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Involvement of the P2X7-NLRP3 axis in Leukemic Cell Proliferation and Death

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ABBREVIATIONS

ADP: Adenosine diphosphate

AML: Acute myeloid leukemia

AMPc: Cyclic adenosine monophosphate

ASC: Apoptosis-associated-speck-like-protein

ATP: Adenosine triphosphate

BIR: Baculovirus-IAP-repeat

BM: Bone marrow

BMDM: Bone marrow derived macrophage

B-PLL: B-cell prolymphocytic leukemia

BSA: Bovine Serum Albumin

BzATP: 2',3'-(4-benzoil)-benzoic-ATP

Ca^{2+}: Ion calcium

CAC: Colitis associated colorectal cancer

CARD: caspase-activation-and-recruitiment-dom

CD: Cluster of Differentiation

CHOP: Cyclophosphamide, Doxorubicin Vincristine and Prednisone

CLL: Chronic Lymphocytic Leukemia

CMV: Cytomegalovirus

COP: Cyclophosphamide, Vincristine, and Prednison

CTLs: C-type lectins

Da: Dalton

DAMPs: Damage-associated molecular patterns

DC: Dendritic cells

DSS: Dextran sodium sulfate

FACS: Flow cytometry

HBV: Hepatitis B virus

HCC: Hepatocellular carcinoma

HCV: Hepatitis C virus

HEK293: Human Embryonic Kidney

IFN-γ: Interferon gamma

IL: Interleukin

KO: Knock out

LPS: Lipopolysaccharide

LRR: Leucine-rich repeats

MHC: Major Histocomptability complex

NF-kB: Nuclear factor kappa light chain enhancer of activated B cells

NK: Natural Killer

NLR: Nod like receptor

NLRP3: NLR family pyrin domain containing 3

PAMPs: Pattern associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate Buffered Saline

PGE2: Prostaglandin E2

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PRRs: Pattern recognition receptors

ROS: Reactive oxygen species

RT-PCR: Reverse transcriptase-polymerase chain reaction

STAT: Signal transducer and activator of transcription

T-ALL: T-cell acute lymphoblastic leukemia

TCR: T-cell receptor

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-alfa

TRX: Thioredoxin

TXNIP: Thioredoxin interacting protein

WBC: Whyte blood count

WT: Wild type

INTRODUCTION

1. Chronic Lymphocytic Leukemia

1.1 Epidemiology and etiology

Chronic Lymphocytic Leukemia (CLL) known as B-Chronic Lymphocytic Leukemia (B-CLL), is a lymphoproliferative disorder characterized by the accumulation of B cells in the bone marrow, lymph node and peripheral blood. Moreover is characterized by the accumulation of small monoclonal B cells CD5 positive, and more of 90% of the cells are blocked in G0/G1 phase of the cell cycle [1]. Furthermore it is known that B lymphocytes are resistant to apoptosis [2]. CLL is the most common adult leukemia in the Western world and is more prevalent in men than in women, the incidence rate in men is nearly twice as high as in woman. CLL mainly affects the elderly population, with a median age at diagnosis of 70 years; sometimes, it is not unusual to diagnose in younger individuals from 30 years of age. The etiology is still unknown; the exposure to common carcinogens does not seem to be associated with the disease progression. Several studies are in progress to assess a potential relation among CLL onset, inflammation, and autoimmune conditions [3], [4]. A familiarity of this pathology is well documented in the 5-10% of cases. Most patients are asymptomatic at diagnosis and in many instances the disease is revealed at a routine blood test when a high white blood cell count is detected [5]. An asymptomatic disease is observed in approximately 40-60% of patients at diagnosis, however duration of this asymptomatic phase varies considerably amongst patients [6].

1.2 Clinical features

CLL is often diagnosed with routine laboratory tests; in other cases, the pathology occurs with asthenia, weight loss, fever, lymphadenopathy, splenomegaly, and hepatomegaly The clinical course and survival of CLL patients varies substantially among patients: some patients stay in asymptomatic for a long time, while others present an aggressive form that is difficult to control with chemotherapy. The typical CLL hypogammaglobulinemia could induce immunodeficiency and high mortality for infections [7],[8]. Even though the causes of death are often attributed to the latent disease in some cases progressing syndromes with a poor prognosis could occur. Another evolution could be the B-cell prolymphocytic leukemia (B-PLL) that is more aggressive and characterized by malignant B cells larger than average. The occurrence of acute lymphoblastic leukemia is very rare, while acute

myeloid leukemia, such as non-hematological disease, could be correlated with B-CLL immunological deficit and chemotherapy [9].

1.3 Diagnosis

It is essential to diagnose the CLL, because sometimes the pathologies is indolent and diagnosis is often performed on asymptomatic patients. Diagnosis of CLL is generally easy and is based on the evaluation of blood count, blood smear, and the immune phenotype of circulating lymphocytes. The National Cancer Instute diagnostic criteria include [5]:

1) Presence of at least $5x10^9$ B lymphocytes/L(>5000/μl) in the peripheral blood. The leukemic cells found in the blood smear are characteristically small mature lymphocytes with a narrow border of cytoplasm and a dense nucleus, lacking discernible nucleoli, with partially aggregated chromatin. According to the FAB classification (French-American-British Group) the number of atypical lymphocytes must not be >10% of the total leukocytes to configure the typical form of CLL. These cells may be found mixed with larger or atypical cells, broken cells, or prolymphocytes which may comprise up to 55% of the blood lymphocytes. Finding a higher percentage of prolymphocytes would favors a diagnosis of prolymphocytic leukemia. Gumprecht shadows, found as cell debris, are other morphological features found in CLL.

2) bone marrow lymphocytic infiltration >30%. This criterion is not essential for the diagnosis but is important for staging, prognosis and for differential diagnosis in case of cytopenia. However, the diagnosis should be confirmed by histopathologic evaluation of lymph node or bone marrow biopsy whenever possible. Marrow infiltration can occur in four forms: diffuse, nodular, interstitial, and mixed. Nodular pattern suggests an early stage of the disease, while diffuse and interstitial patterns are typical of advanced stages.

3) Immunophenotype analysis. CLL phenotype is characterized by three elements:

a) the expression of a single immunoglobulin light chains subtype (κ or λ);

b) co-expression of the T-cell antigenCD5 and the B-cell surface antigens CD19, CD20, and CD23, CD23 [10], [11];

c) low levels of CD79b and surface immunoglobulin (sIg) that in CLL appear to be mainly IgM followed by IgD, IgG, and IgA; it is not unusual to find IgM and IgD co-expression.

In CLL, T lymphocytes, in particular $CD8^+$ T cells, are often increased, with a reduced CD4/CD8 ratio. They show activation markers such as CD25 and HLA-DR. Natural-Killer (NK) cells ($CD16^+/CD56^+$) are present in high levels. Several analyses are performed to confirm the diagnosis and to prevent complications: serum protein electrophoresis, Ig dosage, Coombs test, and analysis of renal and liver function. Before starting an immunotherapy, it is important to assess the absence of viral infection (HBV, HCV, CMV) [11].

1.4 Prognosis

Given the extreme heterogeneity of the disease it is difficult to predict the course of the disease at the time of diagnosis and the value of an early treatment is uncertain. Therapy is currently recommended only for patients with a disease progressive, symptomatic, or both [12]. During the years, different parameters or prognostic factors were proposed to define the clinical course of CLL patients.

1.4.1 Clinical prognostic factors

1) Clinical staging are based on two widely accepted staging methods: the Rai method in USA and the Binet system in Europe. The original Rai classification [13] was modified to reduce the number of prognostic groups from 5 to 3. Both systems now describe 3 major subgroups with different clinical outcomes. These stagies system are currently being applied in clinical practice since they play an important role in estimating the need for therapy and the likely prognosis and survival. These 2 stagies systems are simple, inexpensive, and can be applied by physicians worldwide. Both rely exclusively on physical examination and standard laboratory tests and do not require ultrasound, computed tomography (CT), or magnetic resonance imaging [5].

The Rai system classifies CLL according to the following criteria:

− Low-risk disease (stage 0): absolute lymphocytosis >15,000/mm^3 and marrow lymphocytosis >40%;

− Intermediate-risk disease (stage I or II): lymphocytosis plus lymphadenopathies or splenomegaly and hepatomegaly;

− High-risk disease (stage III or IV): lymphocytosis plus disease-related anemia (Hb<110g/L) or thrombocytopenia (as defined by a platelet count <100 × 10^9/L).

The Binet system is based on the number of involved areas, as defined by the presence of enlarged lymph nodes of greater than 1 cm in diameter or organomegaly, and on whether there is anemia or thrombocytopenia. It is subdivided into 3 stages:

− Stage A. Involvement of up to 2 lymph node areas. Absence of anemia or thrombocytopenia.

− Stage B. Involvement of three or more lymph node areas; absence of anemia or thrombocytopenia lymphoadenopathy.

− Stage C. Anemia and / or thrombocytopenia regardless of the number of lymphoid areas involved.

2) Lymphocyte doubling time: it is less than 12 months and it is associated with a poorer clinical course.

3) Bone marrow infiltration: a spread infiltration pattern correlates with a poor prognosis [14].

1.4.2 Biological prognostic factors

Over the past decade, several prognostic markers based on genetic, phenotypic, or molecular characteristics of CLL cells have been brought forth. These have been valuable adjuncts to the time-honored staging systems of Rai and Binet and other parameters that incorporate patient physical findings and clinical laboratory data. The clinical utility of these prognostic indicators, alone or in combination with each other and the clinical prognostic systems, is still being analyzed [15]. The biological prognostic factors comprehend:

1) Mutational status of the Ig heavy chain variable region (IgVH) genes. Based on the numbers of somatic mutations detected in these genes, the cases are divided into 2 categories: "unmutated" or "mutated". Demonstration that up to 2/3 of CLL cases had less than 98 % sequence homology to the germline gene, [16], [17] indicated that the cell of origin had passed through the germinal center. Conventionally, patients with <2% differences from the most similar germline gene in both the expressed VH and VL genes

were define unmutated; mutated cases were defined as those in which the CLL cells displayed ≥2% differences in either the expressed VH or VL gene [18]. Expression of the immunoglobulin variable region gene V3-21 is associated with poor outcome, regardless of mutational status [19], [20], [21]. Since determination of IgVH mutational status is laborious and not accessible to routine hematological laboratories, a search was initiated for surrogate biomarkers with similar predictive value.

2) β2 microglobulin: this parameter is inversely correlated with the survival. It is related with the lymphocyte doubling time so that an increase in β2 microglobulin indicates an high neoplastic cell proliferation [22]

3) CD38 expression. CD38 is 45 KDa transmembrane glycoprotein highly expressed on the surface of B-cells in a significant percentage of patients with CLL. CD38 is expressed at high levels by B lineage progenitors in bone marrow (BM) and by B lymphocytes in germinal center, in activated tonsils, and by terminally differentiated plasma cells [23], [24].CD38 expression on the surface of CLL cells was the first marker found to correlate with IgVH mutational status [18]. High levels of CD38 expression in CLL cells have been found to be associated with poor prognosis. [18], [25], [26], [27], [28], [29]. A previous study suggested that CD38 expression has prognostic value in CLL. Cases with $CD38^+$ B cells >30% show a bad prognosis. Indeed, the cut-off to discriminate $CD38^+$ to $CD38^-$ is not unanimously defined: some authors place it at 20% [26] or to 7% [30] of $CD38^+$ B cells. CD38 has an independent prognostic value. Moreover, variability is the limit of this marker, in particular after treatment: chemotherapy [30] affects mainly $CD38^-$ cells, determining an increase of $CD38^+$ cells in a second time [18].

3) ZAP-70 expression. Zeta-associated protein of 70 kDa (ZAP-70) is a cytoplasmic tyrosine kinase involved in normal signaling of T-cell and natural killer cells, and is normally absent in mature B-cells, which instead use a related tyrosine kinase, Syk, for signal transduction [31], [32], [33]. ZAP-70 expression may reflect an activation state of the malignant clone associated with progressive disease or may be involved in CLL progression because of its function as a tyrosine kinase that can signal downstream of many surface receptors. About 20% discordance between ZAP-70 expression and IgVH mutational status have been reported in some studies [12], [34] and a high incidence of V3-21 gene usage or adverse genomic aberrations have been found in the ZAP-70 positive discordant cases [35]. The expression of ZAP-70 may change over time in CLL, in particular during clinical progression [18]. ZAP-70 expression analysis can be performed

with different methods: flow cytometry, immunohistochemistry, Western blotting, and Real-Time PCR. Among these, flow cytometry is the most advantageous for its diffusion and easiness of application.

4) Cytogenetic aberrations. Deletions (11q22-23, 17p13, 13q14, 6q21) and chromosome 12 trisomy are the most frequent chromosomal alterations in CLL. Clonal genomic alterations can be characterize in approximately 80% of CLL patients by cytogenetic technique like fluorescence in situ hybridization (FISH). The prevalence of 19 the most common alterations was estimated in a German multicentre study [36]: 13q- 55%, 11q- 18%, +12 16%, 17p- 7%, 6q- 7%. 17p- and 11q- are independent prognostic factors identifying subgroups of patients with rapid disease progression and short survival times in multivariate analysis, whereas 13q- as a single aberration is associated with favorable outcome. In addition, 17p- abnormalities and TP53 mutations have been associated with treatment failure. The presence of chromosome alterations with high risk justifies the use of more aggressive treatment [25]. Chromosome alterations are independent from IgVH mutational status though is evident a more frequency of 11q- and 17p- in unmutated and 13q- in mutated cases. These data show that analysis conducted by cytogenetics could be used as further risk stratification instrument together with the other prognostic factors [37].

1.5 Treatment

Criteria for initiating treatment depend on clinic symptoms, stage and disease activity. In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy unless they have evidence of progression. On the contrary, patients at intermediate (I and II) and high risk stages (III and IV), according to the modified Rai classification or at Binet stage B or C, usually benefit from the start of treatment; some of these patients (in particular Rai intermediate risk or Binet stage B) can be monitored without therapy until they have evidence for progressive or symptomatic disease [5]. Therapeutic possibilities comprehend drugs with different mechanisms of action, up to stem cells auto/allotransplantation. Since CLL is an incurable disease, current therapy is intended control the expansion of the neoplastic clone. The choice of the therapy is linked to patient age and general conditions. In older patients (>65) primary treatment consist of Chlorambucil (10mg/die for 1-4 weeks) associated with Prednisone (25mg/die for 1-4 weeks), while in other cases the therapy is based on Fludarabine, alone or in association with Cyclophosphamide. Chlorambucil treatment

induces a response in 70% of cases, but only 10% shows a complete response and has no effect on survival [36]; these considerations make it suitable for palliative treatment. Steroids have not demonstrated a significant effect on survival, while their side effects, such as opportunistic infections, are well known. However, they are useful to contain autoimmune complications. During the past years, basing on the experience on other lymphomas, therapeutic combinations such as CHOP (Cyclophosphamide, Doxorubicin Vincristine and Prednisone) or COP (Cyclophosphamide, Vincristine, and Prednisone) were tested; although they display a high frequency of complete response (CR) in respect of an increased toxicity, they did not improve survival [38]. From the mid of 1990 years, the first line treatment for CLL was the use of purine analogous. This class of drugs comprehends Pentostatin, an adenosine deaminase inhibitor, Clabridine, Fludarabine, and DNA-polimerase inhibitors. The more valid in CLL treatment is Fludarabine (25-30 mg/die for 5 days, 3-6 month); 80% of cases show global response, and 30% a CR. Moreover, the time of remission is greater than the one obtained with Chlorambucil plus Prednisone [39]. Despite the positive overall response, Fludarabine and purine analogous are not so effective in improving the survival rate than Chlorambucil or alkylating agents [40], [41], [42]. The US Intergroup Trial has recently demonstrated that the combination of Cyclophosphamide and Fludarabine, compared to Fludarabine alone, gives higher overall responses (74.3% vs 59.5%), CR (23.4% vs4.6%), and progression free survival (PFS) (31.6 vs 19.2 months). On the other hand, the combination of the two drugs resulted in a greater bone marrow toxicity, neutropenia, anemia, and thrombocytopenia with infectious complications [43]. Monoclonal antibodies provided a significant support in the treatment of hematological malignancies. CD20, a surface membrane phosphoprotein, has become the preferred target of immunotherapy. The chimeric mouse anti-human monoclonal antibody Rituximab is specific for the CD20 antigen and has been used in clinical trials to treat patients with Non-Hodgkin Lymphoma (NHL). Preclinical studies identified the ability of Rituximab to increase the efficiency of cytotoxic drugs in resistant cell lines, blocking the anti-apoptotic signaling. The combination of Rituximab with other drugs results in a synergistic cytotoxicity and apoptosis [44]; Fludarabine, in fact, downmodulate the complement resistance proteins CD46, CD55, and CD59 on leukemia cells, thereby potentially making cells more vulnerable to Rituximab-induced complement-mediated lysis [45]. The combination of Rituximab with Fludarabine and Cyclophosphamide was evaluated both as initial therapy in those cases of recurrent or refractory to prior therapies. In previously untreated patients, PFS is greater than four years in about 2/3 of cases [46]. Rituximab side effects are essentially related to the intravenous infusion of cytokines

(fever, chills, nausea, and hypotension). Alemtuzumab is a humanized anti-CD52 antibody; CD52 is expressed at high levels on most of normal and malignant mature lymphocytes but not on hematopoietic stem cells. It can be administered subcutaneously and it is very effective in inducing remission in relapsing CLL patients [47]. However, Alemtuzumab may cause a marked immunosuppression that require prophylactic therapy for *Pneumocistis carinii*, Varicella-zoster virus (VZV), and Cytomegalovirus (CMV) infections. The association with Fludarabine and Cyclophosphamide is burdened by significant toxicity to bone marrow, so precautions are necessary during Alemtuzumab administration. The expression of the anti-apoptotic protein Bcl-2 is associated with the pathogenesis of CLL. As negative regulator of the intrinsic apoptotic pathway, overexpression of Bcl-2 confers chemoresistance in a number of hematologic cancers and solid tumors [48]. Although protein levels vary among cells and patients, Bcl-2 is expressed in virtually all patients with CLL and Bcl-2 upregulation plays a critical role in this disease. Allogenic and autologous stem cell transplantation (SCT) are increasingly considered for treatment of CLL patients. With appropriate supportive care, it is safe and can induce a long-lasting clinical and molecular remissions. Feasibility of autologous SCT appears to be best early during the course of the disease, but there is only limited hope that autotransplantation can cure the disease [49]. Cytokines support from the malignant microenvironment prolong CLL cell survival, immune evasion, and resistance to therapy. Interrupting these prosurvival effects from the malignant microenvironment is a potential new approach in treating patients with CLL.

