difference with p<0.05; ** a difference with p<0.01; *** a difference with p<0.001.

Figure 4: absence of anti-inflammatory effects of TNAP in hypertrophic chondrocytes. Mouse primary chondrocytes were differentiated into hypertrophic chondrocytes for 16 days as detailed in the Materials and methods section. A: effect of MLS-0038949 and/or ARL-67156 on ATP-stimulated *Il-6* expression (n=8). To eliminate the inter-experimental variations in *Il-6* levels, *Il-6/Gapdh* ratio in untreated cells was set to 1. B: effect of MLS-0038949 and/or AOPCP on AMP-stimulated intracellular cAMP production (n=4). C: western-blot analysis of TNAP and CD73 in hypertrophic chondrocyte cell membranes, and

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in vesicles released by these cells (n=3, a representative result is shown; the same quantity of protein was used in all conditions). D: effect of a 24-h treatment with IL-1 β on nucleotidase levels, as determined by RT-qPCR (n=4). * indicates a statistical difference with p<0.05; ** a difference with p<0.01.

Figure 5: absence of anti-inflammatory effects of TNAP in MSCs. A: effect of a 7-day treatment with IL-1 β on TNAP activity in MSCs left undifferentiated or differentiated into osteoblasts (n=6). To eliminate the inter-experimental variations, TNAP activity was set to 100% in untreated cells. B: effect of a 7-day treatment with IL-1 β on TNAP activity in murine primary osteoblasts (n=3). C: quantification of nucleotidase mRNA levels in undifferentiated MSCs by RT-qPCR (n=8 independent experiments with MSCs from 4 different donors). D: in undifferentiated MSCs pre-treated for 4 days with 1 ng/mL of IL-1 β , the hydrolysis of 10 μ M of ATP into P_i was measured by the Malachite green assay in the presence or absence of 25 μ M of MLS-0038949 and 100 μ M of ARL-67156 (n=8 with MSCs from 4 different donors). * indicates a statistical difference with p<0.05; ** a difference with p<0.01.

Figure 6: anti-inflammatory ectonucleotidase function of TNAP in neutrophils. A: TNAP activity measured in freshly isolated human neutrophils (n=5 for neutrophils and n=3 for PBMCs). B: effect of 0.5 μ g/mL of LPS on *ALPL* mRNA levels as determined by RT-qPCR (n=6 independent experiments from 4 different donors). C: RT-qPCR analysis of nucleotidase transcripts in freshly isolated human neutrophils (n=5; nd: not detected). D: quantification by ELISA of extracellular levels of IL-1 β in neutrophils primed with 0.5 μ g/mL of LPS for 3 h and further stimulated with 2 mM of ATP during 45 min (n=9 independent experiments from 4 different donors). A representative western-blot experiment of intracellular IL-1 β is shown. E: quantification of cell viability and cell death with MTT and LDH assays respectively in cells treated with 25 μ M of MLS-0038949 for 24 h (n=7 independent experiments from 4 different donors). * indicates a statistical difference with p<0.05; ** a difference with p<0.01; *** a difference with p<0.001.

Table 1: Summary of primers used. Shown are the mouse (m) and human (h) primer sequences (F: forward; R: reverse), annealing temperatures (Ta), base pair (bp) lengths of the corresponding PCR products, and GenBank accession numbers.

