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New role of tumor suppressor PML in the control of the NLRP3-dependent tumor growth

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Index

Abbreviations

AEQ, aequorin;

ANT, adenine nucleotide translocase;

APL, acute promyelocytic leukemia;

ATG, autophagy related genes;

Ca^{2+}, calcium ions;

$[Ca^{2+}]$, Ca^{2+} concentration;

$[Ca^{2+}]c$, cytosolic Ca^{2+} concentration;

$[Ca^{2+}]er$, endoplasmic reticulum Ca^{2+} concentration;

$[Ca^{2+}]m$, mitochondrial Ca2+ concentration;

CABPs, intraluminal Ca2+-binding proteins;

DAG:diacylglycerol;

ER, endoplasmic reticulum;

FACL4, long-chain fatty acid-CoA ligase type 4;

FRET, fluorescence resonance energy transfer;

GFP, green fluorescent protein;

GPCRs: G protein coupled receptors

grp75, glucose-regulated protein 75;

HK, hexokinase;

HIV, human immunodeficiency virus;

IL−1β, interleukin-1 beta;

IL-18, interleukin-18;

IMM, inner mitochondrial membrane;

IMS, intermembrane space;

IP3, inositol 1,4,5-trisphosphate;

IP3R, inositol 1,4,5-trisphosphate receptor;

Letm1, leucine zipper-EF-hand containing transmembrane protein 1;

MAMs, mitochondria-associated membranes;

MAVS, mitochondrial anti-viral signaling;

Mefs: mouse embryonic fibroblasts;

MCU, mitochondrial Ca^{2+} uniporter;

MICU1, mitochondrial calcium uptake 1;

mTOR, mammalian target of rapamycin;

mHCX, mitochondrial H^{+}/Ca^{2+} exchanger;

mNCX, mitochondrial Na^{2+}/Ca^{2+} exchanger;

MOMP, mitochondrial outer membrane permeabilization;

NCX, Na^{2+}/Ca^{2+} exchanger;

NLRP3, NACHT, LRR and PYD domains-containing protein 3;

OMM, outer mitochondrial membrane;

PACS-2, phosphofurin acidic cluster sorting protein 2;

PAMs, plasma membrane associated membranes;

PAS, pre-autophagosomal structure;

PI3K, phosphatidylinositol 3-kinase;

PIP2, phosphatidylinositol 4,5-bisphosphate;

PIP3, phosphatidylinositol 3,4,5-trisphosphate;

PLC-β: phospholipase C-β

PMCA, plasma membrane Ca^{2+} ATPase;

PML, promyelocytic leukemia protein;

PP2a, protein phosphatase 2a;

PTEN, phosphatase and tensin homolog deleted on chromosome 10;

PTP, permeability transition pore;

ROCs: receptor operated Ca^{2+} channels:

ROS, reactive oxygen species;

RyR, ryanodine receptor;

SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase;

Sig-1R, Sigma-1 receptor;

SMOCs: second messenger operated Ca^{2+} channels;

SOCE, store-operated Ca^{2+} entry;

SR, sarcoplasmic reticulum;

STIM: stromal interaction molecule;

STING, stimulator of IFN genes;

TIM: transporters of the Inner Membrane;

TKR: tyrosine-kinase receptors

TOM (Transporters of the Outer Membrane);

UCP, uncoupling protein;

VDAC, voltage-dependent anion channel;

VOCs: voltage operated Ca^{2+} channels.

Abstract

Promyelocytic Leukemia Protein (PML) is a multi-faceted protein, that can be present at nuclear level, where it plays pivotal roles in tumor growth, modulating a wide number of cell activities, including tumor suppression, anti-viral and anti-bacterial responses. Among its very numerous functions, PML can also localize to the ER-mitochondria contact sites (MAMs) where it can control several functions such as the transfer of calcium into the mitochondria, ROS production, apoptosis and autophagy. Recently, MAMs have been shown to function as a platform for inflammatory signaling regulated by the most widely characterized inflammasome, so-called NLRP3. At rest, the NLRP3 protein resides at the ER, whereas upon inflammation activation, both NLRP3 and its adaptor ASC redistribute to MAMs to permit inflammasome formation. Here we discover that the down-regulation of the tumor suppressor PML at the host level can drive an immune response that is able to worsen cancer instead of ameliorate it, enhancing the process of IL-1β release. In particular, we found that the crosstalk between PML and the NLRP3 inflammasome at the ER/MAMs compartments exist and we demonstrated how this molecular pathway in the host could impact cancer development. Furthermore, we revealed that genetic or pharmacological manipulation of NLRP3-inflammasome and of one of its activator, the P2X7 receptor, have the benefit of blocking the release of IL-1β in a PML KO background, reducing tumor growth.

It is well known that even anti-cancer therapies, like radio- and chemotherapy, are able to trigger an immune response. Traditional research on radiotherapy (RT) is focused on increasing treatment doses to target volume and reducing treatment fields to spare healthy tissues. However, radiations interact actively with host's immune system contributing strongly to limit tumor progression. There is some evidence that RT inflammation is related to activation of inflammasome determining an increase of interleukins (IL): IL-1β and IL-18. We demonstrate that low doses of RT induces a hyper-activation of NLRP3 inflammasome through a high IL-1β release in the microenvironment in absence of PML. Although IL-1β can have a potent stimulatory effect on immune response, it can also stimulate the tumor growth. Inhibiting NLRP3 inflammasome activation improve the efficacy of RT protocols in a hyper-inflamed microenvironment by limiting the possible undesirable side effects consisting in the promotion of tumor growth.

Introduction

Mitochondria associated membranes (MAMs)

Specific organization of the intracellular organelles enables direct communication between various compartments within the cell. Among the different direct interactions or "close contacts" between cellular organelles, MAMs have recently attracted the attention of many researchers, as represented by the growing number of publications describing the critical roles of MAMs in physiology and pathology (Giorgi, Missiroli et al. 2015). MAMs consist of regions of the ER involved in direct interactions with the mitochondria. However, proteins from other cellular compartments have also been found in MAMs, suggesting that MAMs also form close contacts with other intracellular structures in addition to the ER. For instance, plasma membrane (PM) proteins are observed in MAMs, indicating the presence of close contacts between the mitochondria and the PM (Poston, Krishnan et al. 2013). According to numerous studies, mitochondria- ER contact sites are dynamic structures. However, because we can isolate these structures, the interactions between these membranes are strong, and they are not destroyed during isolation procedures. Plasma membrane-associated membranes (PAMs) (Suski, Lebiedzinska et al. 2014) and ERmitochondria encounter structures (ERMES) (Kornmann 2013) are other examples of the physical and functional contacts that are isolated during cellular subfractionation.