2. Purinergic Receptors

Purinergic receptors are membrane receptors activated by extracellular nucleotides [50]. The family include P1 receptors that are activated by adenosine, and P2 receptors where the physiological agonist is adenosine-5'-triphosphate (ATP) (Figure 1). The intracellular pathways activated by P2 receptors depend on the cell type, the pattern of receptors expressed and the nature of the stimulation. P2 receptor are divided into 2 subgroups: P2X and P2Y [51]. P2Y receptors also referred to as “metabotropic”, G-protein-coupled receptors whose activation triggers calcium release from intracellular stores. Furthermore the effects of activation depends on the G protein subtype involved. P2Y1, 2,4,6, and 11 are coupled to Gq/11 proteins that trigger the generation of inositol 1,4,5-trisphosphate and

the release of Ca^{2+} from the intracellular stores. P2Y12, 13 and 14 are associated with Gi/0 proteins, which inhibit adenylate cyclases [52], [53].

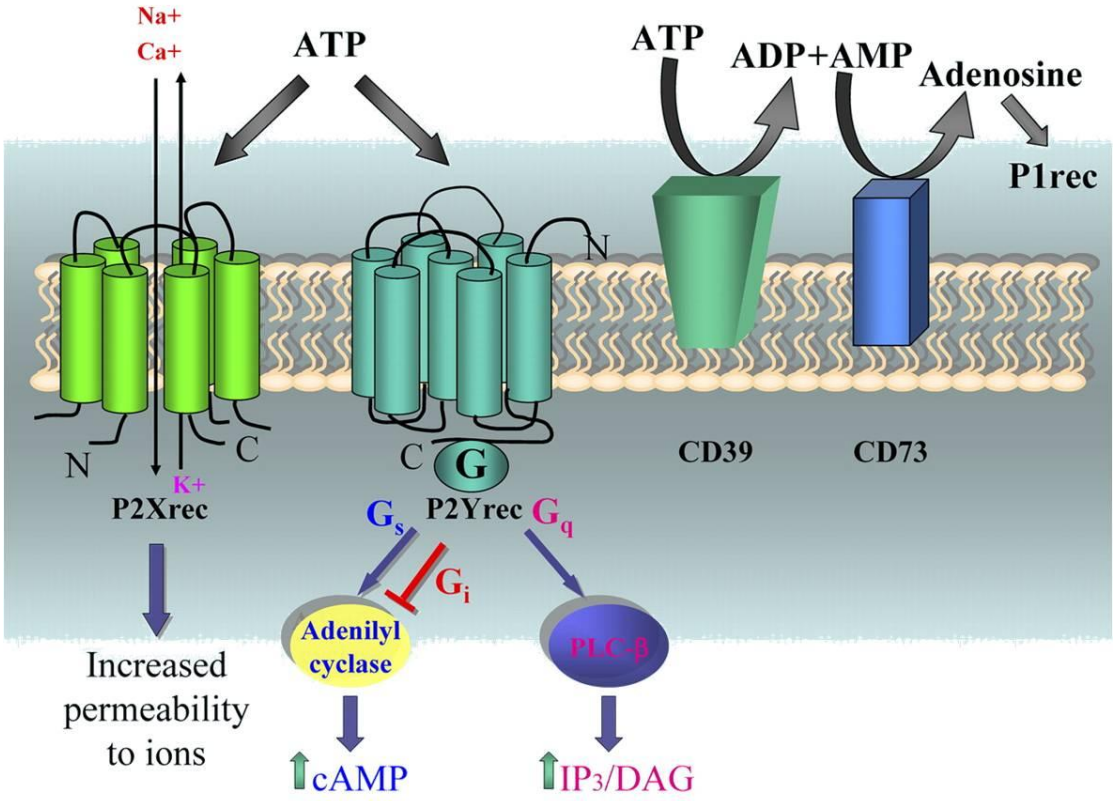

Figure 1. Purinergic receptors. Extracellular ATP exerts its effects on cells by binding to type 2 purinergic receptors. A prototypic P2X receptor is shown. ATP can bind P2Y receptors, which are 7 membrane-spanning, G-protein–coupled receptors. ATP in the extracellular space is metabolized by the plasma membrane-bound ectoenzyme CD39 that catalyzes the hydrolysis of ATP into ADP/AMP and CD73, which subsequently hydrolyzes AMP into adenosine. (from Vitiello L. et al., 2012 [54]).

P2X receptors are ligand-gated ionotrophic channels; receptor activation leads to cationic fluxes trough the plasma membrane for monovalent and divalent cations [55], [56]. P2X receptors are characterized by two hydrophobic transmembrane domains (TM1 and TM2), and a large extracellular region composed, with 10 cysteins and 2-6 sites of glycosilation [57]. Both C-terminal and N-terminal ends are cytoplasmic (Figure 2). Upon activation P2X subunits aggregate to form homo- or in some cases heteromultimers and trigger cation flux, plasma membrane depolarization and increase of intracellular Ca^{2+} concentration [58].

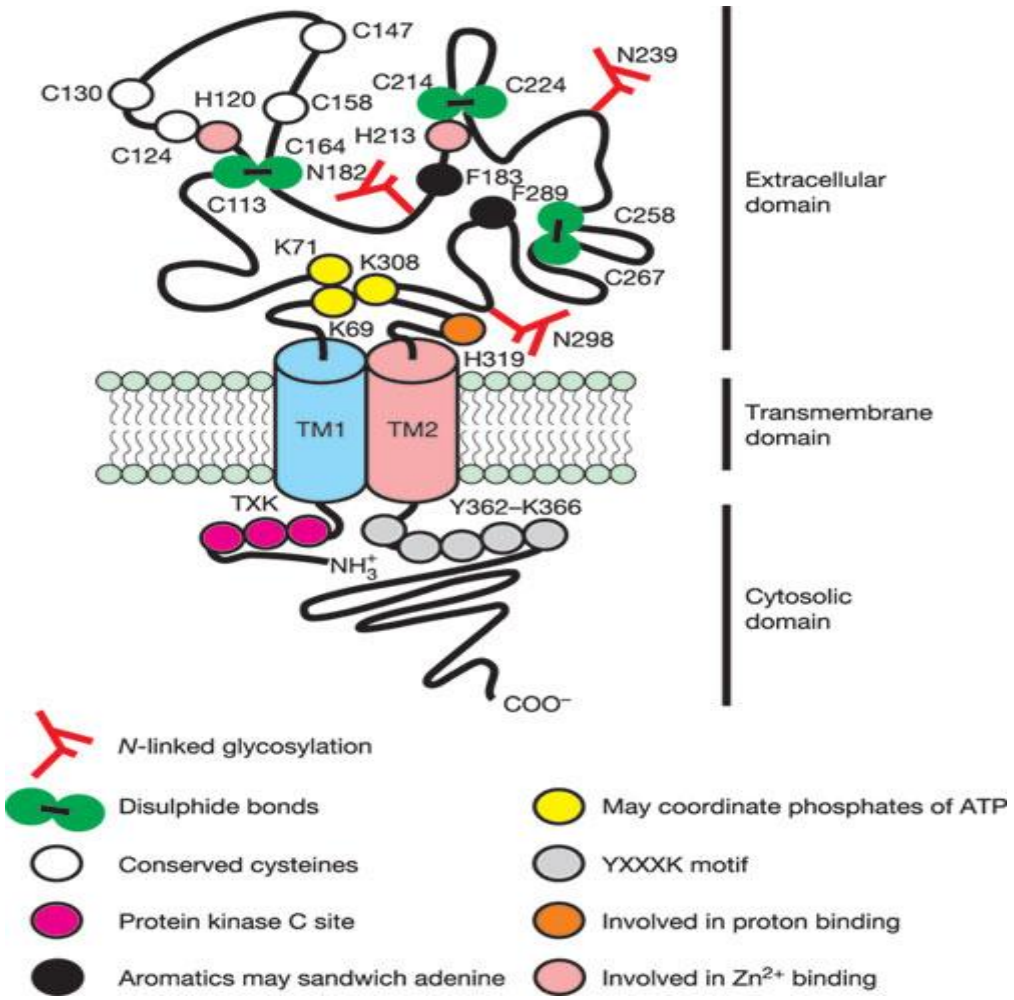

Figure 2. Structure of P2X Receptors. Topology and key features of P2X receptor subunits. In cyan is represented the transmembrane 1 (TM1) and in pink the transmembrane region 2. The cysteine residues that form the disulfide bridges are marked in green, the three sites of N-glycosylation (red) and the binding site with ATP is marked in yellow (from Khakh BS et al., 2006 [57]).

Amino-terminal domain is constituted by twenty-thirty residues and is similar in all P2 subtypes. On the contrary the carboxyl-terminal has a considerable variability in length. Mutations or deletions in the C-terminal domain affects kinetics, permeability and desentization of the ionic channel [59]. The extracellular domain contains two disulfide-bonded loops (S-S) and three N linked glycosyl chains. Between Lys 69 and Lys 71 is localized the ATP binding site. The P2X subfamily is composed of seven members named P2X1-P2X7 [50]. P2X2, P2X4 and P2X7 receptors can undergo a channel to pore transition and have two opening states [60]. The physiological agonist for P2X receptors is ATP, but this subfamily can also be activated by several synthetic pharmacological analogues.

2.1 P2X7R Receptor

P2X7 receptors is one of the most interesting member of the P2X family. P2X7 was cloned in 1996 [61] and was included in the P2X subfamily because, under stimulation in physiological conditions, is selectively permeable to small cations. Furthermore, prolonged stimulation with ATP, is able to induces channel to pore transition (Figure 3), and trigger permeability increase to small molecules as well as ions [62]. The P2X7R gene is made of 13 exons and is located on chromosome 12q24. P2X7R is expressed by various cell types such as neurons, dendritic cells [63], macrophages [64], microglial cells [65], fibroblasts, lymphocytes [66] and endothelial cells the protein of P2X7 receptor can be over-expressed under stimulation with inflammatory mediators like INF-γ, TNF-α and LPS. These observations suggest an involvement of the receptor in inflammatory response. Stimulation with high concentration of ATP or for prolonged period, have been shown to induce apoptosis [67]. P2X7R is also over-expressed by several malignant tumors [68], [69] and it has been shown to induce proliferative effect on T-cells [70]. Over the last years evidence has accumulated in support of an important role of P2X7R as an immunomodulatory receptor involved in IL-1β maturation and release, Ag presentation, graft-versus-host reaction and contact hypersensitivity [71]. The P2X7R receptor is composed by 595 amino acids. The structurally protein is similar to other P2X receptors except for the longer intracellular C-terminal (240 amino acids). P2X7R does not readily desensitize, therefore the pore remains open as long as the agonist is present in the extracellular environment. The molecular mechanism leading the pore formation is still unclear. Surprenant and colleague in 1996 demonstrated the importance of the long C-terminal intracellular domain of which at least the last 177 amino acids are crucial for the induction of the non-selective pore [61]. In human B and T lymphocytes P2X7R stimulation induces Na^+ and Ca^{2+} flux through the plasma membrane and increased permeability to ethidium bromide (314 Da) [72]. In lymphocytes, P2X7R stimulation may cause the formation of a smaller pore than in other cells, permeable to molecules of MW up to 320 Da. Experiments with P2X7R truncated expressed in HEK293 and in *Xenopus Laevis oocytes*, have further stressed the importance of the C-term for the formation of the pore. Channel function is not affected if the receptor is truncated at 380 position. All these data suggest that unlike the pore, the channel formation requires only a short portion of the C-term [73].

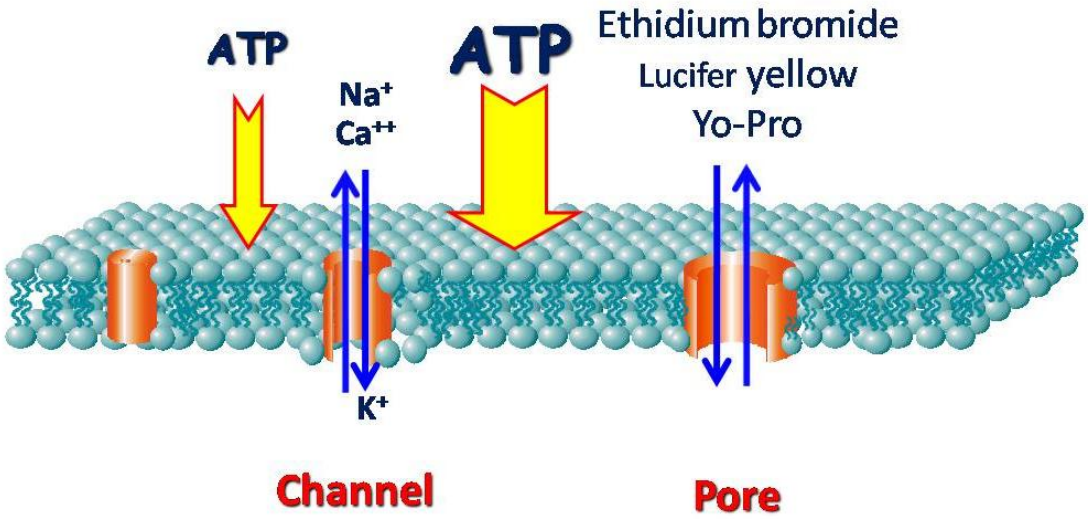

Figure 3. P2X7R Receptor stimulation. In presence of ATP, P2X7R mediates Na^+ and Ca^{2+} influx and K^+ efflux through plasma membrane. Repeated stimulation with the agonist or higher concentrations of the same, result in the opening of a pore that allows the passage of molecules up to 900 Dalton.

2.2 P2X7R in leukemia

In the recent years most studies have been so far concentrated on the effect of purinergic signaling on tumor associated immune response, this is due to the strong effect that nucleotides and nucleosides have on tumor cells [74]. It is known that P2X7R activity depends on the presence of spontaneously ATP released in the milieu by tumor cells, and this observation was confirmed by Morrone and collaborator showing that rats with implanted glioma co-injected with apyrase had a significant reduction in tumors growth [75]. It has been demonstrated that the transfection of P2X7R into human lymphoid cells lines that do not express the receptor sustains their proliferation in serum-free media [70]. This can confers a selective advantage for growth. P2X7R expression has been described in human leukemic lymphocytes, where indirect evidence for a role of P2X7R in proliferation came from the finding that B lymphocyte from patients affected by aggressive variant of chronic lymphocytic leukemia expressed higher level of the P2X7R than patients with indolent variants or healthy controls [68]. In addition there is evidence to suggest that the expression of P2X7R can mediate cell death or proliferation depending on the level of activation [68]. Furthermore In pediatric acute leukemia, P2X7R is overexpressed compared with normal cells [76]. Similarly, Zhang showed a higher expression of P2X7R in acute myeloid leukemia (AML) patients compared to normal controls [77]. In acute lymphoblastic leukemia (T-ALL) elevated expression of functional P2X7R was detected,

at late stage of mouse T-ALL [78]. Elevated expression of functional P2X7R contributed to the massive apoptosis of macrophages at late stage of leukemia. The intercellular communication mediated by nucleotides might orchestrate in the pathological process of leukemia and could be a potential target for the treatment of leukemia [78]. These observations suggest that P2X7R is over-expressed in different types of Leukemia, and this gives a proliferative advantage.