Gene	GenBank	Ta (°C)	Sequence	Length (bp)
<i>mGapdh</i>	NM_008084.3	60	F: 5'-GGCATTGCTCTCAATGACAA-3' R: 5'-TGTGAGGGAGATGCTCAGTG-3'	200
<i>mAlpl</i>	NM_007431.3	60	F: 5'-CAAAGGCTTCTTCTTGCTGGT-3' R: 5'-AAGGGCTTCTTGTCCGTGTC-3'	258
<i>mBglap</i>	NM_001037939.2	60	F: 5'-AAGCAGGAGGGCAATAAGGT-3' R: 5'-CGTTTGTAGGCGGTCTTCA-3'	364
<i>mCol10a1</i>	NM_009925.4	60	F: 5'-CAAACGGCCTCTACTCCTCTGA-3' R: 5'-CGATGGAATTGGGTGGAAAG-3'	129
<i>mIl-10</i>	NM_010548.2	60	F: 5'-CCCTTTGCTATGGTGTCTTTC-3' R: 5'-GATCTCCCTGGTTTCTCTCCC-3'	100
<i>mIl-1β</i>	NM_008361.4	62	F: 5'-GGGCCTCAAAGGAAAGAATC-3' R: 5'-CCACTTTGCTCTTGACTTCTATC-3'	152
<i>mIl-6</i>	NM_031168	60	F: 5'-GTCACAGAAGGAGTGGCTA-3' R: 5'-AGAGAACAACATAAGTCAGATACC-3'	193
<i>mIL1-ra</i>	NM_031167	60	F: 5'-GGGATACTAACCAGAAGACC-3' R: 5'-GACAGGCACAGCTTGCCCCC-3'	157
<i>mTnf-α</i>	NM-013693	60	F: 5'-TGGGACAGTGACCTGGACTGT-3' R: 5'-TTCGGAAAGCCATTTGAGT-3'	67
<i>mNpp1</i>	NM_001308327.1	60	F: 5'-CGGACGCTATGATTCCTTAGA-3' R: 5'-AGCACAATGAAGAAGTGAGTCG-3'	93
<i>mCd39</i>	NM_001304721.1	60	F: 5'-AGGTGAAGAGATTTTGCTCCAA-3' R: 5'-TTTGTCTGGGTGAGTCCCAC-3'	101
<i>mCd73</i>	NM_011851.4	60	F: 5'-GGACATTTGACCTCGTCCAAT-3' R: 5'-GGGCACTCGACACTTGGTG-3'	191
<i>hRPLP0</i>	NM_001002.3	60	F: 5'-CGACCTGGAAGTCCAACACTAC-3' R: 5'-AGCAACATGTCCCTGATCTC-3'	289
<i>hGAPDH</i>	NM_002046.5	60	F: 5'-GTTCCAATATGATTCCACCC-3' R: 5'-AGGGATGATGTTCTGGAGAG-3'	487
<i>hALPL</i>	NM_000478.4	60	F: 5'-CAAAGGCTTCTTCTTGCTGGT-3' R: 5'-AAGGGCTTCTTGTCTGTGTC-3'	258
<i>hNPPI</i>	NM_006208.2	60	F: 5'-CGATTTTGCCGATTGAGGATT-3' R: 5'-AAACTGGTGTGCTGGGAAAGAAGACA-3'	146
<i>hCD39</i>	NM_001776.5	60	F: 5'-CTGATTCCTGGGAGCACATC-3' R: 5'-CTGGGATCATGTTGGTCAGG-3'	99
<i>hCD73</i>	NM_002526.3	60	F: 5'-CTCCTCTCAATCATGCCGCT-3' R: 5'-CAAATGTGCCTCCAAAGGGC-3'	221