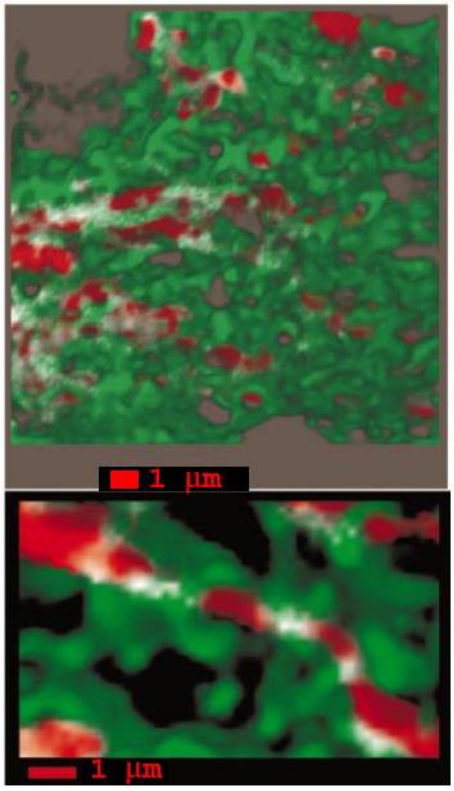


Figure 1 - High-resolution 3D imaging of ER-mitochondria contact sites. Combined 3D imaging of mitochondria and ER in a HeLa cell transiently expressing mtGFP(Y66H, Y145F) and erGFP(S65T). The mitochondrial and ER images are represented in red and green, respectively; the overlaps of the two images are white. On the bottom, a detail of the main image (80-nm pixel) (figure from Rizzuto, R., et al.,Science, 1998).

Most researchers believe that the first evidence showing that the mitochondria and ER are closely positioned at some regions comes from the early 1970s in the studies by Franke and Kartenbeck (Franke and Kartenbeck 1971) and Morre et al. (Morre, Merritt et al. 1971). However, the first reports on the direct association between the mitochondria and ER date back to as early as the late 1950s (Copeland and Dalton 1959). Almost simultaneously with the observations mentioned above from the 1970s, Lewis and Tata (Lewis and Tata 1973) observed that a fraction of the ER was found in low-speed centrifugation pellets containing the mitochondrial portion during subfractionation of rat liver homogenates. Based on this observation, we acknowledge this paper as the first report to describe a MAM isolation procedure. Almost twenty years later, in the early 1990s, the Vance group made significant progress in the MAM field by presenting a detailed protocol describing the isolation of pure MAM fractions in a series of articles published in J. Biol. Chem. (Rusinol, Cui et al. 1994), which was improved upon by Meier et al. (Meier, Spycher et al. 1981) ten years later. Over the years, the MAM isolation method was developed and optimized to enable the isolation of MAMs from different animal tissues and cell cultures (Wieckowski, Giorgi et al. 2009). The existence of the MAM fraction is not an exclusive characteristic of mammalian cells; close interactions between mitochondria and the ER have also been described in yeast. Interestingly, similar contacts between mitochondria and the ER have been described for chloroplasts and the ER in plants. In addition to the development of more refined protocols for isolating pure MAM fractions, the list of proteins present at mitochondria-ER contact sites increases every year. Although many proteins localized at the MAM have been identified, we have not determined which proteins can be used as universal MAM markers because some MAM proteins are only present in specific organs, tissues or cell types. Another problem is the observation that no protein is exclusively localized to the MAM fraction. Instead, the localization of a specific protein at the MAM is only appropriately termed as enriched because these proteins are also present in other cellular compartments. Regarding the molecular composition of the MAM fraction, an article by Poston et al. presents a detailed proteomic analysis of the MAM (Poston, Krishnan et al. 2013). These authors detected and classified approximately 1200 proteins from the MAM fraction isolated from a mouse brain and confirmed that the MAM fraction contains proteins characteristic of the PM and the Golgi apparatus (24% and 6%, respectively, of the total proteins detected in MAMs). Based on the long list of proteins found in the MAM fraction or that translocate to the MAMs under certain conditions, MAMs seem to play essential roles in various processes. Initially, the MAM fraction was considered necessary for lipid synthesis and trafficking (long-chain fatty acid coenzyme A ligase-1 (FACL-1) and -4, phosphatidylserine synthase-1 (PSS-1) and -2, serine active site containing 1 (SERAC1), fatty acid transport protein 4 (FATP4), acyl-CoA desaturase,

phosphatidylethanolamine N-methyltransferase 2 (PEMT2) and many other proteins) and Ca^{2+} handling (e.g., IP3R, ryanodine receptor, sigma-1receptor (SIG1R), and promyelocytic leukemia protein (PML)). MAMs were later linked to the modulation of mitochondrial morphology (mitochondria-shaping proteins and chaperone proteins (MFN-1 and -2)), apoptosis (Bcl-2, hematopoietic cell-specific Lyn substrate1 (HCLS1)-binding protein 3 (HS1BP3)), mitochondrial contact site formation (VDAC and adenine nucleotide translocase (ANT)), protein folding (calnexin (CNX)) and sorting (phosphofurin acidic cluster sorting protein 2 (PACS-2)), ER stress (glucose-regulated protein 75-kDa (GRP75) and endoplasmic reticulum resident protein 44 (ERp44)), inflammation (inflammasome components: NALP3, adaptor ASC and thioredoxin interacting protein (TXNIP)), autophagy (pre-autophagosome/autophagosome markers (ATG14 and ATG5), and p66Shc) and the cellular response to oxidative stress (p66Shc protein and Ero1α). The presence of these critical proteins involved in crucial cellular processes explains why alterations in MAM composition are related to the pathogenesis of different disorders (Raturi and Simmen 2013), including type-2 diabetes (mTOR complex 2 (mTORC2) and MAM-associated Akt), and several neuronal-based diseases, such as Parkinson's disease and Huntington's disease, and neurodegenerative diseases, such as schizophrenia, dementia, and seizures. Moreover, MAMs have been proposed to be involved in familial Alzheimer's disease (FAD) and GM1-gangliosidosis.