3 Inflammasome

The inflammasome is a molecular platform of approximately 700 kDa, activated upon cellular infection or stress that triggers the maturation of proinflammatory cytokines such as interleukin-1β. It is responsible for the activation of caspases 1 and is able to cause the maturation and secretion of pro-inflammatory cytokines such as IL-1β and IL-18. The inflammasome can be activated by different signals, such as infection or cellular stress that lead to the recruitment of innate immune defenses [79]. The innate immune system engages an array of germline-encoded pattern-recognition receptors (PRRs) to detect microbial conserved motifs. PPRs are expressed by cells at the front line of defense against infection, including macrophages, dendritic cells, monocytes epithelial cells and neutrophil cells as well as in cells adaptive immunity. Immune cells are responsible for the identification of "pathogen associated molecular patterns" (PAMPs). The PRRs include Toll-like receptors (TLRs) and C-type lectins (CTLs), which scan the extracellular medium and endosomal compartments to detect the presence of molecular profiles expressed by pathogens (PAMPs) such as LPS, flagellin, peptidoglycan, dsRNA [79]. PAMPs are recognized first by the host cell, and are potent innate immune stimuli. They are referred to as exogenous signals of danger. However cellular stress or tissue damage can trigger inflammation even in the absence of pathogens. In different studies it has been suggested that the immune system cells are able to react to the release, induced by damage, of molecules that are normally present within the cell. These molecules are part of a widespread surveillance system designed to alert the organism in the presence of damage and are of endogenous danger signals, called "damage-associated molecular patterns" (DAMPs). PAMPs and DAMPs work closely together and give rise to an efficient immune response [80], [81]. The Toll-like receptors (TLRs) are the best characterized family among the pattern recognition receptors (PRRs). Once activated, they start a cascade of inflammatory reactions that culminate in the activation of transcription factors, including

NF-kB and AP-1, which in turn induce the production of proinflammatory cytokines and transcription factors, members of the IRF family, that mediate type I interferon (IFN)-dependent antiviral responses. Recently other PRRs families including the "NOD-like receptors" (NLRs) have been described. Phylogenetic analysis reveals 3 distinct subfamilies within the NLR family: the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA), the NLRPs (NLRP1-14, also called NALPs) and the IPAF subfamily, consisting of IPAF (NLRC4) and NAIP (Figure 4 A). The phylogenetic relationships between subfamily members (Figure 4 A) are also supported by similarities in domain structures (Figure 4 B). This is particularly clear for the NLRPs, which all contain PYD, NACHT, and LRR domains, with the exception of NLRP10, which lacks LRRs [79].

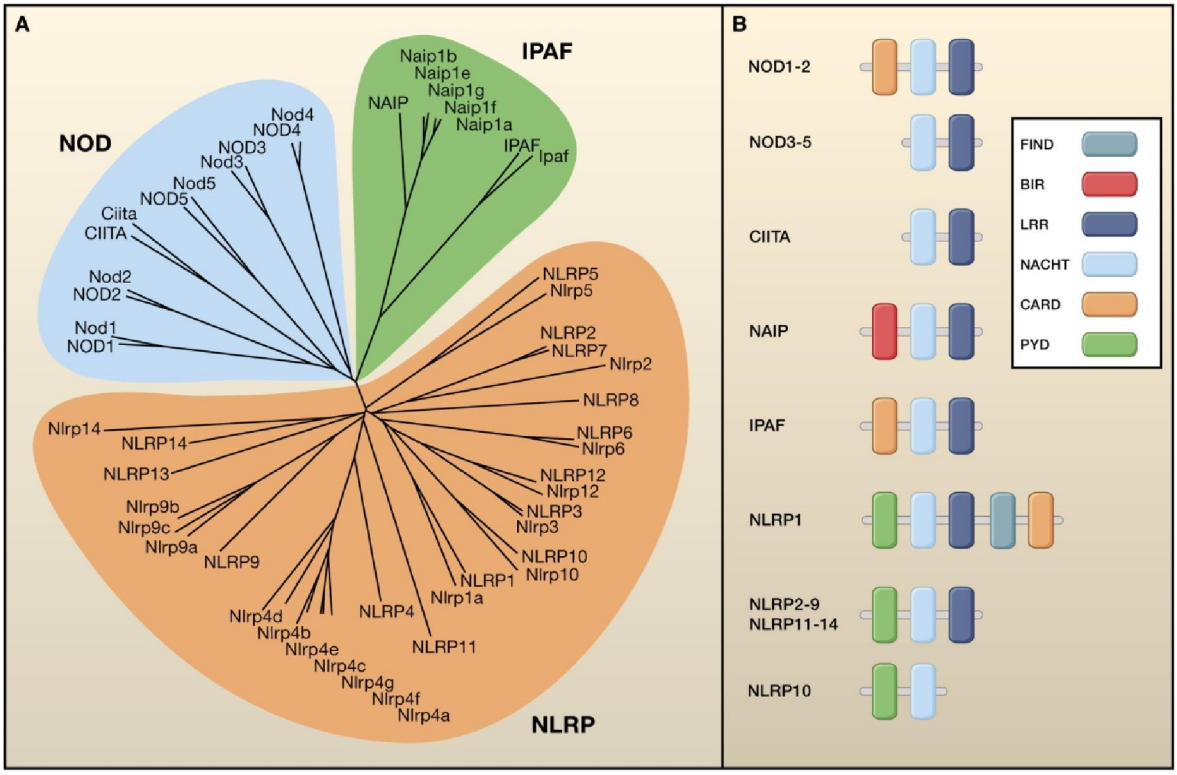

Figure 4. Human and Mouse NLR Family Members. (A) Phylogenetic relationships between NACHT domains of each human (uppercase) and mouse (lowercase). (B) Domain structures for human NLRs reveal commonalities within the subfamilies. Domains are classified according to the NCBI domain annotation tool for the longest human protein product, with the exception of the FIIND domain that was identified independently of NCBI ([82]). Domains: BIR, baculoviral inhibition of apoptosis protein repeatdomain; CARD, caspase recruitment domain; FIIND, domain with function to find; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerizationdomain; PYD, pyrin domain. Figure from Schroder K. et al., 2010 [79].

The NLR family is characterized by the presence of a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. LRRs are believed to function in ligand sensing and autoregulation, whereas CARD and

PYD domains mediate homotypic protein-protein interactions for downstream signaling. The NACHT domain, which is the only domain common to all NLR family members, enables activation of the signaling complex via ATP-dependent oligomerization. The LRR domain is characterized by the presence of several repetitions (2 to 40) of a typical region LXXLXL that is responsible for the interaction with the bacteria and harmful chemical agents [83], [84], [85]. The N-terminal motif is designated for the recruitment of caspase-1 or caspase-5 based on the type and species of inflammasome (caspase 5 is not present in mouse).

3.1 NLRP3 Inflammasome

The inflammasome NLRP3 has become the center of interest for its ability to modulate cellular responses to a wide range of exogenous or endogenous harmful agents. The NLRP3 inflammasome is probably the most versatile inflammasome subtype poised to react to a multiplicity of potentially harmful biologic and chemical agents and so far is the best characterized molecular trigger of IL-1β and IL-18 maturation and release. The NLRP3 protein contains a central NACHT, a series of C-terminal LRRs, and a N-terminal PYD. The NACHT domain allows ATP-dependent oligomerization, and the LRR domain is thought to be responsible for PAMP/DAMP sensing and autoinhibition, while PYD mediates homotypic protein-protein interaction. It is thought that LRRs fold on the NACHT domain, thus preventing oligomerization and subsequent activation. Keeping NLRP3 in an inactive state may be helped by interaction with the chaperone proteins SGT1 and HSP90 [86], [87]. Upon stimulation, NLRP3 is activated and via its own PYR domain interacts with PYR domain present on the adapter protein ASC. The scaffold protein NLRP3 lacks a CARD sequence; therefore, it has to rely on ASC to recruit caspase-1.The interaction of the ASC CARD domain with the same domain of pro-caspase-1 enzyme promotes self cleavage and the formation of active caspase-1. Subsequently immature forms of the cytokines IL -1β and IL-18 are processed into their mature form and secreted [79] (Figure 5).

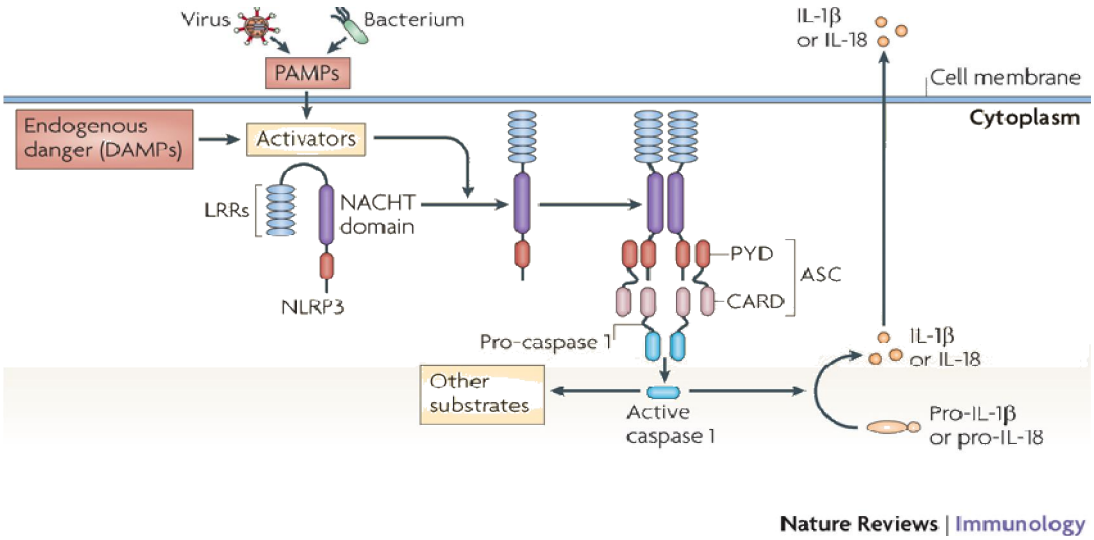

Figure 5. Mechanism of NLRP3 complex formation. Under healthy cellular conditions, NLRP3 is auto-repressed owing to an internal interaction between the NACHT domain and LRRs. In the presence of PAMPs the auto-repressed is removed from microorganism or DAMPS from endogenous danger signals. This result in exposure of NACHT domain. In turn, NLRP3 oligomerizes and recruits ASC and pro-caspase-1, triggering the activation of caspase-1 and the maturation of IL-1β and IL-18. (Figure from Tschopp J. et al., 2010 [88]).

Many different PAMPs and DAMPs activate -the NLRP3 inflammasome: LPS, MDP, PGN, bacterial and viral RNA, viral DNA, the dinoflagellate toxin maitotoxin, ATP, monosodium urate (MSU), calciumpyrophosphate dihydrate (CPPD), amyloid β (Ab), cholesterol crystals, the glycosaminoglycan hyaluronan, alum, asbestos, silica, and metal alloy particles [89], [90]. Furthermore, physical agents such as ultraviolet radiation can also stimulate the NLRP3 inflammasome.

3.2 Models for NLRP3 Inflammasome activation

The sequence of molecular events that lead to the NLRP3 activation has not been completely elucidated. In the literature, three distinct models of activation have been proposed (Figure 6).

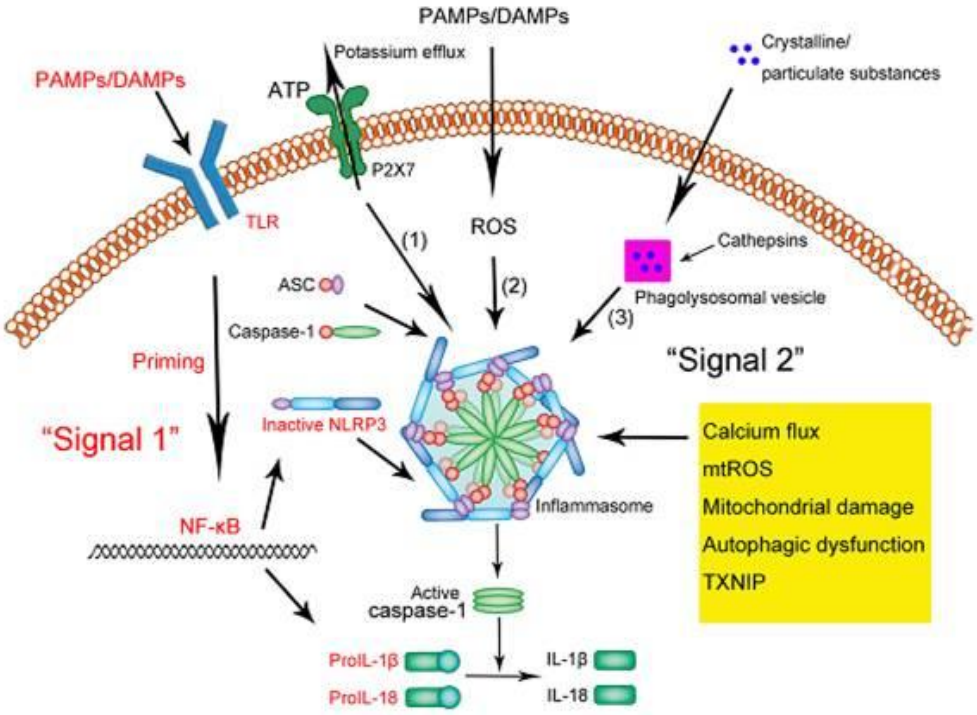

Figure 6. Schematic illustration of the NLRP3 inflammasome activation. Three models have been proposed to describe the inflammasome activation. (from Shao B.Z. et al., 2015 [91]).

1. The first model suggests that extracellular ATP, released from damaged tissue, stimulates the purinergic receptor P2X7R [92], triggering K^+ efflux and inducing gradual recruitment of the pannexin-1 membrane pore [85]. Potassium efflux leads to calcium influx and activation of various phospholipases involved in the sequence of events culminating in IL-1β maturation and secretion [93], [94].

2. The second model has been proposed for activators forming crystalline or particle structure, such as MSU, silica, asbestos, alum and β-amyloid aggregates [95]. Based on this model, the inefficient elimination of these particles, internalized by phagocytes, leads to phagosomal destabilization and release of lysosomal contents into the cytosol. NLRP3 can sense phagosomal or lysosomal disruption as a danger signal and thereby indirectly sense excessive crystal phagocytosis or escape of microbes from endo-lysosomal compartments into the cytosol. The exact nature of ligand formed after lysosomal damage remains unknown. Lysosomal contents could be a danger signal sensed by NLRP3, or lysosomal proteases released in the cytosol could cleave a substrate, which, in turn, could be a ligand for the inflammasome. A role in this process for cathepsin B, a lysosomal protease, has been suggested [96]. The inhibition of this protease led to a substantial decrease of IL-1β maturation and cathepsin B-deficient macrophages show partially reduced IL-1β secretion upon NLRP3 activation. In addition, the activation of pannexin-1

pores might, not only induce K^+ efflux or microbial products access in the cytoplasm, but also destabilize lysosomes and lead to release of lysosomal content into the cytoplasm [97].
3. The third model proposed that reactive oxygen species (ROS) act on a target upstream of the NLRP3 inflammasome and indirectly cause its activation. The involvement of redox reactions in inflammasome activation has been suggested by several studies. Many known NLRP3-activating stimuli, such as ATP, MSU and alum, generate ROS. Although consensus exists on the importance of the redox pathway in inflammasome activation, how and by which ROS are generated and their role in specific activation of caspase-1 by pathogen or damage associated molecular patterns remain unclear. It is believed that NADPH oxidase or mitochondria could be involved. ROS generation is frequently associated with potassium efflux, demonstrating that the two models can be connected [97], [88]. ROS might induce directly the assembly of inflammasome or indirectly via cytoplasmatic proteins that modulate inflammasome activity. It has been demonstrated that the ROS production, ATP mediated, led to PI3K-pathway activation and that pharmacological inhibition of PI3K results in prevention of caspase-1 activation induced by ATP [98], [99]. Mechanism directing ROS-dependent NLRP3 inflammasome activation remain to be characterized in details; however Zhou and co-worker have identified thioredoxin (TRX) -interacting protein (TXNIP) as a redox-sensitive regulator of inflammasome activation induced by MSU. In normoxic homeostasis TXNIP is bound to TRX and inhibits the reducing activity of TRX; after oxidative stress, the TXNIP-TRX complex dissociates and the unbounded TXINP interacts with NLRP3. This interaction is necessary for inflammasome activation in response to oxidative stress in vitro. Accordingly, $Txnip^{-/-}$macrophages treated with ATP or MSU crystals show less caspase-1 maturation and IL-1β production [100]. The role of ROS in the activation of inflammasome and IL-1β maturation and release is controversial. Tassi and collaborators proposed that IL-1β maturation and release are regulated by a biphasic redox response, characterized by two stages: the oxidative stress and then the antioxidant response. They demonstrated that different PAMPs induce the generation of ROS by NADPH oxidase in human monocytes. Subsequently, an antioxidant pathway is engaged to abolish the oxidative stress, via up-regulation of enzymes, such as thioredoxin, and a high increase of activity of cystine/cysteine redox cycle. Antioxidant response is slightly delayed compared to ROS generation and is concomitant to IL-1β release. The oxidative stress could be necessary to induce the antioxidant response and is essential for IL-1β cleavage and release; in fact, the reduction of extracellular medium with exogenous reducing agents (e.g. DTT) rescues IL-1β release in the presence of inhibitors of ROS production [101].