Figure 1

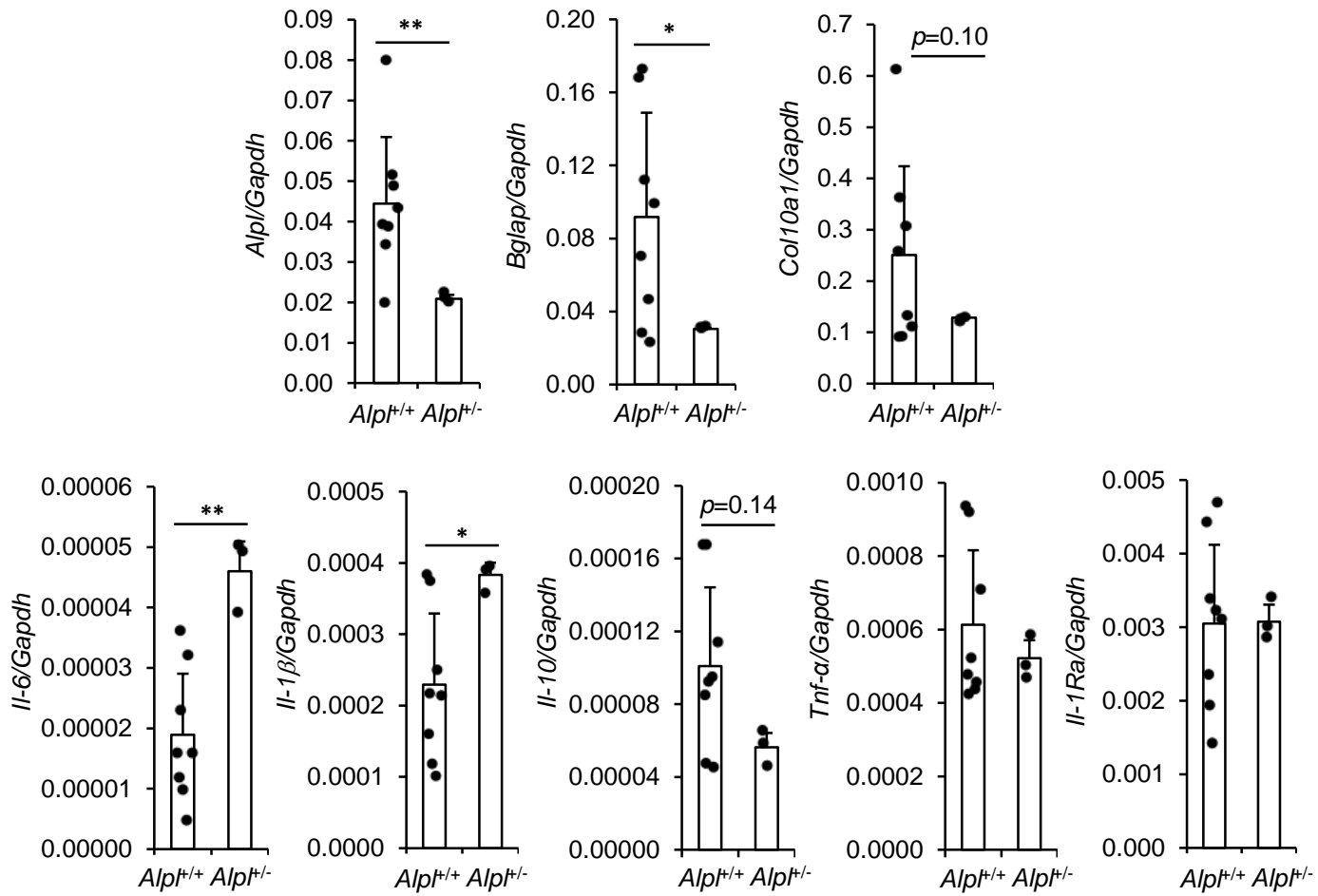
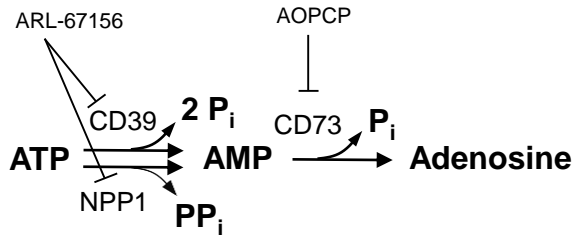
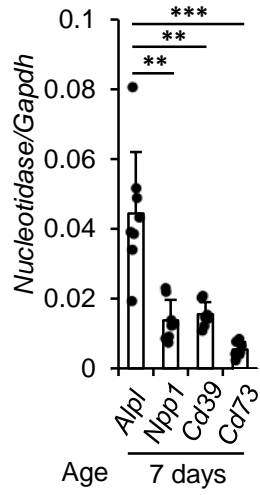