The inflammasomes

Inflammation is a protective immune response mounted by the evolutionarily-conserved innate immune system to harmful stimuli, such as pathogens, dead cells, or irritants, and is tightly regulated by the host. Traditionally, innate immunity has been viewed as the first line of defence discriminating ‘‘self’’ (e.g., host proteins) from ‘‘nonself’’(e.g., microorganisms). However, emerging literature suggests that innate immunity actually serves as a sophisticated system for sensing signals of ‘‘danger,’’ such as pathogenic microbes or host-derived signals of cellular stress, while remaining unresponsive to non-dangerous motifs, such as normal host molecules, dietary antigens, or commensal gut flora. The innate immune system engages an array of germline encoded pattern-recognition receptors (PRRs) to detect invariant microbial motifs. PRRs are expressed by cells at the front line of defence against infection, including macrophages, monocytes, dendritic cells, neutrophils, and epithelial cells, as well as cells of the adaptive immune system. Activation of PRRs by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) triggers downstream signaling cascades and leads to production of type I interferon

(interferon-α and interferon-β) and proinflammatory cytokines. Of note, DAMP-triggered inflammation, which is particularly important in inflammatory diseases, is termed sterile inflammation when it occurs in the absence of any foreign pathogens (Chen and Nunez 2010).

A further set of intracellular PRR are the NOD-like receptors (NLRs) that recognize PAMPs, as well as DAMPs. The NLRs are comprised of 22 human genes and many more mouse genes because of gene expansion since the last common ancestor. 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. In addition to the NLRs, recent studies have revealed additional families of genes that can nucleate the assembly of inflammasomes: the AIM2-like receptor (ALR) family, which possesses a HIN200 DNA binding domain instead of an LRR (Schattgen and Fitzgerald 2011), and the RIG-I-like receptor (RLR) family (Ireton and Gale 2011). Several NLRP proteins (NLR subfamily with N-terminal PYD domain) and the protein NLRC4 (NLR family CARD domain-containing protein 4) have been shown to form an inflammasome upon stimulation with the respective activator. Hereby, oligomerization of the inflammasome sensors NLRP1, NLRP3, NLRP6 or AIM2 (HIN200 protein family) allows interaction of the respective N-terminal PYD with the PYD of the protein ASC (Apoptosis associated speck-like containing a CARD domain)(Bauernfeind, Ablasser et al. 2011) (Elinav, Strowig et al. 2011). ASC itself then recruits pro-caspase-1 via CARD-CARD interactions. According to its bipartite structure, composed of a PYD and CARD, ASC is often referred to as a bridging or adapter protein. The interaction of NLRPs, ASC and caspase-1 is based on homotypic interactions, which are common for death fold domains of the same subfamily, usually forming dimers or multimers. On the other hand, interactions across death domain subfamilies (e.g. CARD with PYD) are uncommon, even though somewhat surprising given their overall structural similarities.

Caspases are cysteine proteases that initiate or execute cellular programs, leading to inflammation or cell death. They are synthesized as inactive zymogens, and their potent cellular activities are tightly controlled by proteolytic activation. Caspases are categorized as either proinflammatory or proapoptotic, depending upon their participation in these cellular programs. The proinflammatory caspases are comprised of caspase-1, -11 and -12 in mouse and caspase-1, -4, and -5 in human

(Martinon and Tschopp 2007). Of the proinflammatory caspases, caspase-1 is the most fully characterized. Its catalytic activity is tightly regulated by signal-dependent autoactivation within multiprotein complexes called ‘‘inflammasomes’’ that mediate caspase-1-dependent processing of cytokines such as IL–1β (Martinon, Burns et al. 2002). Activation of the inflammasome is a key function mediated by the innate immune system, and recent advances have greatly increased our understanding of the macromolecular activation of inflammasomes. Inflammasomes have been linked to a variety of autoinflammatory and autoimmune diseases, including neurodegenerative diseases (multiple sclerosis, Alzheimer’s disease, and Parkinson’s disease) and metabolic disorders (atherosclerosis, type-2 diabetes, and obesity)(Strowig, Henao-Mejia et al. 2012). In inflammatory disease initiation, inflammasomes play either causative or contributing roles, and also exaggerate the pathology in response to host-derived factors.

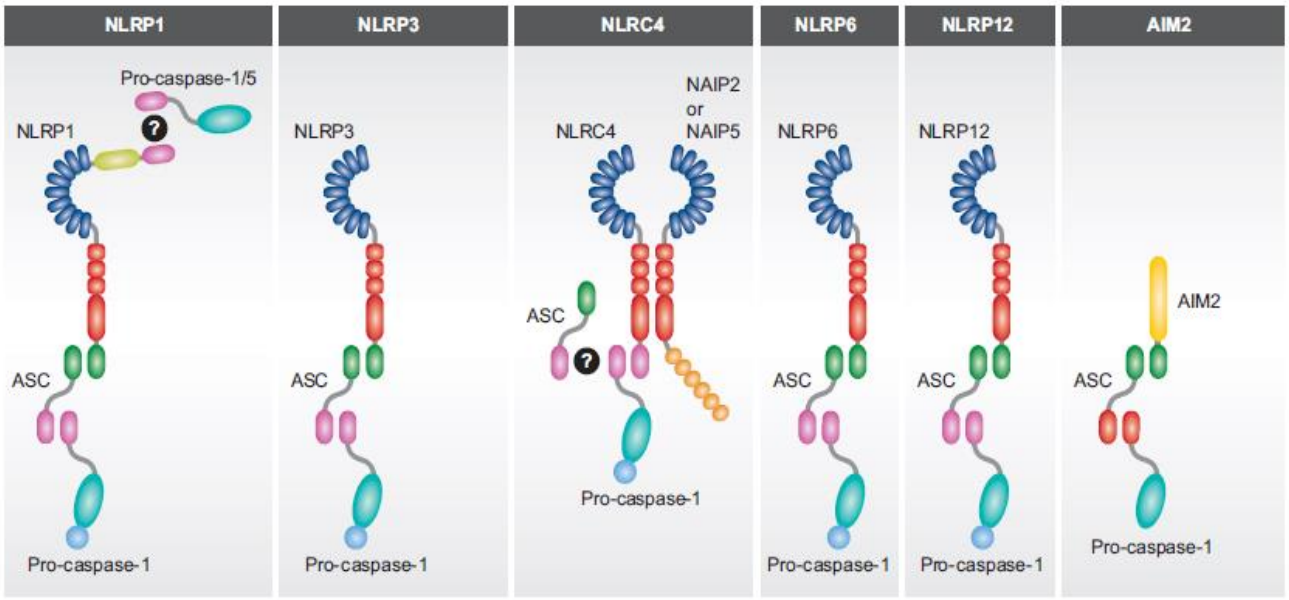


Figure 2: Assembled inflammasomes from (Lamkanfi and Dixit 2014)