These three models for NLRP3 inflammasome activation are different, but considering these as being exclusive of one another is probably an oversimplification, as crosstalks among these pathways probably occur. It is possible that ROS generation contributes to lysosomal destabilization and that bacterial products indirectly contributes to NLRP3 activation [102]. It is also reasonable that the activation of NLRP3 is a multistep process that requires more than one factor. Recent studies have demonstrated that priming via transcriptionally PRRs or via cytokines is required for subsequent activation of NLRP3 inflammasome by ATP, pore-forming toxins, or crystals in macrophages. These studies have demonstrated that cell stimulation via PRRs or cytokine receptors leads to high cytosolic levels of NLRP3; when a critical NLRP3 concentration was reached, NLRP3 inflammasome assembly was induced by stimulation with ATP, nigericin or crystalline activators. Many of the reported NLRP3 stimuli are not direct activators, but rather they are required for the transcriptional regulation of the inflammasome components.

3.3 NLRP3 Inflammasome and cancer

Two families of receptors of innate immunity, the Toll-like receptors (TLRs) and NOD-like Receptors (NLRs), have emerged as mediators for homeostasis of gastro-intestinal inflammation. NLRP3, is important for prevention of colitis associated colorectal cancer (CAC) development in the azoxymethane and dextran sodium sulfate (AOM-DSS) model [103], [104], [105]. Nlrp3 knock out (KO) mice had increased polyp numbers and size and worsened pathology compared to wild type mice. This phenotype was also seen in Asc and caspase-1 KO mice, indicating that the NLRP3 inflammasome is important in suppressing CAC development. Principally, it was shown that the presence of NLRP3 in hematopoietic cells was needed for the tumor-suppressing effect in response to AOM-DSS challenge [104]. In addition, IL-18 levels were dramatically reduced in the colon of Nlrp3 KO and caspase-1 KO mice. Treatment of caspase-1 KO mice with recombinant IL-18 led to a reduction in disease demonstrating a crucial role of IL-18 in protection against CAC development [103], [105]. Partial improvement of disease in caspase-1 KO by administration of exogenous IL-18 was also demonstrated in a separate study [106]. In agreement with these data, Myd88 KO, Il-18 KO, and Il-18r KO mice were also shown to be more responsive to DSS-induced colitis and colon cancer [107]. IL-18 was proposed to have a role in IFN-γ mediated activation of STAT1, which is known to have a role in tumor suppression [108]. These data imply that the absence of NLRP3 results in decreased IL-18 production and STAT1 signaling that are necessary for protection against CAC

potentially by playing a role in epithelial repair. Treatment with recombinant IL-18 did not fully alleviate symptoms indicating that some other mechanisms may be at play. It should be noted that in another study utilizing the AOM-DSS model, Nlrp3 KO mice exhibited a similar tumor load as WT mice [109]. The difference in phenotypes may be attributed to alterations in mouse intestinal microbiota or differences in experimental procedures. Although, either NLRP3 is important for protection in colitis-associated cancer still remains unclear. In contrast to the protective role of NLRP3 in CAC, NLRP3 was shown to promote tumor formation in a chemical-induced fibrosarcoma model [110]. Nlrp3 KO mice treated with methylcholanthrene (MCA) exhibited prolonged tumor-free survival compared to wild-type mice. In concert with this data, Nlrp3 KO mice challenged intravenously with B16F10 melanoma and RM-1 prostate carcinoma cells had significantly fewer metastasis compared to wild-type mice. Reduced pulmonary metastasis was also seen in Nlrp3 KO mice using orthotopic transplant of E0771 mammary adenocarcinoma cells [110]. The decrease in tumor formation was attributed to an increase in natural killer (NK) cells and CD11b+Gr-1int myeloid cells seen in Nlrp3 KO mice. The CD11b+Gr-1int myeloid cells secreted CCL5 and CXCL9 that were important for recruiting NK cells into the tumor microenvironment [110], demonstrating a role for NLRP3 in the suppression of NK cell activation and promotion of a suppressive tumor environment. In an interesting in vitro study of late stage metastatic melanoma cell lines, HS294T and 1205Lu cells were shown to constitutively produce IL-1β in culture [111]. In contrast, non metastatic melanoma cell lines secreted less or no IL-1β. HS294T and 1205Lu cells also expressed NLRP3, ASC, and caspase-1 and secreted factors that resulted in increased macrophage chemotaxis and angiogenesis [111]. Other recently studies have been conducted, where they observed a down-regulation of NLRP3 inflammasome connection in hepatocellular carcinoma (HCC). Hepatocellular carcinoma is one of the most common malignancies in worldwide, and is mostly the result of chronic hepatitis and liver cirrhosis. This study was conducted to investigate the role of NLRP3 in the development and progression of HCC. The expression of NLRP3 inflammasome components were analyzed in the tissues of liver carcinoma and corresponding liver tissues healthy. The data show that the expression of all components of NLRP3 inflammasome connection was either completely lost or significantly down-regulated in human HCC, and that deregulation significantly correlates with pathological advanced stages. In fact the expression of NLRP3 in normal liver tissue was low, and the deregulation of NLRP3 in malignant parenchymal cells indicate the loss of the protective role of NLRP3 inflammasome connection [112]. Finally, in another tumor model NLRP3 is down modulated in Malignant Mesothelioma (MM) compared with the

cell line control; and chemotherapeutic drugs can activate NLRP3 inflammasomes in MM tumors resulting in caspase activaction and increase cell death [113]. Westbom and collaborators suggest that a combination of chemotherapeutic drugs and IL-1R antagonist could be a possible strategy for inhibiting MM tumor growth [113]. These data merit further study on the role of inflammasome activation in metastatic tumor cells, and how inflammasome activation is altered in tumor cells as they progress from non-metastatic cells to ones with metastatic capacity.

4. AIM OF WORK

NLRP3 inflammasome in recent years it is a focus of interest for its ability to modulate cellular responses to a wide range of exogenous and endogenous harmful agents. The most important plasma membrane receptor responsible for activation of NLRP3 inflammasome is the P2X7R. Which is the mechanism between P2X7R is able to recruiting NLRP3 at the intracellular sites is still unclear.

The most important pathophysiological response triggered by activating the P2X7R/NLRP3 axis is the release of pro-inflammatory cytokine IL-1β. We have previously shown that P2X7R is overexpressed in peripheral lymphocytes of patients with Chronic Lymphocytic Leukemia [68]. In addition, in vivo studies show that the overexpression of P2X7R accelerates while its down-modulation inhibits tumor growth [114]. Recently it has been shown that the constituents of NLRP3 inflammasome play a protective role in acute and recurrent forms of colitis and even in cancer associated with inflammatory bowel disease. In fact on mouse models have shown that in case of low levels of il-18 and il-1β in tumor site due to the absence of NLRP3, occurs higher growth of the tumour [104]. In the present study we investigated the P2X7R/NLRP3 inflammasome axis in CLL lymphocytes from a cohort of 23 CLL patients. P2X7R, ASC and NLRP3 were investigated by Western blot, PCR and transfection techniques. To further investigate the correlation between P2X7R, NLRP3 and cell growth, we silenced NLRP3 in the leukemia cell line THP-1, that natively expresses both NLRP3 and P2X7R. We also used a cellular model, HEK293, which does not express NLRP3 and P2X7R, in order to verify the contribution of NLRP3 to proliferation. We performed overexpression experiments in two human leukemia cell lines, Ramos and THP-1 to clarify the role of NLRP3 in cell death by apoptosis.

In addition, the role of NLRP3 in cell death and proliferation was further investigated in cells from Nlrp3-KO mouse.

5. MATERIALS AND METHODS

5.1 Cell cultures and transfections

Ramos and THP-1 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Celbio, Euroclone, Milano, Italy). NLRP3-silenced THP-1 cell clones were transfected by electroporation with Neon® Transfection System for Electroporation (Lifetechnology, Grand Island, NY, USA) with predesigned HuSH-29 targeting the following sequences:

-AGAGAAGGCAGACCATGTGGATCTAGCCA (shRNA1, c.n. GI321521);

-CAGTCTGATTCAGGAGAACGAGGTCCTCT (shRNA2, c.n. GI321522);

-GTACGTGAGAAGCAGATTCCAGTGCATTG (shRNA4, c.n. GI321524);

or with scrambled RNA cassette (c.n. TR30013), all from OriGene (Rockville, MD, USA). Stably transfected cell lines were obtained by selection with puromycin (0.5-1 μml/L, Sigma-Aldrich, Milan, Italy).

HEK293 were cultured in DMEM-F-12 (Sigma-Aldrich) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. HEK293-NLRP3 cells and HEK293 GFP were obtained by transfection with lipofectamin 2000 (Lifetechnologies) as per manufacturer's instructions. NLRP3 plasmid (c.n. RG220952) and the empty vector plasmid (c.n. PS10010) carried the GFP used as a control were provided by OriGene. Stably-transfected cell lines were obtained by selection with G418 sulphate (0.8 mg/ml) (Calbiochem, La Jolla, CA, USA). Single cell-derived clones, were obtained by limiting dilution.

For transient overexpression experiments, 6 x 10^6 of Ramos and THP-1 were plated in 6-well plates and were transfected by electroporation system. We used the NLRP3 plasmid obtnaied from Origene, and a pCMV6-AC-GFP as a empty vector. Experiment were performed 48 h later.

Cells were plated in 75 cm^2 Falcon flasks (Microtech, Naples, Italy) and incubated at 37°C in humidified incubator in the presence of 5% CO_2.

5.2 Patients

We analyze 23 CLL patients (13 males and 10 females), aged between 46 and 84 years, enrolled by the Hematology Clinic of Ferrara University Hospital. Clinical characteristics of patients are listed in Table I. In particular, for each patient we reported: the number of white blood cells (WBCs), the number of lymphocytes, mutational status of IgVH genes, the expression of ZAP-70, the value of β2-microglobulin, the positive or not for the CD38 marker and the presence or not for chromosome 12 trisomy.

All patients gave their informed consent. All experimental protocols were approved by the Ethical Committee of the Ferrara University Hospital and all experiments were performed in accordance with relevant guidelines and regulations.

5.3 PBMC and lymphocytes isolation from CLL patients and healthy donors

Blood samples from CLL patients and healthy donors were obtained from the Section of Hematology, S.Anna Hospital, Ferrara, or from the local blood bank. B lymphocytes and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood. From a sample of heparinized venous blood, mononuclear cells were obtained proceeding with a layering on Ficoll/Hypaque (F/H) (GE Healthcare Life Sciences, Milan, Italy). This method exploits the difference of density of mononuclear cells (lymphocytes and monocytes) with respect to the other elements of the blood. Mononuclear cells, which have lower density, focus on the layer of F/H while the red blood cells and granulocytes are collected on the bottom of the tube. Considered the high WBC count in CLL patients, peripheral blood was first diluted in 1:2 ratio with PBS (Celbio) at room temperature, gently agitated, and later layered slowly over F/H solution. We proceeded with a centrifugation at 900g for 20 min at 20°C, without brake. The ring of mononuclear cells formed at F/H interface was aspirated and subjected to three successive washes with PBS by centrifugation at 400g for 10 minutes at 20°C (Figure 6). The pellet was resuspended in a adequate amount of RPMI medium and the cells were counted in a Burker chamber.

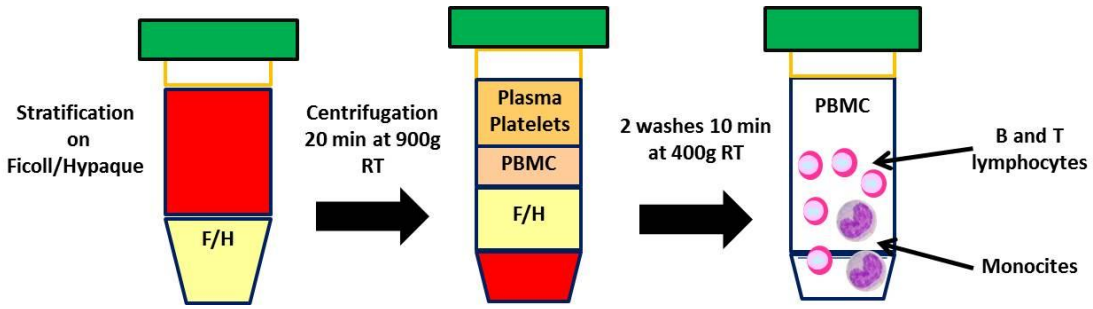

Figure 6. **Isolation of mononuclear cells from peripheral blood by stratification on Ficoll/Hipaque.** By centrifugation on F/H, mononuclear cells were isolated from peripheral blood. Mononuclear cells and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cells (RBC) and granulocytes (PMN) have a higher density than the F/H and collect on the bottom of the tube.

5.4 B lymphocytes isolation from healthy donors

Blood samples of healthy individuals were obtained through the blood bank of the University of Torino. Peripheral blood mononuclear cells (PBMCs) were obtained as described above. B lymphocytes were purified through a first step of negative selection, using anti-CD3, anti-CD16, and anti-CD14 monoclonal antibodies (produced locally) and immunomagnetic bead separation Dynabeads Sheep anti-Mouse IgG (Life Tecnologies), followed by positive purification using the CD19 microbeads kit [iltenyi Biotec S.r.l., Caldererara di Reno (BO), Italy]. Flow cytometric analysis showed that these cells were more than 95% $CD19^{+}$.

Table 1: Clinical characteristics of analyzed B-CLL patients

ID PATIENT	SEX	AGE	CD 38	ZAP 70	IgVH	WBC/mm^3	LYMPHOCYTES/mm^3	β2-microglobulin mg/L	TRISOMY 12
CLL 01	M	62	+	+	unmutated	12050	5330	5,6	+
CLL 02	F	70	+	-	unmutated	19360	14470	3,6	-
CLL 03	M	84	-	-	unmutated	74040	66640	4,04	-
CLL 04	F	58	+	+	unmutated	14160	8830	5,6	+
CLL 05	F	70	+	+	unmutated	79430	65550	4,1	-
CLL 06	M	62	-	-	mutated	98780	94400	2,04	-
CLL 07	M	61	-	+	unmutated	13470	8690	4,6	-
CLL 08	F	77	+	+	unmutated	15900	9920	6,6	+
CLL 09	F	68	+	+	unmutated	21950	18440	9	+
CLL 10	M	54	+	-	mutated	13700	7110	2,7	+
CLL 11	M	64	-	+	unmutated	14000	10220	3,1	+
CLL 12	M	75	+	+	unmutated	34310	22490	4	-
CLL 13	F	79	+	+	unmutated	14800	7460	2,3	+
CLL 14	F	74	-	-	mutated	39000	29220	2,5	-
CLL 15	F	77	+	-	unmutated	14160	8300	5,3	+
CLL 16	M	54	-	+	unmutated	12452	108330	2,2	+
CLL 17	M	72	-	-	mutated	36850	27070	6,2	-
CLL 18	M	79	-	+	unmutated	17477	16401	11	-
CLL 19	F	69	+	+	unmutated	7520	5930	2	-
CLL 20	M	70	-	+	unmutated	20810	12960	2,2	-
CLL 21	F	65	+	+	unmutated	39360	31060	2	-
CLL 22	M	46	-	+	unmutated	15770	10450	3,2	-
CLL 23	M	84	+	+	unmutated	11956	9627	5	+

5.5 RNA isolation and qRT PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Life Technology, Grand Island, NY, USA), and then purified with the Pure link RNA Mini Kit (Invitrogen) according to manufactures instruction. RNA content was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Milan, Italy). Reverse transcription was performed by using 1 µg of total RNA extract, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Carlsbad, CA, USA). qRT PCR was performed in a Step One Real Time PCR System (Applied Biosystem).

Two µl of cDNA were used as a template. Amplification was performed using predesigned Taqman probes (Applied Biosystem) for P2X7R, NLRP3, ASC, casp-3, casp-9 and GAPDH as a housekeeping gene. A comparative analysis using the ΔΔCt method was used to quantitate fold increase of target cDNA relative to THP-1 wild type reference sample.

Gene	Assay ID	Description
P2X7R	Hs00175721_m1	Purinergic receptor P2X, ligand-gated ion channel 7
NLRP3	Hs00366461_m1	NLR family, pyrin domain containg 3
PYCARD	Hs00203118_m1	PYD and CARD domain containing
CASP3	Hs00234387_m1	Caspase 3, apoptosis-related cysteine peptidase
CASP9	Hs00154261_m1	Caspase 9, apoptosis-related cysteine peptidase
GAPDH	Hs99999905_m1	Glyceraldehyde-3-phosphate dehydrogenase

Table 2: List of the human target genes analyzed

Gene	Assay ID	Description
P2x7r	Mm00440578_m1	Purinergic receptor P2X, ligand-gated ion channel 7
Nlrp3	Mm00840904_m1	NLR family, pyrin domain containg 3
Gapdh	Mm99999915_g1	Glyceraldehyde-3-phosphate dehydrogenase

Table 3: List of the mouse target genes analyzed

5.6 Proliferation assay

For study the involvement of NLRP3 in cell proliferation in vitro we used THP-1 silenced for NLRP3 and HEK 293 GFP and NLRP3. Cell proliferation was assessed by direct cell counting. Cells were seeded in 6-well plates in complete medium at the concentration of $5x10^4$ cells/well. Pictures was taken every 24 hours until 96 hours. Four separate fields per well were captured and analyzed with ImageJ software (Wayne Rasband, NIH, Bethesda, USA). Proliferation rate was expressed as fold increase over time zero.