Figure 2

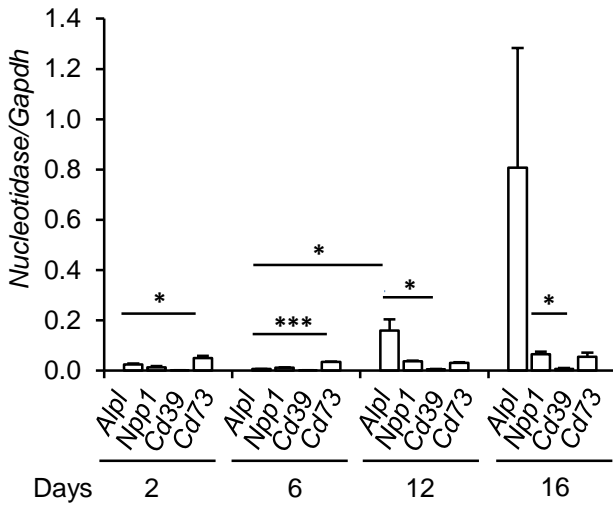
A



B



C



D

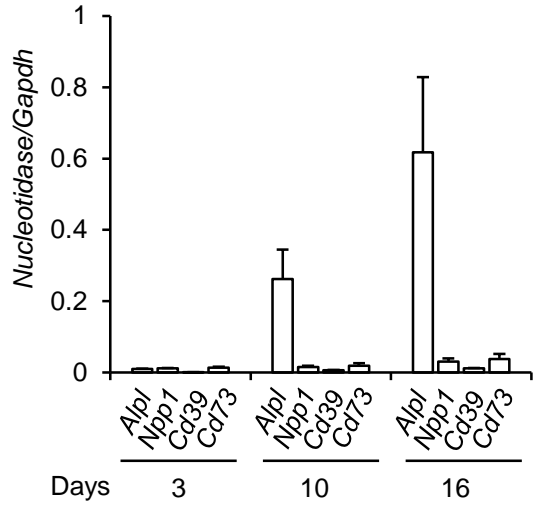