NLRP3 inflammasome

The NLRP3 inflammasome (Figure 3) is activated in response to the widest array of stimuli. While all inflammasomes recognize certain PAMPs or DAMPs, the characteristic of NLRP3 is to be activated by extremely diverse stimuli making NLRP3 the most versatile inflammasome. The mechanisms of activation supported by the most studies include potassium efflux out of the cell, the generation of mitochondrial reactive oxygen species (ROS), translocation of NLRP3 to the mitochondria, the release of mitochondrial DNA or cardiolipin, or the release of cathepsins into the cytosol after lysosomal destabilization (Sutterwala, Haasken et al. 2014). However, not all of these events are induced by all NLRP3 agonists, so the precise mechanism of NLRP3 activation is still debated. Additionally, increases in intracellular calcium can activate the NLRP3 inflammasome (Katsnelson, Rucker et al. 2015), (Murakami, Ockinger et al. 2012).

In the ion flux model, changes in cytosolic levels of specific cations, such as K^+, Ca^{2+}, and H^+, are proposed to play a critical role in NLRP3 activation. Several NLRP3 activators were shown to directly induce potent ion fluxes. Extracellular ATP activates the ATP-gated ion channel P2X7 and triggers rapid K^+ efflux (Franchi, Kanneganti et al. 2007),(Petrilli, Papin et al. 2007). Nigericin creates a K^+ pore in the cell membrane (Mariathasan, Weiss et al. 2006); the influenza M2 protein triggers export of H^+ ions from the Golgi complex into the cytosol (Ichinohe, Pang et al. 2010); and high concentrations of extracellular Ca^{2+}, increase cytosolic Ca^{2+}, and cAMP(Lee, Subramanian et al. 2012), (Murakami, Ockinger et al. 2012) (Rossol, Pierer et al. 2012). Oxidative stress in the form of ROS has been widely implicated in NLRP3 activation and many NLRP3-activating stimuli, including ATP, alum, uric acid, and Nigericin, all induce ROS production(Schroder and Tschopp 2010). However, the precise role of ROS in NLRP3 inflammasome activation remains somewhat controversial. Initially, intracellular ROS produced via the NADPH oxidase system were thought to activate NLRP3; however, both mouse and human cells defective in NADPH oxidase show normal NLRP3 activation (Latz 2010) (van Bruggen, Koker et al. 2010). In addition, although ROS inhibitors were shown to inhibit NLRP3 activation, this effect was likely because of the role of ROS in NF-kB mediated up-regulation of NLRP3 and pro- IL–1β transcription (signal one) rather than its role in NLRP3 inflammasome activation itself (Bauernfeind, Horvath et al. 2009). Recently, a report suggested that increased amounts of ROS were sensed by a complex of Thioredoxin and Thioredoxin-interacting protein (TXNIP), with the latter binding to NLRP3 in response to oxidative stress (Zhou, Tardivel et al. 2010). Tw other studies suggest an alternative role for ROS in

inflammasome activation, in which oxidized mitochondrial DNA that is released from dysfunctional mitochondria can directly bind to and activate the NLRP3 inflammasome (Nakahira, Haspel et al. 2011)(Shimada, Crother et al. 2012). In contrast, ROS-independent mitochondria-derived cardiolipin was shown to bind and activate NLRP3 (Iyer, He et al. 2013). Finally, the NLRP3 inflammasome was proposed to sense lysosomal rupture during "frustrated" phagocytosis of crystalline or large particulate molecules, such as uric acid crystals, alum, silica, malarial hemozoin, hydroxyapatite, and amyloid-b (Schroder and Tschopp 2010)(Jin, Frayssinet et al. 2011).

Despite considerable efforts, there is currently no agreement in the field on a universal mechanism by which the NLRP3 inflammasome is activated. One likely reason for this is the diversity of stimuli that can lead to NLRP3 activation, many of which have multiple effects on cell physiology. Recently, NLRP3 activation was shown to be even more complex with the discovery of noncanonical inflammasome activation (Kayagaki, Warming et al. 2011). Although the canonical pathway activates NLRP3 directly, noncanonical activation involves the activation of caspase-11 by intracellular LPS released by rupture of bacteria-loaded phagolysosomes or by bacteria that actively enter the cytosol (Kayagaki, Wong et al. 2013) (Hagar, Powell et al. 2013). Notably, the initial discovery of caspase-11-dependent inflammasome activation resulted from the realization that the caspase-1-deficient mice used by most researchers are in fact double knockouts, as they also lack a functional caspase-11 owing to a passenger mutation in the 129 mouse strain, from which the embryonic stem cells to generate the mice were derived. Although its nature is currently unknown, presumably the formation of an inflammasome-like complex containing a cytosolic LPS-receptor precedes caspase-11 activation. Also, the mechanism through which caspase11 interplays with the NLRP3 pathway remains to be elucidated. A possible pathway is that activated caspase-11, which initiates pyroptosis in a similar manner as caspase-1, induces signals that are subsequently sensed by NLRP3.