5.7 MTT assay

Growth rate of control and NLRP3-silenced cells was measured by MTT assay (Invitrogen). For MTT assay, 5 x 10^4 cells were plated into each well of a 96-multiwell plate. Twenty four, 48, 72 and 96 hours after plating 10 µl of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] was added to each well and incubated at 37°C for additional 4 h. Finally, 100 µl of SDS-HCl was added to each well to solubilize the precipitate. Optical density was measured at 544 nm and data were expressed as a fold increase of THP-1 scrambled control.

5.8 Interleukin-1β secretion measurement

PBMCs from CLL patients or healthy donors (HDs) (5x 10^4 cells/ml) were plated in a 24-multiwell plate in complete medium, and left untreated or primed with 1μg/ml of LPS (Sigma-Aldrich, Milan, Italy) overnight at 37°C, 5% CO_2. After stimulation the whole medium was collected and centrifuged at 160 g for 5 minutes, to remove any detached cells, followed by transfer of the supernatant to a fresh tube. Supernatants were stored at -80°C and assayed with Human IL-1 β, Quantikine ELISA (R&D Systems, Minneapolis, MI, USA).

5.9 Western blot analysis

Total cell lysates were prepared in a lysis buffer (300 μM sucrose, 1mM K2HPO4, 5,5 mM D-glucose, 20 mM Hepes) supplemented with proteases inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) (all by Sigma-Aldrich,Milano, Italy). Proteins were quantified using the Bradford method. Samples were loaded into 4-12% Bolt-SDS pre cast-gel (Life Technologies, USA), and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Milan, Italy). Membranes were incubated overnight with the primary Abs at 4 °C. The anti-P2X7R Ab (Sigma-Aldrich) was used at a dilution of 1:200 in TBS-t (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) supplemented with 3% non- fat milk (Biorad, Copenhagen, Denmark) and 0.5% BSA (Sigma-Aldrich). The anti-ASC Ab (MBL, Woburn, MA,USA) was diluted 1:200 in PBS supplemented with 1% skimmed milk. The anti-NLRP3 Ab (Abcam, Cambridge, UK) was diluited 1:500 in PBS. The anti-caspase-3 Ab (Cell Signalling, Milano, Italy) was diluited 1:1000 in TBS-t supplemented with 5% non-fat milk (Biorad). The anti-actin Ab (Sigma-Aldrich) was diluited 1:1000 in TBS-t. Membranes were incubated with secondary goat anti-rabbit or anti-mouse HRP-conjugated Abs (Biorad) at a 1:3000 dilution in TBS-t buffer, or in PBS-t (PBS supplemented with 0.1% Tween 20) for 1h at room temperature. Detection was performed with the Luminata™ Western chemiluminescent HRP kit (Merck S.p.A., Milan, Italy). Densitometric analysis of the protein bands was performed with ImageJ software. Band density was normalized over the actin band.

5.10 Measurement of Apoptosis

Forty eight hours after transfection of THP-1 and Ramos, with NLRP3 and GFP empty vector, cells were centrifuged 160 g for 5 minutes, washed with PBS (Celbio), and the cell pellet was resuspended in 500 µl of labelling medium containing 5 µl of Annexin V-Cy3 solution (Biovision, Milpitas,CA,USA). After 10 min of incubation, according to manufacturer's instructions, cell fluorescence was measured with Tali™ image-based cytometer (Invitrogen).

5.11 Isolation and differentiation of murine bone marrow macrophage

Bone marrow cell suspension were isolated by flushing femurs and tibia of 4-to 8-week old mice with complete RPMI 1640. The cell suspension was collected and washed with RPMI by 10-minute centrifugation at 160 g. Cells were incubated for 5 minutes at room temperature in red blood cell lysis buffer (Sigma-Aldrich). Reaction was stopped by adding RPMI, cells were centrifuged, resuspended in M-CSF–supplemented (200 ng/ml) RPMI, and seeded in Petri dishes at a concentration of 2.5×10^5cells/ml. After 7 days cells were detached using cold PBS supplemented with 2mM EDTA.

5.12 Isolation of murine spleen cells

Spleen were isolated from mouse C57BL/6 wild type and $Nlrp3^{-/-}$, and spleen cells were aseptically prepared. Spleen were homogenized by pulping. Single spleen suspension were collected and treated with red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes at room temperature for removed any erythrocytes present. After the suspension were blocked adding RPMI, cells were centrifuged for 10 minutes at 160 g, and passes through at 70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ). The cell suspension was washed twice with RPMI and then resuspended at $20*10^6$ cells/ml in culture medium.

5.13 MLR

Spleens of C57BL/6 wild type and $Nlrp3^{-/-}$ were aseptically removed and mechanically disrupted. The spleen cells were isolated as previously described. In 96-well were plated

$2*10^5$ per wells of spleen cells from C57BL/6 (responder) and an equal number of spleen cells obtained from BALB/CJ γ irradiated (3000 rad) (stimulator). The cultures were then incubated at 37°C in an atmosphere saturated with water vapor and containing 5% CO_2.

5.14 Cell viabilty assay using flow citometry

Spleen cells isolated as previously described were plated into 96-well plate at the density of $2*10^5$ cells per well. Cells after 72 and 96 hours were harvested and washed twice. Then cells were resuspended into 200µl of PBS and stained with 0.25µg/$10^{\wedge 6}$ cells 7-AAD (BD Pharmingen™) according to the manufacture instruction. At least 50,000 FACS events were collected on a FACS Calibur flow cytometer (BD, San Jose, CA) and data were analyzed using FlowJo Software (TreeStar, Ashland, OR, USA).

5.15 Statistical analysis

All data are shown as means ± standard error of the mean (SEM). Test of significance was performed by Student's t-test using GraphPad InStat software (GraphPad, San Diego, Ca, USA).

6. RESULTS

6.1 P2X7R is overexpressed in CLL patients

We previously demonstrated that PBMCs from CLL patients up-modulate P2X7R mRNA and protein in evolutive form compared to indolent form [68]. This observation suggested that the P2X7R might be involved in leukemogenesis, and might be a marker of the disease [74]. According to previously published data, RT-PCR result showed that the lymphocytes purified from CLL patients overexpresed P2X7R as compared to normal PBMC from healthy donors (HD), and HD B lymphocytes, that was considered the normal counterpart of CLL disease (Figure 7 A). Moreover, we observed that the highest expression of P2X7R was specifically detected on CLL patients both by RT-PCR and by Western blot analysis (Figure 7 B-C).

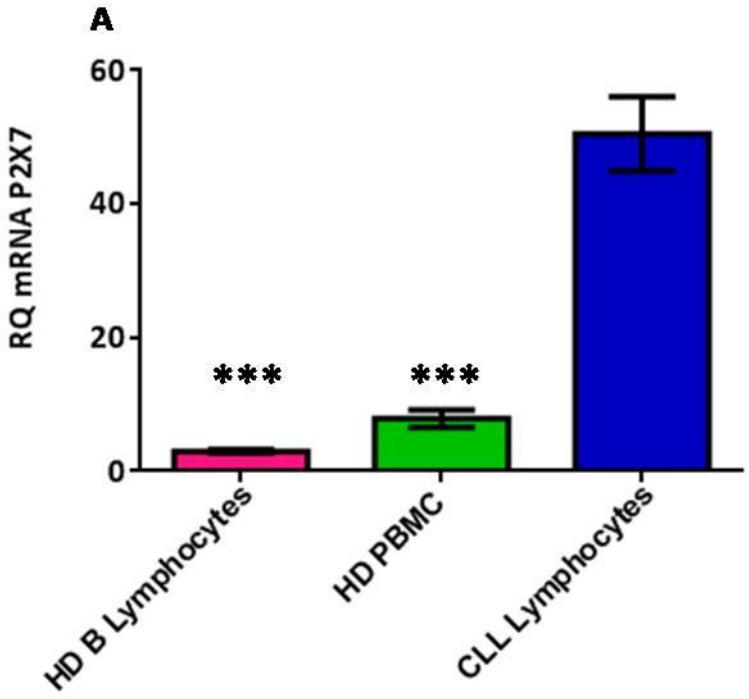

Figure 7. P2X7R expression in CLL patients and HD control. (A) Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH. P2X7R mRNA level was evaluated by Real-Time PCR. Data are show as mean ± SEM, n=3,***p<0.001.

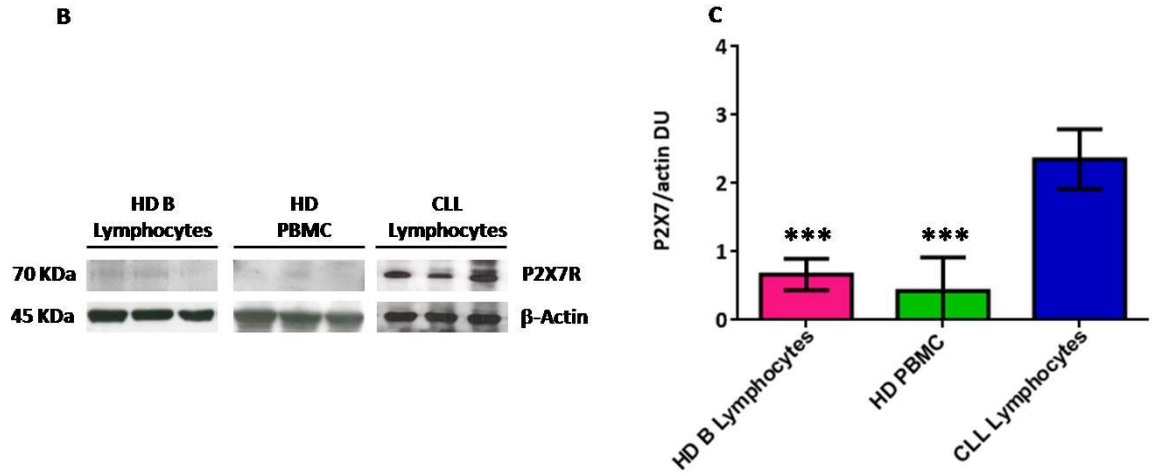

Figure 7. P2X7R expression in CLL patients and HD control. (B) Representative P2X7R Western blot from three subjects representative of each population. (C)Densitometry of P2X7R normalized on the housekeeping β-actin level. N=3,***p<0.001.

6.2 Overexpression of P2X7R correlate with chromosome 12 trisomy in CLL patients

Trisomy 12 is the third most frequent chromosomal aberration in CLL and often appears as the sole cytogenetic alteration [115]. Trisomy 12 has been traditionally considered an intermediate-risk prognostic factor in CLL [116], [117]. The P2X7R gene is localized on chromosome 12q24, therefore we analyzed whether P2X7R overexpression was associated with chrosome 12 trisomy. To evaluate the possible up-regulation, CLL patients were divided into two group: with or without trisomy 12. By Real Time PCR, we analyzed the P2X7R content of the two groups; as expected there was a statistically significant different between CLL patient with trisomy 12, and CLL patients without trisomy 12 (Figure 8), suggesting a gene-dosage effect.

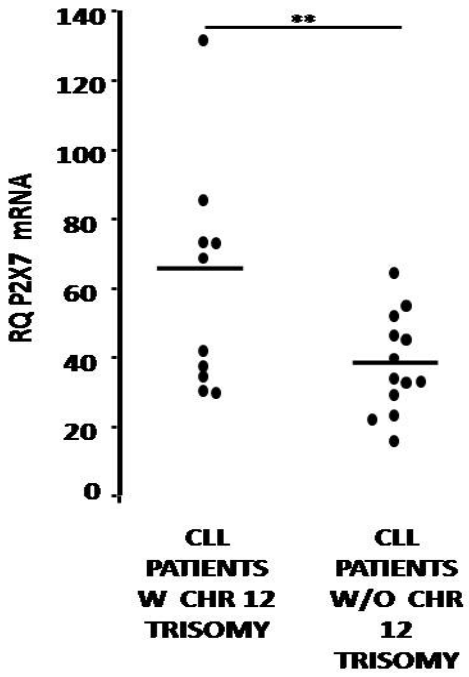

Figure 8. Correlation between chromosome 12 trisomy and P2X7R overexpression. mRNA expression levels of P2X7R in CLL patients with or without trisomy 12, measured by Real-time PCR. Gene expression data were normalized to the expression levels of the housekeeping gene GAPDH. ** $p<0.01$

6.3 Release of Interleukin-1β in CLL patients

The P2X7R is a main switch for processing and release of the potent pro-inflammatory cytokine IL-1β, thus we asked whatever CLL lymphocytes, due to increased P2X7R expression might be endowed with IL-1β releasing activity. Previous literature reports suggested that this might not be the case as IL-1β plasma levels are reported to be lower in CLL patients than in healthy controls [118]. For this reason we stimulated B lymphocyte from CLL patients and PBMC from healthy donors with 1 μg/ml overnight with LPS. As shown in Figure 9, LPS-stimulated release was dramatically lower in CLL lymphocyte than in HD PBMC despite much higher P2X7R expression in these cells.

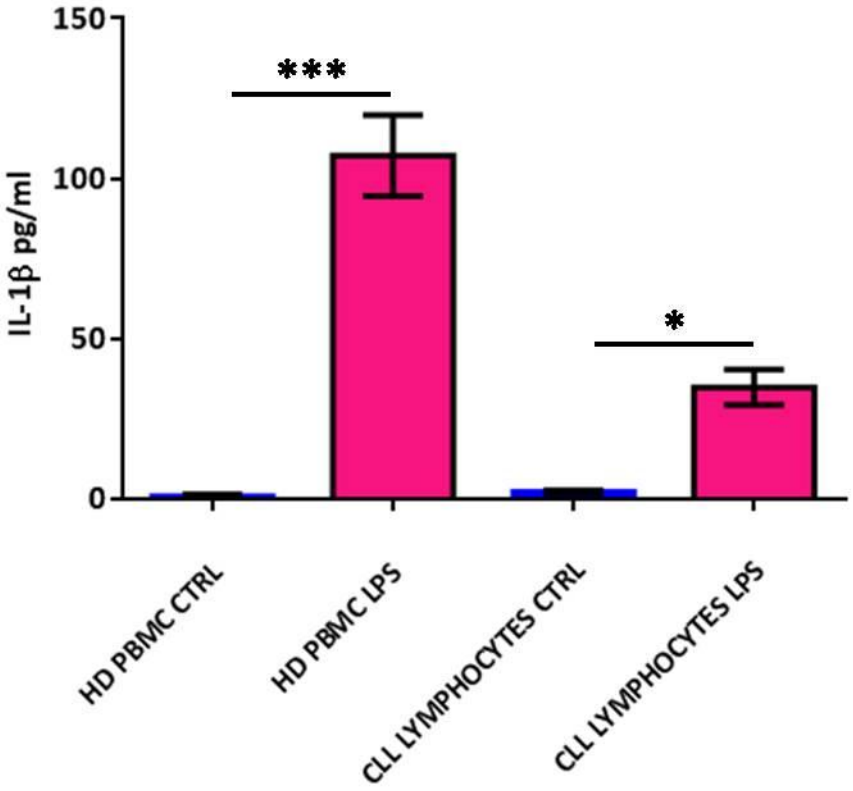

Figure 9. IL-1β release in CLL patients versus HD control. Cells were primed overnight with 1μg/ml LPS. At the end of this incubation time, cell free supernatants were assayed for IL-1 concentration with Human IL-1 beta Quantikine ELISA kit (R&D Systems, Minneapolis, MI, USA). * $p<0.05$, *** $p<0.001$.

6.4 NLRP3 is dowmodulated in CLL patients

The P2X7R is closely functionally associated to the NLRP3 inflammasome, the major intracellular molecular platform responsible for IL-1β processing and release [86], for this reason we decided to investigated if NLRP3 expression paralleled P2X7R expression level. On the contrary to our prevision, NLRP3 mRNA levels of CLL lymphocytes were dramatically reduced compared to HD PBMC and B lymphocytes respectively (Figure 10 A). NLRP3 protein levels at the same time, were also significantly lower in CLL lymphocytes compared to Healthy donors PBMC or B lymphocytes (Figure 10 B-C).