Figure 3

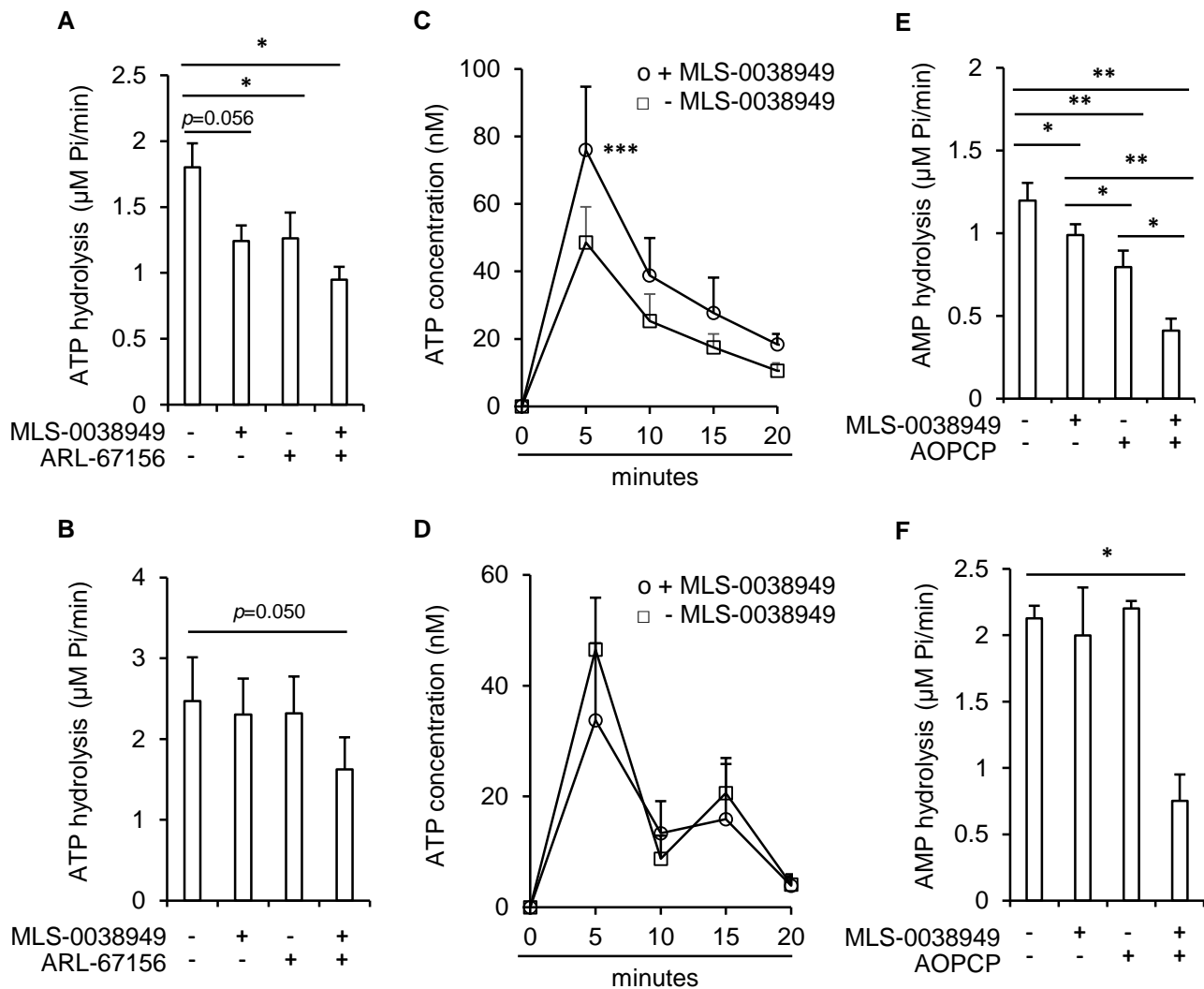


Figure 4