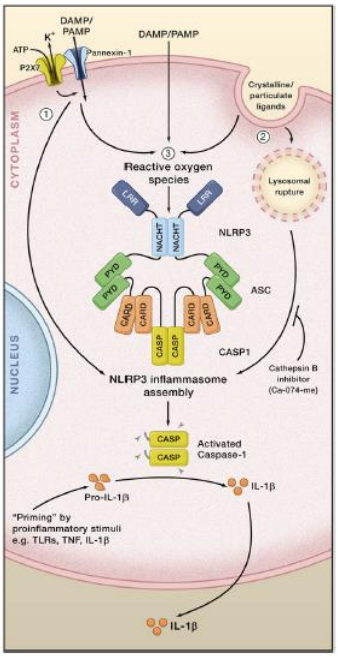


***Figure 3**: NLRP3 activation from(Schroder and Tschopp 2010)*

In most cell types, NLRP3 must be primed, and a prototypical example of such a priming event is the binding of LPS to TLR4. Priming has long been known to increase cellular expression of NLRP3 through NF-κB signaling (Bauernfeind, Horvath et al. 2009). However, recent findings have shown that priming rapidly licenses mouse NLRP3 inflammasome activation by inducing the deubiquitination of NLRP3 independent of new protein synthesis, while inhibition of deubiquitination inhibits human NLRP3 activation (Juliana, Fernandes-Alnemri et al. 2012), (Py, Kim et al. 2013). Once primed, NLRP3 can respond to its stimuli and assemble the NLRP3 inflammasome. Additionally, ASC must be linearly ubiquitinated for NLRP3 inflammasome assembly (Rodgers, Bowman et al. 2014). Current stimuli recognized as NLRP3 agonists that induce NLRP3 inflammasome formation include ATP, pore-forming toxins, crystalline substances, nucleic acids, hyaluronan, and fungal, bacterial, or viral pathogens (Lamkanfi and Dixit 2014). These stimuli can be encountered during infection, either produced by pathogens or released by damaged host cells.

MAMs as a critical site for inflammasome formation

In resting state, NLRP3 localizes at the endoplasmic reticulum (ER) and cytosol but when activated it relocates at MAMs (mitochondria associated membranes) (Zhou, Yazdi et al. 2011). Misawa et al. have observed that during inflammasome formation microtubules drive the perinuclear migration of mitochondria that results in subsequent apposition of ASC on mitochondria to NLRP3 on the endoplasmic reticulum (Misawa, Takahama et al. 2013). MAMs are the contact regions between ER and mitochondria and are considered specialized microdomains for Ca^{2+} transfer. Recently, an intense study of this subcellular fraction has revealed the identification of its role as platform for different proteins that control many cellular processes. Among these, inflammasome components and other inflammatory factors play a critical role in initiating inflammatory responses at the MAM interface. ASC protein is predominantly localized to the cytosol, but a small percentage is at the ER in unstimulated conditions. After stimulation with MSU (monosodium urate) or nigericin, ASC traslocates at the MAMs and also to a lesser extent in mitochondria in a NLRP3-dependent manner. Using PSORT bioinformatics analysis Subramanian and collagues have predicted that human NLRP3 should localize to mitochondria and N-terminal amino acids 2-7 are fundamental for association of NLRP3 at mitochondria (Subramanian, Natarajan et al. 2013). However, murine NLRP3 differs in N-terminal sequence and PSORT predicts it will localize in the cytosol, but it traslocates at mitochondria upon activation (Park, Juliana et al. 2013).

At the MAMs, NLRP3 detects signals from damaged mitochondria (Zhou, Yazdi et al. 2011) which are responsible of its activation. In particular, Zhou and colleagues have demonstrated that respiratory chain inhibitors (like complex I inhibitor rotenone) activate the inflammasome implicating loss of ΔΨm and ROS accumulation. Several studies have confirmed that NLRP3 agonists decrease ΔΨm and induce the mPTP opening through Ca^{2+} accumulation in mitochondria (Murakami, Ockinger et al. 2012). Moreover, calcium accumulation is the major cause of mPTP opening and consequent release of mitochondrial components that activate the inflammasome via NLRP3. Consistent with these observations, defective mitophagy has been shown to enhance inflammasome activation. Infact, inhibition of mitophagy/autophagy by 3-methyladenine (3MA) treatment or silencing the autophagy regulator beclin 1 and autophagy protein 5 (ATG5) in macrophages display an increased NLRP3 activation and IL-1β release upon stimulation with MSU and nigericin due to accumulation of damaged mitochondria and increased ROS generation (Wu, Li et al. 2016). However, treatment with the antioxidant 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC) blocked NLRP3 inflammasome activation and IL-1β secretion. Together with mtROS, also mitochondrial DNA (mtDNA) is one of the mitochondrial danger signals that interacts directly with NLRP3 and AIM2 and oxized mtDNA (ox-mtDNA) interacts specifically with NLRP3 in cells with ATP and nigericin treatment (Shimada, Crother et al. 2012). Moreover, IL-1β release is drastically reduced in AIM2 KO macrophages in response to mtDNA. Importantly, IL-1β release is higher after ox-mtDNA stimulation than mtDNA and AIM2 does not seem to be involved in ox-mtDNA–mediated inflammasome activation (Zhou, Tardivel et al. 2010).

Although these numerous studies support the idea that mitochondrial dysfunctions and reactive oxygen species (ROS) is closely associated with inflammasome assembly and activation, there is a lack of understanding how these factors trigger NLRP3 activation. Furthermore, accumulation of mitochondrial ROS is accompanied by oxidation of thioredoxin-1 (TRX-1) which dissociates from thioredoxin-interacting protein (TXNIP also called VDUP1). The consequence is the interaction of TXNIP with NLRP3 (via its NBD and/or LRR domain) and NLRP3 activation. In addition to this interaction, Zhou and collagues have been demonstrated that upon stimulation with uric acid crystals TXNIP redistributes to MAMs/mitochondria where it binds NLRP3. TXNIP is induced by endoplasmic reticulum (ER) stress, which further demonstrates a potential role for ER stress in innate immunity. In particular, the expression of TXNIP induced by ER stress is under the control of the IRE1α and PERK-eIF2α pathways of the UPR (Oslowski, Hara et al. 2012). In different studies it is proposed that ER stress activates the NLRP3 inflammasome in a K+ efflux- and ROS-

dependent manner that may also affect the mitochondria suggesting the critical role played by the MAM as a site for signals exchange between the two organelles.

Additionally, mitochondrial anti-viral signaling protein (MAVS) is suggested to recruit NLRP3 to mitochondria in response to viral infection. MAVS is a crucial regulator of mitochondrial innate immune system. In resting state, MAVS expression in macrophages is not involved in maintaining mitochondrial architecture considering that MAVS deficient cells have normal ER mitochondria-associated membranes (Subramanian, Natarajan et al. 2013), although MAVS could form prion-like aggregates via engaging RIG-I (RNA-trapped retinoic acid inducible gene I) which may facilitate the NLRP3 oligomerization and consequent assembly of inflammasome. Recently, multiple works focus on the other inflammasomes studing the possible pathway and activation mechanisms. For instance, D'Osualdo et al. show that expression of NLRP1, a core inflammasome component, is specifically up-regulated during severe ER stress conditions in human cell lines suggesting a probable involvement of MAMs.