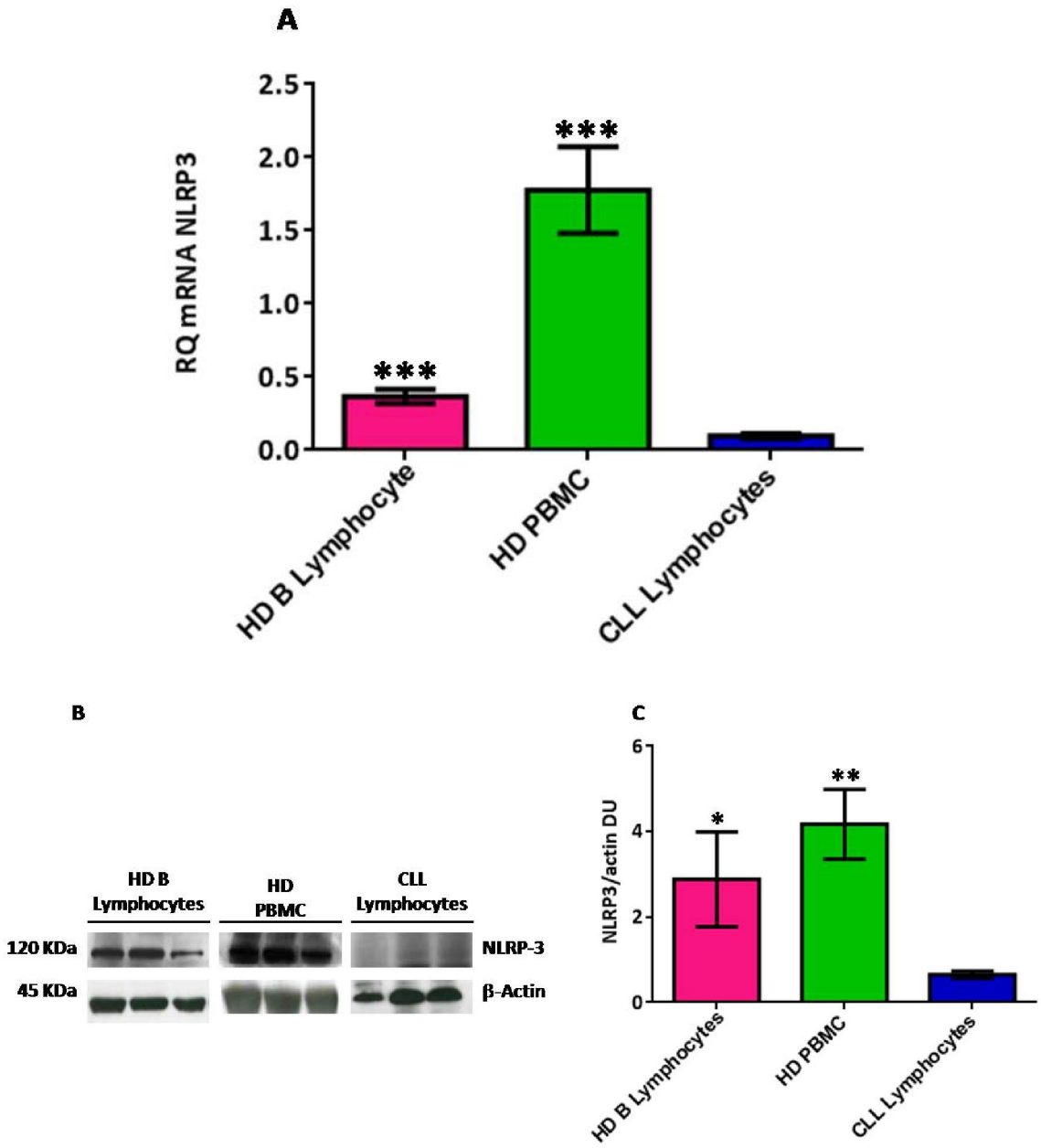

Figure 10. NLRP3 downregulation in CLL patients. (A) NLRP3 expression in HD B lymphocytes, PBMCs and CLL lymphocytes were isolated and qRT-PCR analysis was performed. Data are expressed as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) Representative Western of, HD B lymphocytes HD PBMC and CLL lymphocytes from three subjects representative of each population are shown. (C) Protein bands were quantified by densitometry and normalized onto endogenous b-actin band.

6.5 ASC in CLL

Finally we analyzed the other component of NLRP3 inflammasome ASC, that is responsible of the recruitment and the activation of the enzyme pro-caspase 1 that in turn when activated is able to secrete mature IL-1β [86]. In a first time, we analyzed the expression of mRNA by Real-Time PCR in the three different populations. Figure 11A

shows that ASC mRNA expression in CLL lymphocytes was slightly and not significantly higher than in HD PBMCs, but several fold higher relative to HD B lymphocytes. Furthermore, we assessed ASC expression on the same samples by immunoblotting (Figure 11 B). also in this case, the level of ASC was overexpressed in CLL lymphocytes (Fig. 11 B-C).

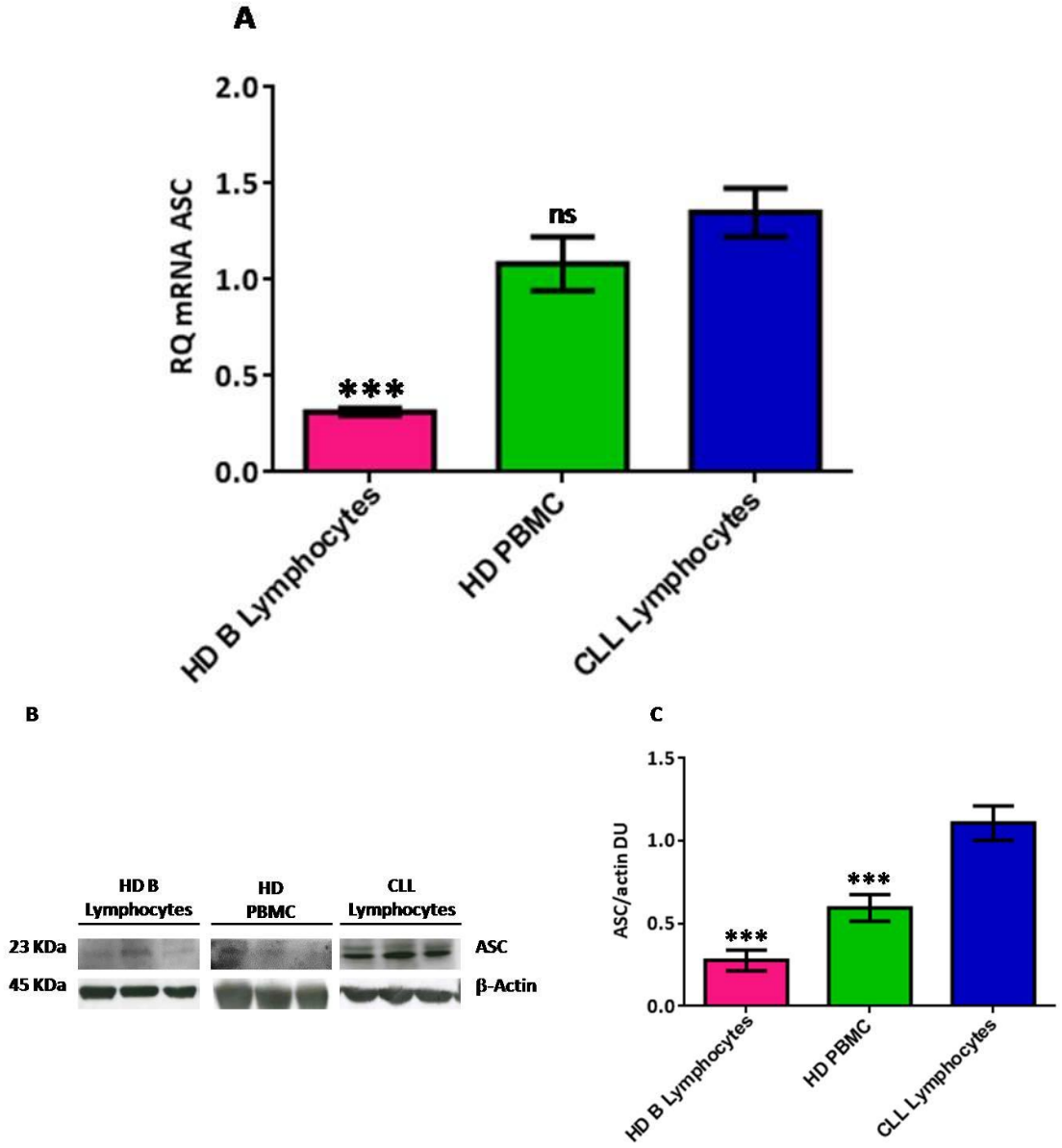

Figure 11. ASC expression is not changed in CLL. (A) Expression levels of ASC was determinated by qRT-PCR. Data are expressed as mean ± SEM. ns, not significantly;***, $p < 0.001$. (B) Representative Western blot of protein expression of ASC as shown in figure. Sample from CLL lymphocytes, HD PBMC and HD B lymphocytes from three subjects representative of each population are shown. (C) Densitometry of ASC normalized on b-actin. Data are averages ± SEM. ***, $p < 0.001$.

6.6 NLRP3 silencing induces P2X7R overexpression

Our previous findings showed that the P2X7R promotes growth *in vitro* and *in vivo* [70], [114], and in a number of experimental tumors its expression positively correlates with proliferation. Contrary to P2X7R, present data show that NLRP3 is down-modulated in leukemic cells and negatively correlates with proliferation. For this reason, we decided to mimic *in vitro* conditions, using the human leukemic cell lines THP-1, that can be easily transfected and has a fast *in vitro* growth rate and natively expressed NLRP3 and P2X7R. To down-modulate NLRP3, cells were transfected with three different NLRP3 *short hairpin RNA* (Figure 12 A-B). Thus we investigated if the response of *in vitro* models was comparable to B leukemic cells. Very interestingly, P2X7R expression in these NLRP3 silenced cell clones was strongly enhanced at protein level (Figure 12 C-D).

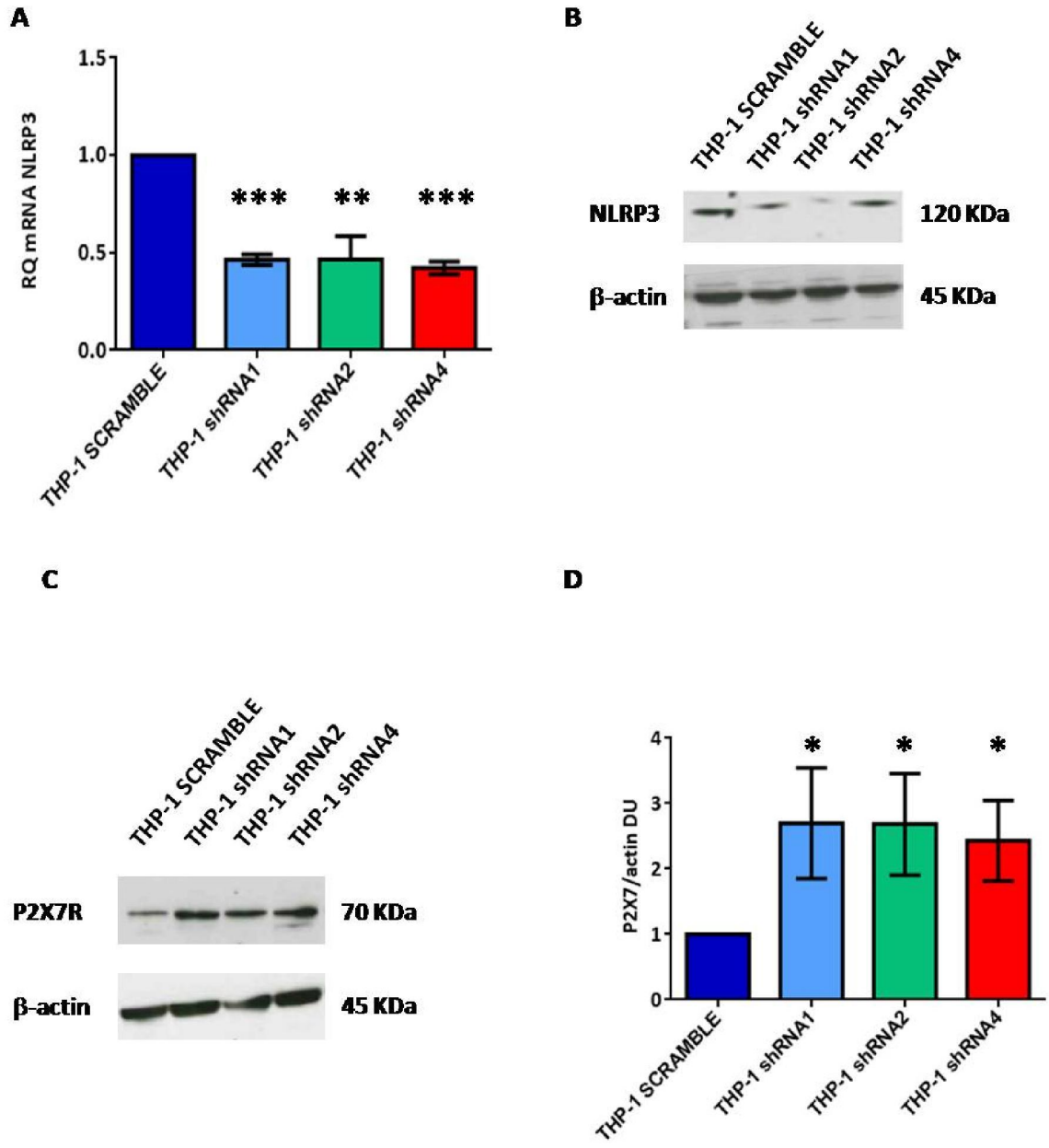

Figure 12. NLRP3 silencing in THP-1 cells. (A) THP-1 cells were silenced by HUSH-29 transfection as described in Methods; and analyzed for NLRP3 mRNA and protein (B) expression. (C) Up-regulation of P2X7R protein in NLRP3-silenced THP-1 cells. (D) Densitometry of P2X7R protein bands as shown in panel.

6.7 NLRP3 silencing in THP-1 cells accelerates cell growth

In order to test our anticipation that NLRP3 silencing promotes cell growth, we evaluated if stable THP-1 clones were able to accelerate proliferation *in vitro* using two different methods: cell counting and MTT assay. Cells were plated and the proliferative rate was measured over 96 hours. As expected, the cells silenced for NLRP3 showed a faster growth

rate relative to scramble shRNA control (Fig. 13 A-B). Higher silencing levels of the clone shRNA2 correlated with faster growth and an high proliferative rate, as shown in Figure 13 A-B. These observations on the one hand showed that inhibition of NLRP3 expression promoted growth, and on the other suggested that NLRP3 overexpression might negatively correlate with growth.

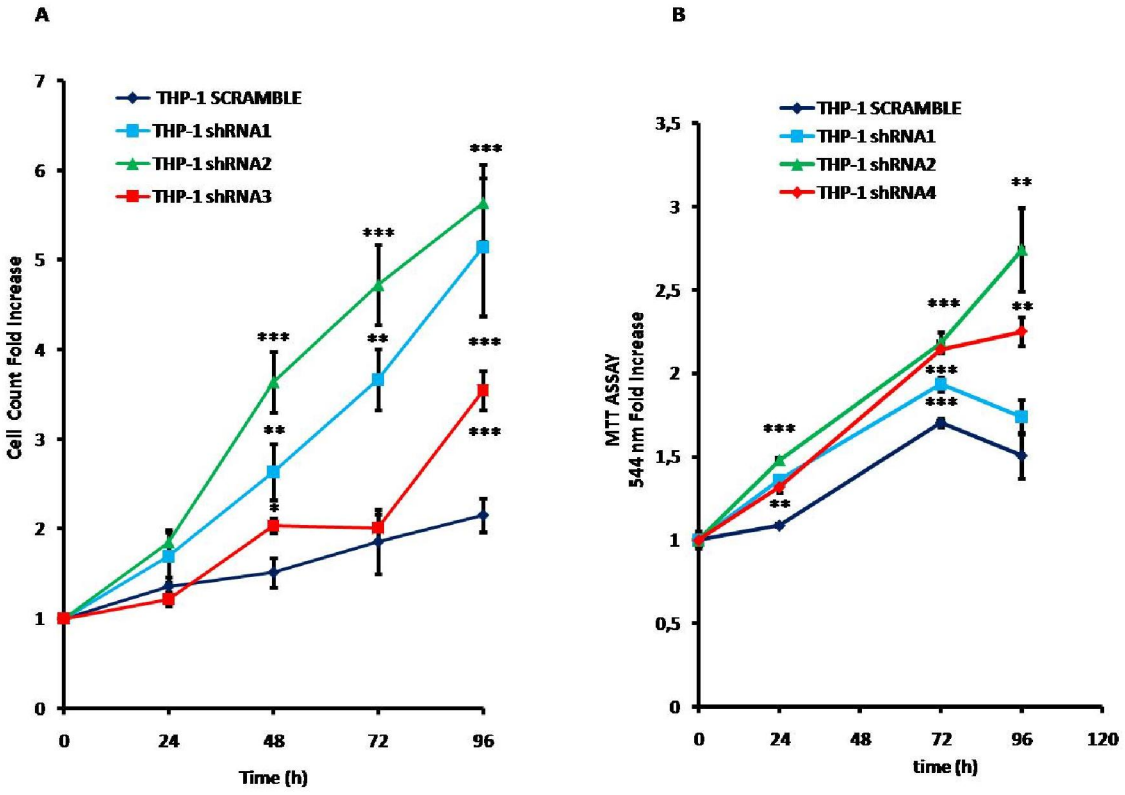

Figure 13. NLRP3 silencing accelerates cell growth. (A) For cell proliferation, 5 x 10^4 cells were plated in 6-well plates in complete medium and counted at the indicated time points. (B) THP-1 silenced were tested using MTT assay. Data from triplicate determinations are shown in panels A and B. Data are averages ± SEM. **, $p < 0.01$; ***, $p < 0.001$.