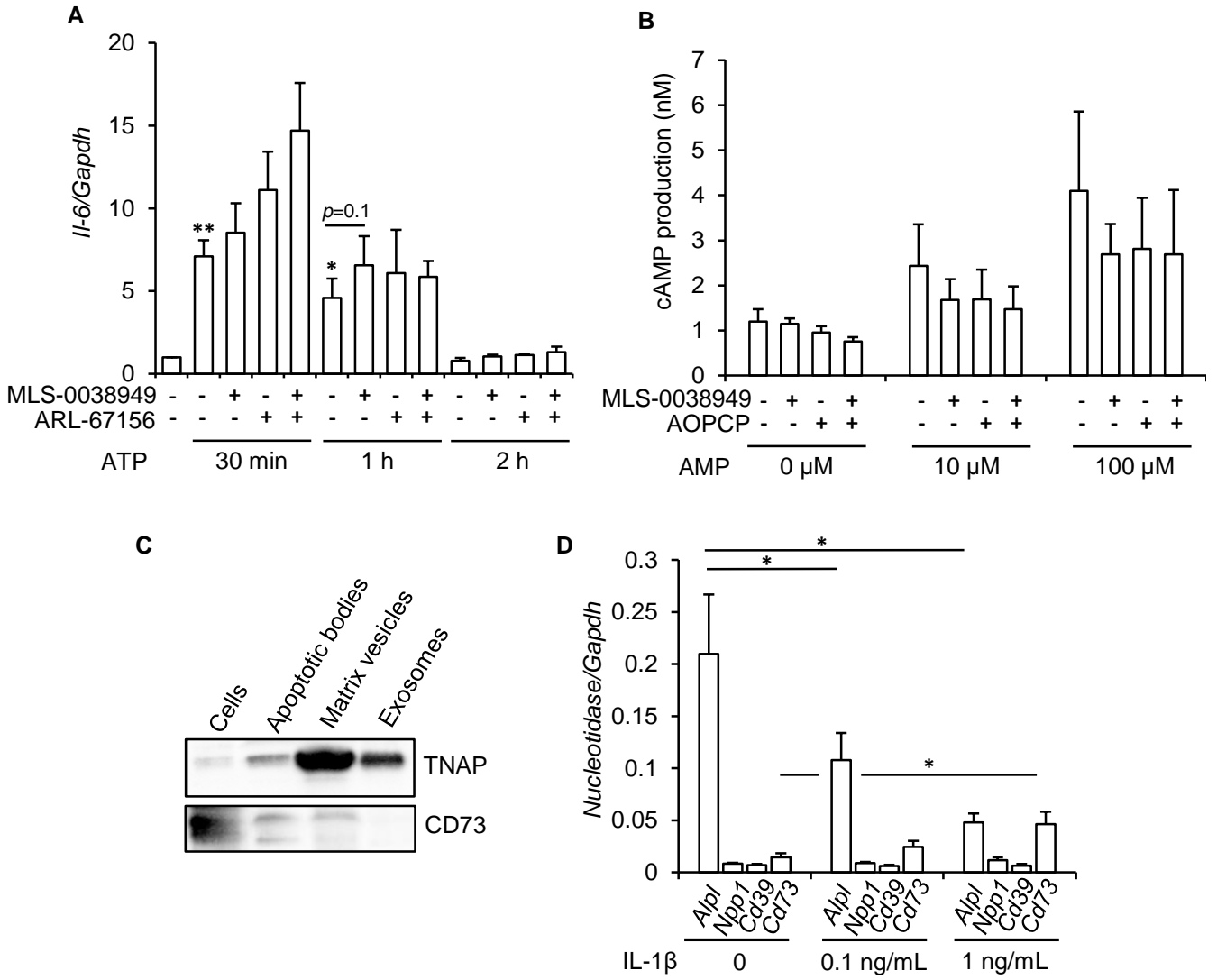


Figure 5

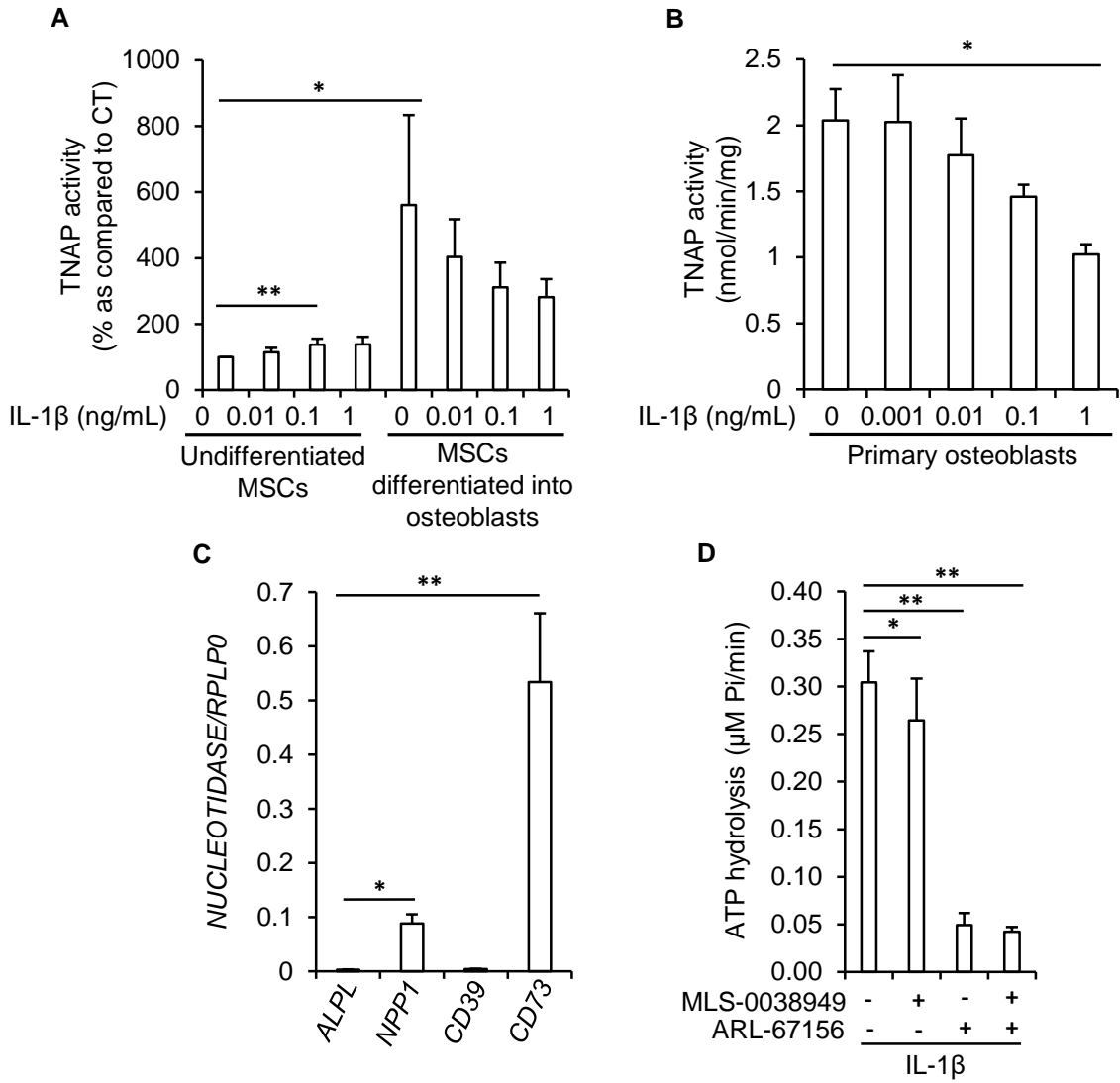


Figure 6