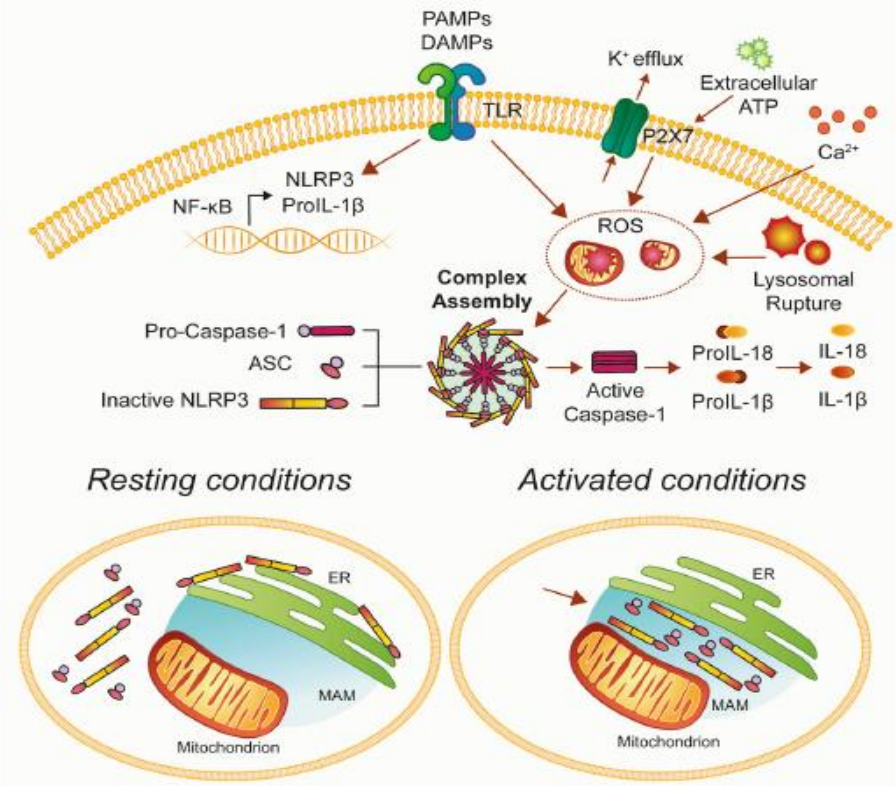


***Figure 4**:* Mitochondria-ER associated membranes are important sites for NLRP3 activation from (Missiroli, Patergnani et al. 2018)

NLRP3 inflammasome in cancer

Inflammation plays an important role in many cancers. However, the role of inflammasomes in cancers is controversial. This is probably due to heterogeneity of cancer cells and the different cell types associated with cancer such as cancer-associated fibroblasts, tumor-infiltrating immune cells, endothelial cells, adipocytes and pericytes as well as various chemokines and cytokines (Grivennikov, Greten et al. 2010), (Coussens, Zitvogel et al. 2013). Moreover, hypoxic microenvironment affects the function of inflammasomes in cancer. Several reports indicate that inflammasomes and IL-1 promote inflammation-induced skin cancer in a two-stage carcinogenesis-induced papilloma model. Drexler et al. found that IL-1R- and caspase-1-deficient mice were partially protected against skin cancer induced by 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) treatments (Drexler, Bonsignore et al. 2012). Caspase-1 and IL-1R KO mice have a later onset and reduced incidence of tumors after DMBA/TPA treatments, compared with WT mice. In an independent study, Chow et al. also found that NLRP3 KO mice exhibited reduced skin papilloma lesions in the inflammation-induced skin cancer model (Chow, Tschopp et al. 2012). These data suggest that inflammasome-dependent IL-1β production contributes to the development of epithelial skin cancer.

A recent study has demonstrated that inflammasome and IL-1β play a critical role in promoting tumor growth and metastasis in breast cancer (Guo, Fu et al. 2016). These results show that tumor growth is associated with elevated levels of IL-1β in tumor microenvironments in mouse mammary tumor models and human breast cancer tissues. Moreover this study also shows that inflammasome activation promotes the infiltration of myeloid cells such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells. Daley et al. show that NLRP3 signaling drives macrophage-induced adaptive immune suppression in pancreatic carcinoma and that NLRP3 promotes the expansion of immunosuppressive macrophages (Daley, Mani et al. 2017). Inflammasome activation and IL-1 signaling are also implicated in the development of asbestos-induced mesothelioma (Kadariya, Menges et al. 2016), (Thompson, MacPherson et al. 2017). On the other hand, Wei et al. found that inflammasomes might suppress the development of human liver cancer (Wei, Mu et al. 2014). Emerging evidence indicates that inflammasomes play an important role in non-alcoholic fatty liver disease, a spectrum of metabolic disorders ranging from steatosis (NAFL) to steatohepatitis (NASH) to cirrhosis.

Moreover, caspase-1, IL-1β, and IL-18 are overexpressed in lung cancer tissues and AIM2 inflammasome is upregulated in NSCLC, whereas high levels of NLRP3 inflammasome are reported in small-cell lung cancer (SCLC) (Kong, Wang et al. 2015). Therefore, inflammasomes are