6.8 Overexpression of NLRP3 induce cell death

In order to test our prediction that NLRP3 was involved in cell growth, we made several attempts to generate stable THP-1 or Ramos cell clones overexpressing NLRP3. However we were consistently unable to expand these NLRP3-overexpressing cell cultures as they systematically stopped proliferating and died few days after transfection. For this reason we decided to study several marker of cell death and in particular of apoptosis. THP-1 and Ramos cells were transiently transfected with NLRP3 and after 48 hours of transfection they were screened for NLRP3 protein and mRNA expression (Figure 14 A-B and 15 A-B). In parallel, these cells were also analyzed for caspase-3 and caspsase-9 expression

(Figures 14 C-E and 15 C-E), showing that both caspases were up-regulated, and in addition caspase-3 was also activated. Finally we evaluated the Annexin V positive cells after NLRP3 transfection (Figure 14 F and 15 F). THP-1 and Ramos cells overexpressing NLRP3 also showed an increase in Annexin V positive cells, suggesting that NLRP3 overexpression can trigger cell death.

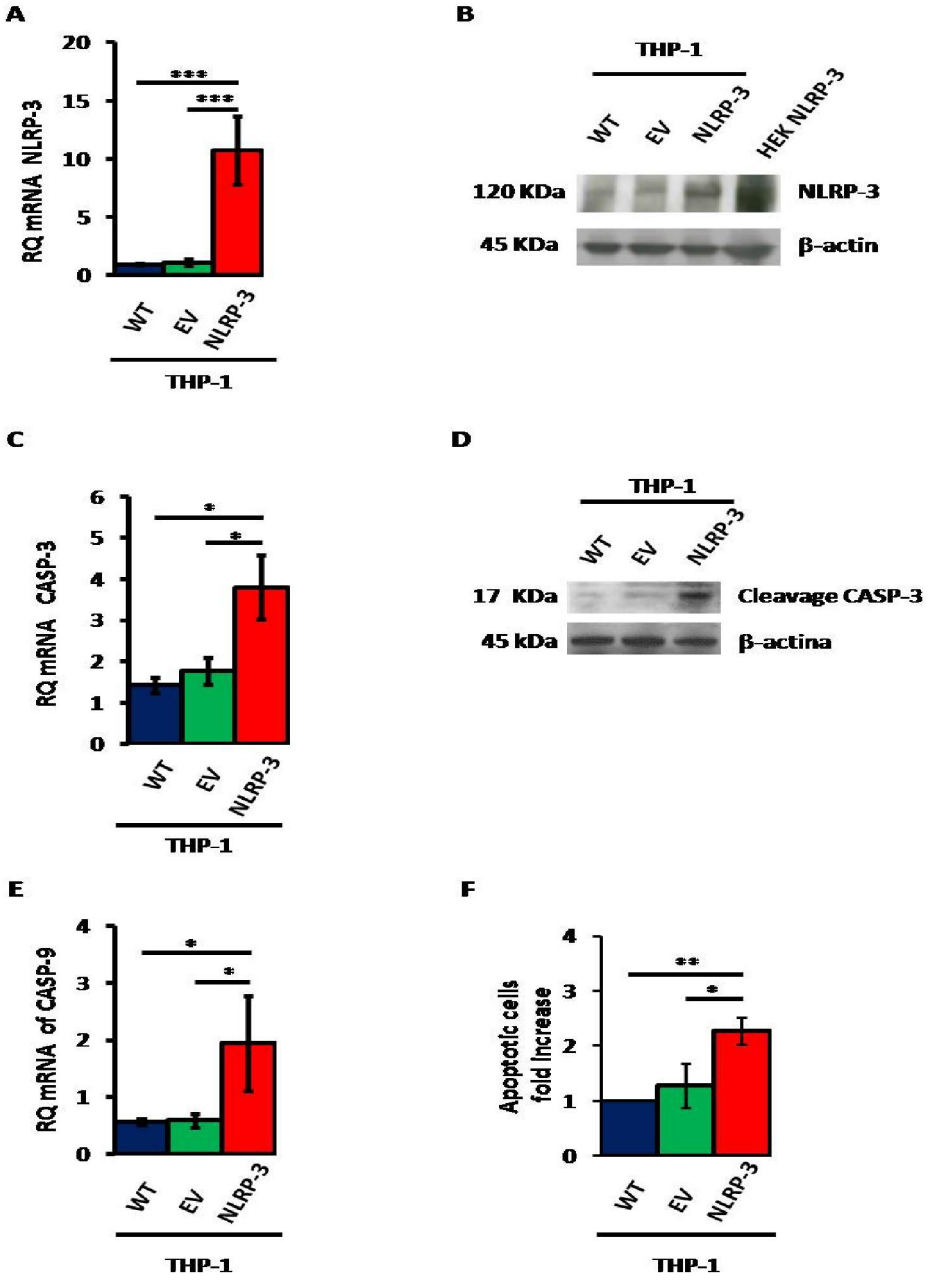

Figure 14. NLRP3 overexpression in THP-1 cells triggers cell death. (A) THP-1 cells were transfected with a plasmid encoding NLRP3 and by Real Time-PCR analyzed for NLRP3 mRNA expression. (B) NLRP3 protein expression; empty vector (EV) transfected cells and a HEK293 clone stably transfected with NLRP3 (HEK293-NLRP3) are shown as control. (C) Caspase-3 mRNA expression is shown in panel. (D) Effect of NLRP3 transfection on caspase-3 activation (cleavage) is shown in panel. (E) Caspase-9 mRNA expression. (F) Apoptotic cell number (Annexin V-positive cells) determinated by Tali image, and express as fold increase. Data are averages ± SEM from triplicate independent determinations. *, $p < 0.05$; **, $p < 0.01$; ***, ***, $p<0.001$.

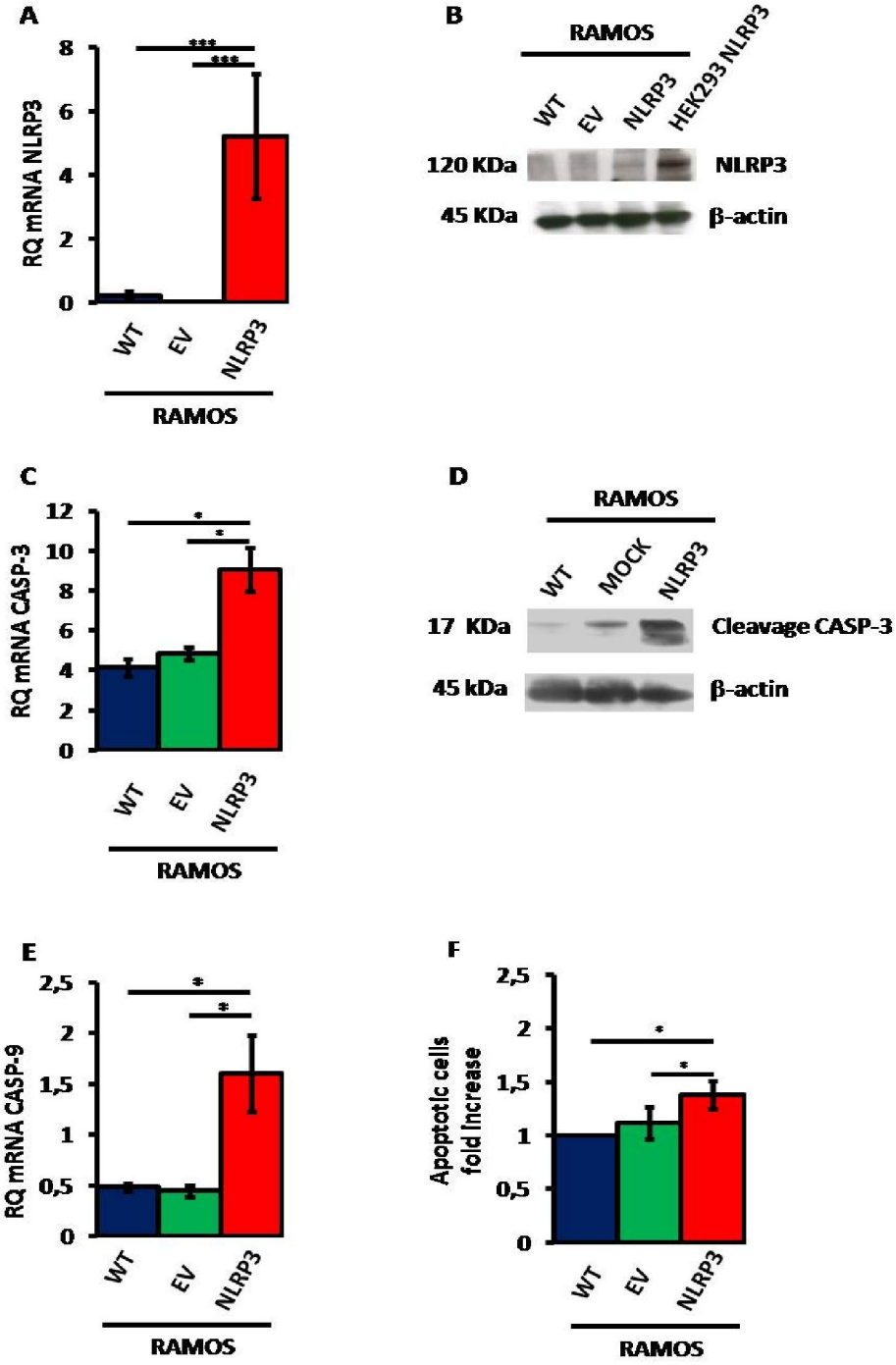

Figure 15. NLRP3 overexpression in Ramos cells triggers cell death. (A)Ramos cells were transiently transfected with NLRP3 plasmide, and then analyzed for NLRP3 mRNA expression. (B) NLRP3 protein expression was confirmed by Western Blot analyze. (C) Effect of NLRP3 over-expression on caspase-3 mRNA. (D) Panel show the Caspase-3 protein activation. (E) Effect of NLRP3 transfection on caspase-9 expression. (F) Apoptotic cell number (annexin V-positive cells) is shown in panel. Data are averages ± SEM from 3 independent determinations. *, $p < 0.05$; ***, $p < 0.001$.

6.9 NLRP3 transfection inhibits cell growth

Since both THP-1 and Ramos cells proved to be extremely sensitive to the adverse effects of NLRP3 overexpression, we investigated the long-term effects of NLRP3 transfection in the human cell line HEK293. We were able to select several stable NLRP3-expressing HEK293 clones, one shown in Figure 16 A and B. Growth rate of these stable NLRP3-expressing clones, as shown in Figure 16 C, was significantly slower (** $p < 0.01$) than that of HEK293 cells transfected with the empty vector (EV). Interestingly, not only NLRP3-transfected HEK293 cells had a slower growth rate, but also showed substantial shape changes relative to mock-transfected cells. In fact, mock-transfected cells showed the spindle-like morphology typical of wild type HEK293 cells, while on the contrary HEK293 overexpressing NLRP3 were rounded with numerous dendrite-like protrusion (Figure 16 D-E).

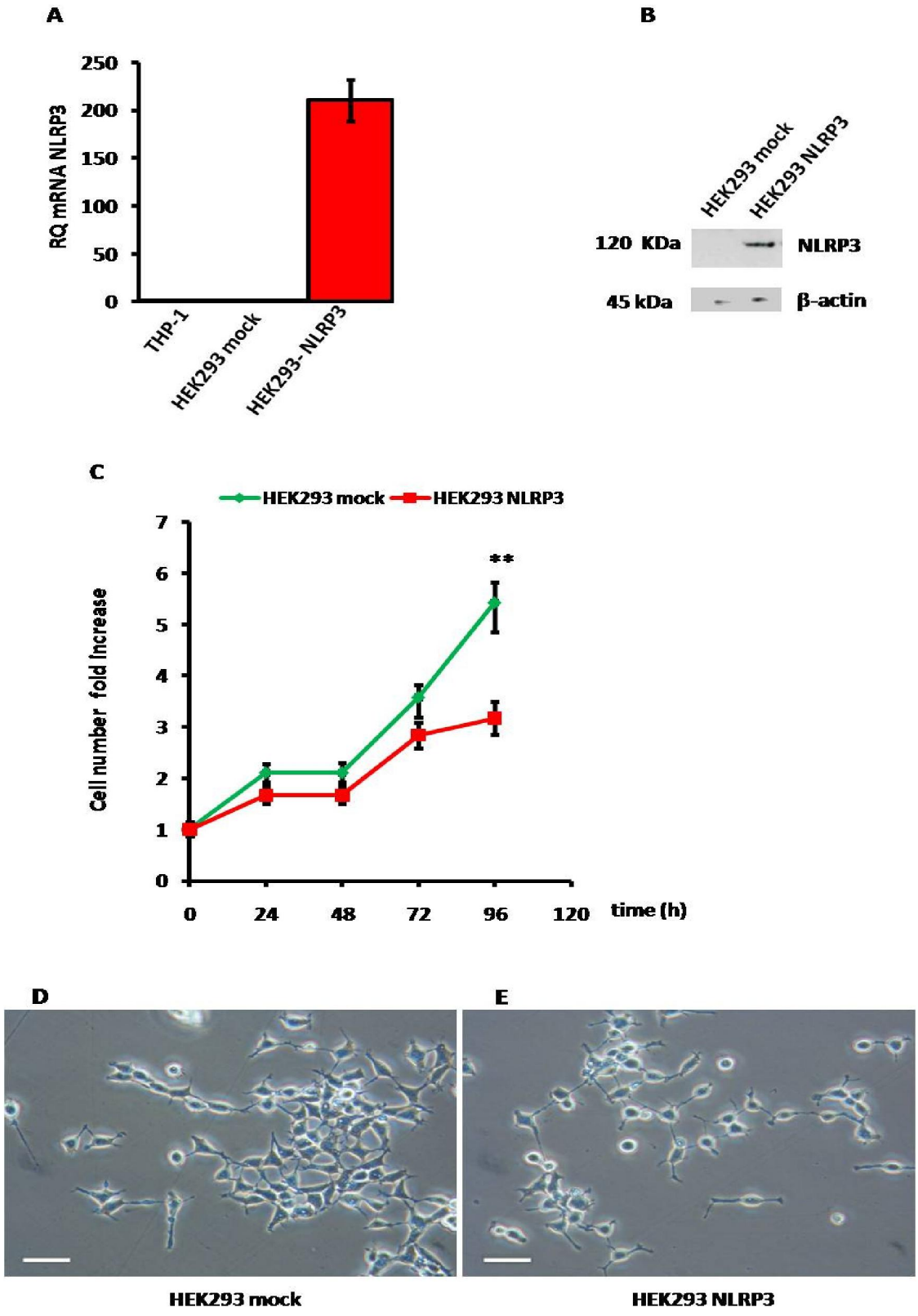

Figure 16. NLRP3 overexpression inhibits proliferation of HEK293 cells. (A) HEK293 cells transfected with a NLRP3 vector. Panels show level of mRNA expression respectively, in mock- and NLRP3-transfected cells. THP-1 cells are shown as a control for baseline NLRP3 expression. (B) Western blot analysis of HEK293-mock and HEK293-NLRP3, β-actin s as a loading control. (C) Panel show growth kinetics of NLRP3- and mock-transfected HEK293 cells, respectively. (D-E) Morphology of HEK293 mock (D) and HEK293 NLRP3-transfected (E) HEK293 cells. Bar = 40 μm. **, $p < 0.01$

6.10 Nlrp3$^{-/-}$ mouse cells overexpress P2X7R

In order to understand if was present an inverse correlation between NLRP3 and P2X7R, similar to other data obtained from *ex-vivo* –CLL cells and *in vitro* model, we decided to assayed different cell type originated from Nlrp3$^{-/-}$ C57BL/6 mice. Single cell suspension obtained from spleen, macrophage derived from bone marrow (BMDM), and bone marrow (BM), from Nlrp3 wild type e knock-out mice, were analyzed for expression levels of P2X7R and NLRP3 by Real time PCR (Figure 17 A-F). In general, it was possible to observe a good inverse correlation between the absence of NLRP3 and a higher expression of P2X7R in particular in spleen cells and BMDM. Furthermore data obtained from spleen cells were corroborated from immunoblotting analyses that confirmed of P2X7R is overexpressed in the absence of NLRP3 (Figure 17 G).

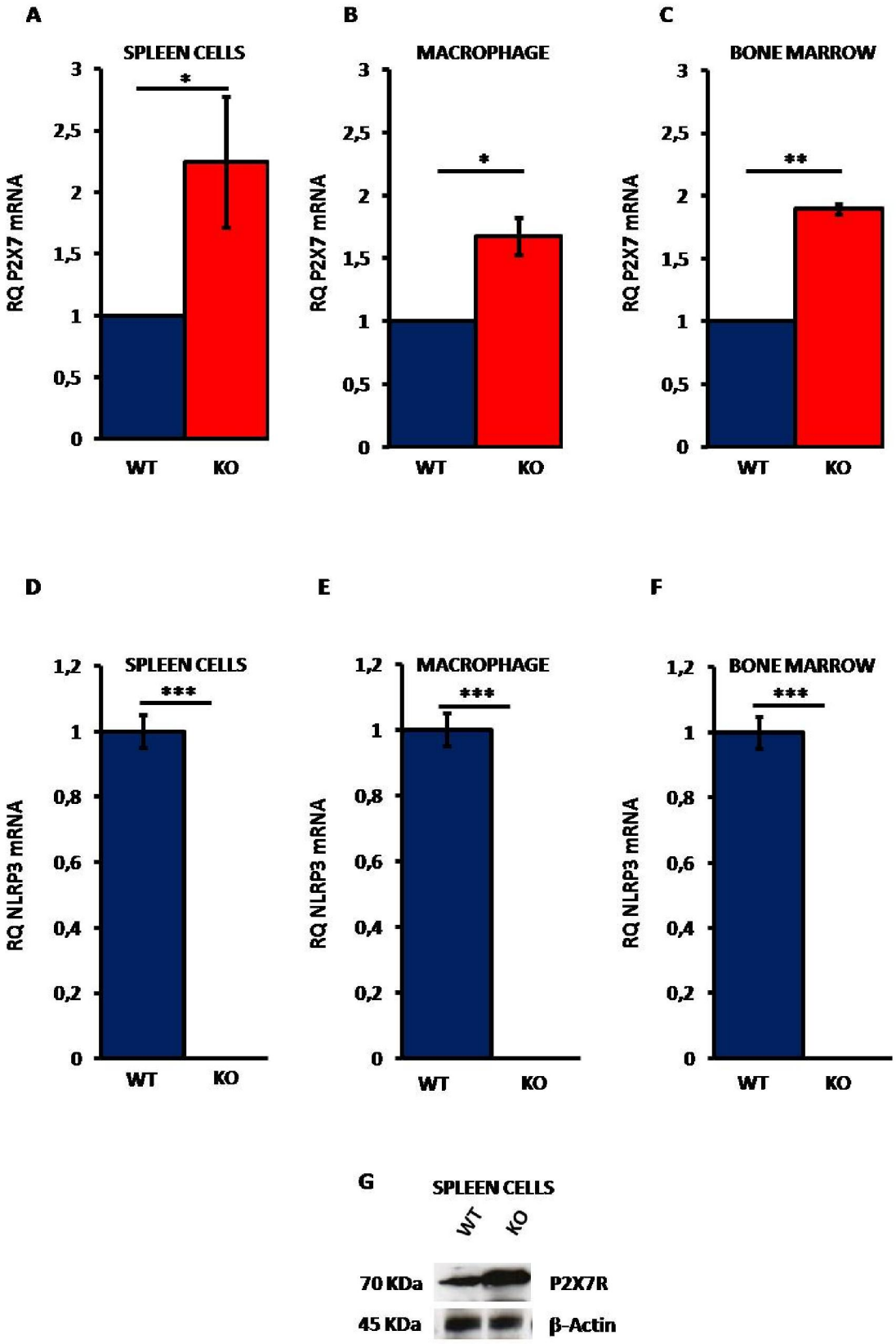

Figure 17. P2X7R is overexpressed in Nlrp3$^{-/-}$ mouse. (A) P2X7R mRNA expression in murine spleen cells. (B) Graphs show P2X7R expression in macrophage from BM. (C) P2X7R expression levels into bone marrow. (D) NLRP3 expression in murine spleen cells. (E) Real-Time PCR expression of NLRP3 in macrophage from BM. (F) NLRP3 expression level obtained from bone marrow. (G) P2X7R expression obtained by Western Blot analysis of spleen cells. *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$.

6.11 Reduced cell death in Nlrp3$^{-/-}$ spleen cells

We previously demonstrated that overexpression of NLRP3 in *in vitro* model was able to induced cell death. We then decided to test the effect of NLRP3 in a primary culture of spleen cells derived from mice Nlrp3$^{-/-}$compared to Nlrp3 wild type. We investigated two conditions: 1) mixed lymphocyte reaction MLR (obtained as a reported in Material and Methods), where spleen cells from Nlrp3$^{-/-}$ and Nlrp3-WT were co-cultured for 72 or 96 hours; 2) spleen cells obtained from Nlrp3$^{-/-}$ and Nlrp3-WT were cultured in presence of 5 µg/ml of phytohemoagglutinin (PHA), a well known mitogen, and incubated for 72 or 96 hours. At the end of incubation, spleen cells were collected and stained with 7-AAD (a fluorescent DNA intercalator of DNA, impermeant in live cells), and analyzed by FACS. As shown in Figure 18, viability of cells from Nlrp3$^{-/-}$ mice was higher compared to Nlrp3-WT, at all time points analyzed.

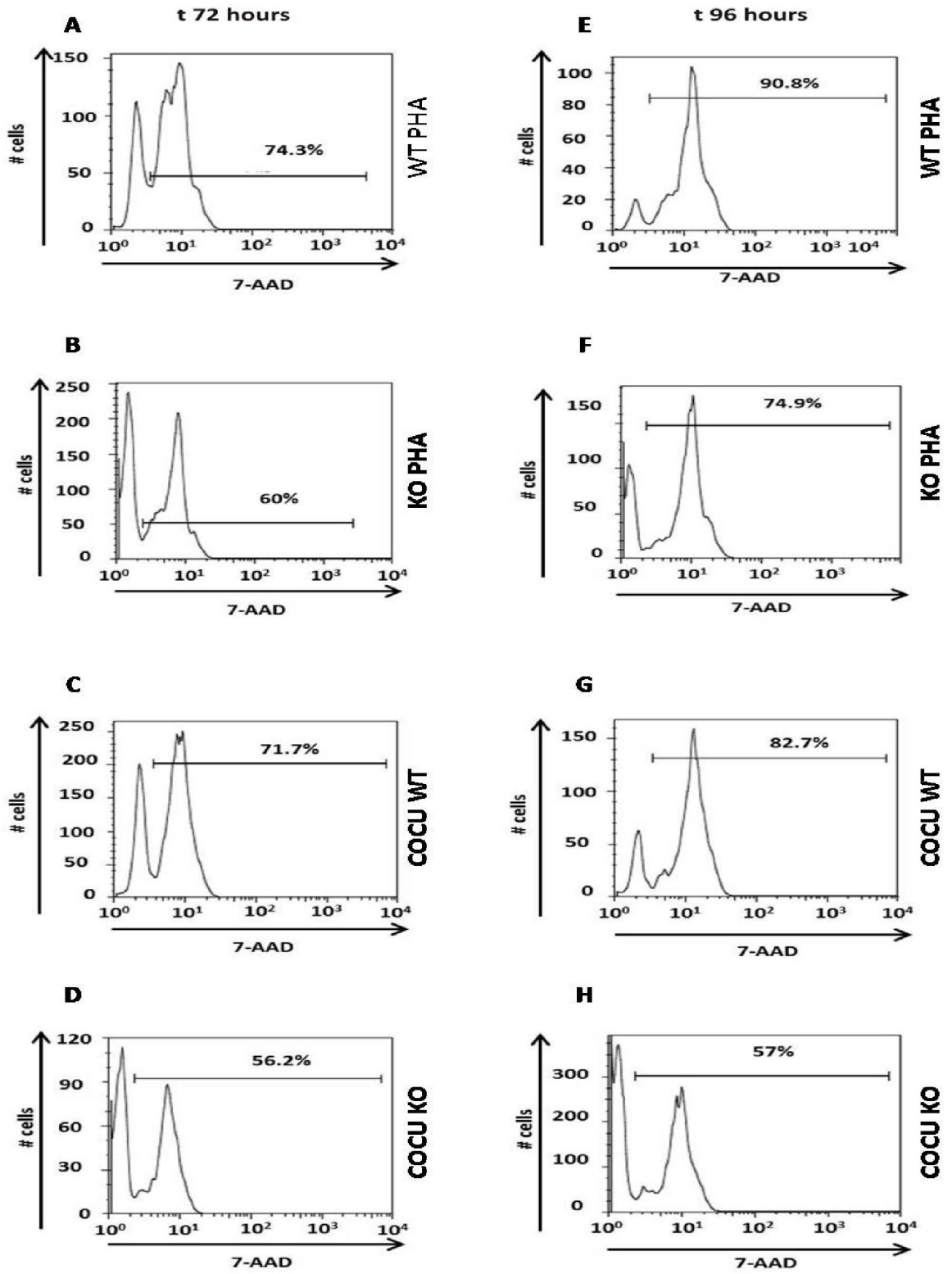

Figure 18 NLRP3 is involved in cell death. (A) Spleen cells WT survive after 72 hours with PHA. (B) Spleen cells KO surviving after 72 hours with PHA. (C) Co-Culture of spleen cells WT after 72 hours of stimulation. (D) Co-Culture of Spleen cells KO after 72 hours of stimulation. (E) Spleen cells WT surviving after 96 hours with PHA. (F) Spleen cells KO survive after 96 hours with PHA. (G) Co-Culture of Spleen cells WT after 96 hours of stimulation. (H) Co-Culture of Spleen cells KO after 96 hours of stimulation.

DISCUSSION

Inflammasomes have become a focus of hot interest in cell biology and immunology for their crucial role in several cell functions, most notably cytokine secretion and host-pathogen interaction [119]. Among the different subtypes so far described, the NLRP3 inflammasome is attracting increasing attention as it is a main target of DAMPs and the most active intracellular organelle responsible for maturation of cytokines of the IL-1β family. NLRP3 is functionally closely associated to the P2X7R, and in fact P2X7R is the most potent NLRP3-activating plasma membrane receptor [86]. P2X7R makes the NLRP3 inflammasome sensitive to extracellular ATP, one of the major DAMPs released during inflammation [120]. Furthermore, other early pro-inflammatory agents, some themselves considered DAMPs such as the cathelicidin LL37 and amyloid-β, may directly interact with and stimulate P2X7R [121], [122], and by consequence activate the NLRP3 inflammasome. Besides its crucial role in IL-1β processing and release, P2X7R is also well known for its cytotoxic activity and for its counterintuitive trophic activity. We and others have extensively shown that tonic, low-level, P2X7R stimulation, far from being cytotoxic, is beneficial for cell growth and proliferation [123], [124], [114]. In fact, and not surprisingly, most human malignant tumors overexpress P2X7R, and targeting this receptor with selective blockers slows down *in vivo* growth of several transplanted tumors. Overexpression of P2X7R in CLL is of particular interest since a known adverse prognostic factor in this leukemia is chromosome 12 trisomy [125]. Albeit chromosome 12 is known to host several genes related to cancer 16541075 [126], a pathogenetic explanation for the adverse prognostic significance of chromosome 12 trisomy in CLL is lacking. Present and previous reports documenting an increased expression and activity of the P2X7R in CLL provide a pathophysiological explanation given the compelling evidence supporting a role of this receptor in supporting cell proliferation and metastatic spreading. Given the close functional association between P2X7R and NLRP3 and the known, albeit controversial, role of the IL-1β in tumor progression, we initially hypothesized that NLRP3 followed in CLL lymphocytes a similar expression pattern to P2X7R, and thus was overexpressed in CLL leukemic B lymphocytes. However, contrary to this anticipation, our present data show that NLRP3 was surprisingly down-regulated at the mRNA and protein level in CLL lymphocytes compared to HD PBMCs or tonsil B lymphocytes. The ASC protein on the other hand followed a different expression pattern, as like P2X7R was up-regulated in CLL patients B cells. The inverse P2X7R/NLRP3 correlation suggested that high NLRP3 levels might negatively affect cell proliferation,

contrary to high P2X7R levels that support proliferation. Since primary human B lymphocytes are not manageable to transfection studies, we turned as a model for transfection to THP-1 cells, a human leukemic cell line that expresses both P2X7R and NLRP3. In agreement with our prediction, NLRP3 down-modulation caused a strong acceleration of cell proliferation paralleled by an increase in P2X7R protein expression. Given the known growth-promoting effect of P2X7R, it is highly likely that the proliferative advantage conferred by NLRP3 suppression was due to the enhanced P2X7R expression. Identification of P2X7R overexpression as a mechanism by which NLRP3 down-modulation supports growth provides also hints as to the metabolic pathways involved as we have previously demonstrated that P2X7R overexpression increases mitochondrial matrix Ca^{2+} and potential, intracellular ATP stores, activates NFATc1 and protects from apoptosis [123], [127]. We then investigated the effect of NLRP3 overexpression. To this aim, NLRP3 was also transfected into the Ramos a human leukemic cell line that natively does not express P2X7R and NLRP3; and besides into THP-1 cells. It was rather difficult to prove that NLRP3 overexpression had an adverse effect on cell growth as its mere transfection proved to be cytotoxic on most THP-1 and Ramos cells, making it impossible to select stably- transfected clones, as we did for the NLRP3-silenced cells. Thus, we were forced to investigate a few and more important apoptotic markers in the 48 hours following transfection. During this time, apoptosis was accelerated in both THP-1 and Ramos cells, and apoptotic caspases-3 and -9 were up-regulated and activated. After 48-72 hours very few transfected cells survived, thus making additional studies impossible. However, we were able to verify the long term effects of NLRP3 transfection in HEK293 cells. This cell type turned out to be much more resistant to the adverse effects of NLRP3 transfection compared to THP-1 or Ramos cells. A few stably-transfected clones were selected and their growth kinetics could be investigated. In agreement with our prediction of the anti-proliferative role of NLRP3, NLRP3-transfected cells showed a much slower growth rate compared to empty vector- transfected or wild type cells, and a strikingly different morphology. Finally we decided to investigate if P2X7R is overexpressed in different primary culture obtained from NLRP3 knockout mice compared to wild type. Our prevision was confirmed and P2X7R is up-regulated in murine spleen cellss, macrophages obtained from bone marrow and bone marrow. Furthermore we demonstrated that murine spleen cellss from NLRP3 knockout mice were more inclined to survive, these suggested that these cells was less sensitive to cell death. Our findings raise the provocative issue of the direct participation of the NLRP3 inflammasome in the control of cell proliferation. In principle, it is not surprising that NLRP3 overexpression stimulates

cell death as it is well known that inflammasome activation may trigger pyroptosis, a form of lytic cell death mediated by inflammasome activation and IL-1β release [128]. However, it is indeed surprising that NLRP3 down-modulation is growth-promoting in a cell-autonomous fashion, and in murine model support cell survival, as shown by our results. It was shown that NLRP3 is repressed in human hepatocellular carcinoma compared to healthy liver cells, and that NLRP3 expression inversely correlates with liver cancer progression [112]. This finding was interpreted by the Authors as a proof that low NLRP3 levels impaired liver cell ability to sense cytoplasmic danger signals and react accordingly, thus enhancing the cell susceptibility to undergo malignant transformation. While we confirm in the present study that tumor cells have low NLRP3 levels, contrary to the interpretation put forward by Wei et al [112] we show that NLRP3 expression directly affects cell growth, in a cell autonomous fashion. Finally very recently show, that NLRP3 is down modulated in different Malignant Mesothelioma (MM) cell lines compared with the cell line control; and chemotherapeutic drugs can activate NLRP3 inflammasomes in MM tumors resulting in caspase activaction and increase cell death [113].Westbom and collaborators suggest that a combination of chemotherapeutic drugs and IL-1R antagonist could be a possible strategy for inhibiting MM tumor growth [113]. This data support our hypothesis an increasingly strong involvement of NLRP3 in cell death. The mechanism responsible involves the up-regulation of the P2X7R, a receptor that is known to have a dual role in cell physiology as on one hand it has an undisputed cytotoxic effect when overstimulated, while on the contrary exert a trophic activity when it is tonically activated by endogenously-released ATP [129]. These findings help place the NLRP3 inflammasome in an entirely novel context in cancer. Current view holds that NLRP3 interferes with tumor progression in virtue of its ability to [130] modulate cytokine release and cell death (pyroptosis), and thus control inflammation. In this context, the outcome can then be different since in certain experimental settings this activity is protective against cancer development, while in other settings NLRP3 activation and IL-1β secretion are associated to a more malignant tumor phenotype. Our data expand the role of NLRP3 in cancer as a factor that is directly controlling growth. In conclusion, the P2X7R-NLRP3 axis appears to have an increasing importance at multiple steps involved in tumor progression, from to modulation of inflammation to control of cell proliferation and cell death. These findings might be of potential interest for the identification of novel therapies against cancer.

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