associated with the biological features of lung cancer. Besides, different inflammasomes are expressed in distinct cell lines and tumor tissues suggesting that their transcription was cell and tissue specific. Therefore, inflammasomes in different subtypes of lung cancer are associated with their histological classification, grading, tumor invasion and chemoresistance (Kong, Wang et al. 2015). The interaction between the tumor microenvironment and various cytokines and cell types is vital for lung cancer growth and progression. In C57BL/6 mice model with murine Lewis lung carcinoma cell xenograft, secretion of IL-1β from the tumor cells induced abundant vasculature and cytokines such as vascular endothelial growth factor (VEGF), macrophage-inflammatory protein-2 (CXCL2) and hepatocyte growth factor (HGF). This contributes to angiogenesis (Vempati, Popel et al. 2014) and chemotactic migration of macrophages(Sherry, Horii et al. 1992). While cultured mouse Lewis lung carcinoma cells do not secrete HGF, coculturing with stromal fibroblasts and tumor infiltrating cells produces HGF (Saijo, Tanaka et al. 2002). This suggests that the tumor microenvironment as well as stromal fibroblasts and tumor infiltrating cells are crucial in cancer progression. In patients with glioblastoma, the expression of NLRP3 inflammasome predicts poor survival in patients that have undergone radiotherapy (Li and Liu 2015). Caspase-1 is increased in both glioblastoma tissues and glioma cell lines, U87 and T98G, whereas miR-214 inhibits glioblastoma cell proliferation and migration by suppressing caspase-1 (Jiang, Yao et al. 2017). However, the role of IL-1β in regulating glioblastoma progression is still controversial. IL-1β promotes proliferation, migration, and invasion of human glioma cells (Fathima Hurmath, Ramaswamy et al. 2014) and increases the cancer stem cell phenotype in murine and human proneural glioma stem cells. However, IL-1β inhibits the transactivation activity of hypoxia-inducible factor 1 (HIF-1), thereby downregulating adrenomedullin (AM) expression and inducing glioma cell apoptosis (Sun, Depping et al. 2014). Therefore, cancer microenvironment such as degree of hypoxia can influence cancer outcomes.

In the future, the mechanism of the activation of the various inflammasomes and their effect on the immune system needs to be addressed in greater detail. The various signaling mechanisms that regulate the activation of different inflammasomes also need to be defined in greater detail. While pyroptosis is vital in anticancer treatment, oxidative stress, mitochondrial dysfunction and release of inflammatory factors are also involved in carcinogenesis.

P2X7R in inflammation

P2X7 as NLRP3 inflammasome activator

The association of the P2X7R with inflammation and immunity is long standing. Before it was cloned in 1996, investigators were intrigued by its dramatic effects on lymphocyte and macrophage responses (Steinberg and Silverstein 1987), (Di Virgilio, Bronte et al. 1989), until the discovery of the NLRP3 inflammasome has finally placed P2X7R in the appropriate context. However, evidence suggests that role of P2X7R might be more profound. For example, genetic ablation of P2xr7 dramatically ameliorates all functional and disease parameters in a mouse model (the MDX mouse) of Duchenne muscular dystrophy (Sinadinos, Young et al. 2015). Furthermore, P2X7R blockade inhibits allograft rejection (Vergani, Tezza et al. 2013), graft-versus-host disease (Wilhelm, Ganesan et al. 2010), choroidal neovascularization, geographic atrophy, sterile liver inflammation (Fowler, Gelfand et al. 2014) and retinal ganglion cell loss due to experimentally elevated intraocular pressure (a model of glaucoma) (Resta, Novelli et al. 2007). Macrophage P2X7R might also be implied in spreading of human immunodeficiency virus (HIV) infection because its stimulation by autocrine ATP promotes release of HIV-laden microvesicles (Graziano, Desdouits et al. 2015). Finally, the P2X7R has been suggested to bridge inflammation and coagulation since stimulation of macrophage P2X7R drives enhanced expression and release of microvesicle-associated tissue factor (TF), thus producing a high pro-thrombotic response (Moore and MacKenzie 2007), (Baroni, Pizzirani et al. 2007). The P2X7R promotes release of pro-inflammatory factors, such as IL-6 from human fibroblasts, mouse microglia and mouse mast cells (Solini, Chiozzi et al. 1999), (Kurashima, Amiya et al. 2012), (Shieh, Heinrich et al. 2014), TNF from human dendritic cells (DCs) and mouse microglia (Ferrari, La Sala et al. 2000), and activation and shedding of matrix metalloproteases from human peripheral-blood mononuclear cells (Gu and Wiley 2006) (Gu, Bendall et al. 1998). Converging evidence shows that P2X7R promotes expression of several chemokines, such as monocyte chemoattractant protein 1 (MCP-1, CCL2) in rat astrocytes and mouse mast cells (Panenka, Jijon et al. 2001),IL-8 in rat C6 glioma cells CC-chemokine (Wei, Ryu et al. 2008) ligand 3 (CCL3) in mouse microglial MG-5 cells (Shiratori, Tozaki-Saitoh et al. 2010), and CXCL2 in mouse microglia and mast cells (Kurashima, Amiya et al. 2012). Several intracellular signal transduction pathways in addition to ion fluxes are activated downstream of P2X7R, e.g. mitogen-activated kinases (MAPK), (Bradford and Soltoff 2002)phospholipase C, D, and A2, and neutral and acidic sphingomyelinases (Humphreys and Dubyak 1996), (Alzola, Perez-Etxebarria et al. 1998), (Garcia-Marcos, Pochet et al. 2006). But

more importantly, P2X7R has a special place in IL-1β release for its role in NLRP3 inflammasome activation.

P2X7R was initially described as ATP^{4-} receptor or P2Z in mastcells, lymphocytes, and macrophages and thought to be mainly expressed in immune cells (Bianco, Pravettoni et al. 2005), (Cockcroft and Gomperts 1979), but this view has substantially changed as it is clear that most mouse and human cells express the P2X7R. Nevertheless, immune cells express this receptor to a high level and it is in the immune system that its function is best understood. Early experiments by Gabel and co-workers showed that extracellular ATP is a strong stimulus for the release of mature IL-1β from mouse macrophages. This was thought to be due to the potent cytotoxic effect caused by ATP on these cells. A few years later, P2X7R was identified as the receptor mediating the ATP effect (Di Virgilio, Bronte et al. 1989), (Perregaux and Gabel 1994, Ferrari, Villalba et al. 1996) and highlighted the key role of K^+ efflux in ATP-mediated caspase-1 activation and therefore in pro-IL-1β processing. The discovery of the NALP1 (NLRP1) inflammasome and the identification of additional members of the family (namely NLRP3) finally placed P2X7R in a logical pathophysiological context providing the molecular mechanism that couples P2X7R activation to IL-1β processing (Ferrari, Villalba et al. 1996). These observations prompted the proposal of the ‘‘two signal model’’ for IL-1β release, the first being stimulation of Toll-like receptors (TLRs) leading to accumulation of cytoplasmic pro- IL1β and the second ATP-dependent stimulation of P2X7R promoting inflammasome-mediated caspase-1 activation (Ferrari, Chiozzi et al. 1997), (Martinon, Burns et al. 2002), (Ferrari, Pizzirani et al. 2006). In LPS-primed mouse peritoneal and bone marrow-derived mouse macrophages as well as in mouse microglia cells, ATP stimulation of P2X7R strongly accelerates mature IL-1β release (Perregaux and Gabel 1994). This is also true of human monocyte and macrophage cells (Perregaux, McNiff et al. 2000) and of mouse and human DCs, although more recent findings suggest that in mouse DCs the second stimulus (P2X7R activation) is dispensable during prolonged incubations in the presence of LPS. Requirement of P2X7R expression for in vivo IL1β release has been tested in a few mouse models. In P2X7-deficient mice intraperitoneally (i.p.) injected with LPS, no IL1β was detected in the peritoneal fluid (Solle, Labasi et al. 2001).

Other investigators observed reduced levels of brain IL1β in P2X7R-deficient mice systemically stimulated with LPS, and have lower serum and intratumor IL1β levels in tumor-bearing P2X7R-deficient mice compared to wild-type (Adinolfi, Capece et al. 2015). Interleukin-18 secretion is also promoted by P2X7R activation, but the coupling mechanism is less clear. Pore function is a requirement for NLRP3 activation suggesting that a large reduction of intracellular K^+ is needed.

The accompanying intracellular Ca^{2+} increase has been implicated, but it is doubtful whether Ca^{2+} as well as Na^{+} influx have any role in this process, and thus the decrease of intracellular K^{+} is a major driver of P2X7R-dependent NLRP3 inflammasome activation but the molecular mechanism involved is unknown (Munoz-Planillo, Kuffa et al. 2013).

Recent evidence suggests that P2X7R and NLRP3 might physically interact at discrete subplasmalemmal cytoplasmic sites in both mouse microglia and mouse peritoneal macrophages. The crucial role of intracellular ion changes in NLRP3 inflammasome activation is supported by the recent observation that the intracellular K^{+} decrease caused by opening of plasma membrane channels (P2X7R included) enhances NLRP3 interaction with the Nimarelated kinase (NEK)7 protein, which is a non-dispensable NLRP3 inflammasome activator. Macrophage stimulation in high (50 mM) K^{+} medium prevents NEK7 association with NLRP3 and formation of high-molecular-mass inflammasome complex. NEK7 is required for P2X7R-driven inflammasome assembly as ATP-stimulated NLRP3 activation is abrogated in NEK-deficient bone-marrow-derived macrophages (He, Zeng et al. 2016). Other inflammasome subtypes (e.g., NLRP2,NLRC4, NLRP6, and AIM2), with the possible exception of NLRP1 and NLRP2, are not activated by P2X7R or by a K^{+} drop to any significant extent (Di Virgilio 2013).

Other agents reported to trigger NLRP3 activity, such as reactive oxygen species and cathepsin, are also produced by P2X7R stimulation, but K^{+} depletion is the only stimulus which in most experimental settings appears to be both necessary and sufficient, as NLRP3 inflammasome activation is fully obliterated by an increase in the extracellular K^{+} concentration, i.e., by the reduction of the driving force for K^{+} efflux. The molecular mechanism by which K^{+} depletion causes inflammasome assembly is obscure.

Since several microbial toxins are known to activate the inflammasome by causing depletion of intracellular K^{+} (Dinarello 2002), a perturbation in the homeostasis of this cation might be a general mechanism for alerting immune cells to the presence of pathogens. P2X7R has a special association with IL1β since it is not only a major trigger of its processing, but also a strong stimulus for externalization. Interleukin-1β is an atypical cytokine that lacks a secretory piece, thus it does not follow the canonical endoplasmic reticulum route for release into the extracellular environment.

Several alternative mechanisms have been suggested, such as passive release following cell death and secretion via modified lysosomes, exosomes, or plasma membrane-derived microvesicles (MacKenzie, Wilson et al. 2001),(Lopez-Castejon and Brough 2011, Piccioli and Rubartelli 2013). P2X7R is a main driver for all these routes further emphasizing its fundamental contribution to the

release of bioactive IL1β.The ability of P2X7R to trigger the release of multiple proinflammatory factors, some of them bonafide endogenous pyrogens, raises the issue of its involvement in a complex systemic response such as fever. An early paper by Gourine et al. showed that P2X7R blockade attenuated the febrile response induced in rats by LPS inoculation (Gourine, Poputnikov et al. 2005).

P2X7 receptor in carcinogenesis

A large body of literature has investigated the role of P2X7R in cancer (Roger, Jelassi et al. 2015), in particular the crucial role of P2X7R in immunogenic cell death. It is a cancer cell death triggered by some anti-cancer drugs or by radiotherapy whereby dying cells express and release factors that potentiate DC ability to present tumor antigens to T lymphocytes (Galluzzi, Buque et al. 2017). It was initially shown by Kroemer and coworkers that tumor cells killed by anthracyclin (but not other anti-cancer drugs such as mitomycin C) administration elicited a strong caspase-dependent anti-tumor response that favored tumor regression (Casares, Pequignot et al. 2005). Immunogenicity of anthracyclin-stimulated cell death depends on the release of intracellular molecules such as calreticulin, high mobility group box 1 (HMGB1) protein, and ATP (Obeid, Tesniere et al. 2007, Ghiringhelli, Apetoh et al. 2009). Lack, or pharmacological blockade, of P2X7R severely hampers immunogenic cell death in vitro and in vivo, likely due to inability of P2X7R-less DCs to release IL1β. A feature of some cancer cells is the high level of expression of P2X7R, which, depending on the tumour type, can mediate either proliferation or cell death(Ferrari, Malavasi et al. 2017). Thus, P2X7R activation may have effects on antitumour immunity that are opposed to a direct effect on tumour growth. It was also pointed out that there is increased release of ATP, promoting cancer cell migration and metastasis. High levels of extracellular ATP accumulate in tumour interstitium and high ATP doses inhibit migration of endothelial cells from human breast carcinoma, via the activation of P2X7R (Avanzato, Genova et al. 2016). Human pancreatic duct adenocarcinoma cells in vitro express high levels of P2X7R protein and AZ10606120 (a potent P2X7 antagonist) inhibited cell proliferation (Giannuzzo, Saccomano et al. 2016).

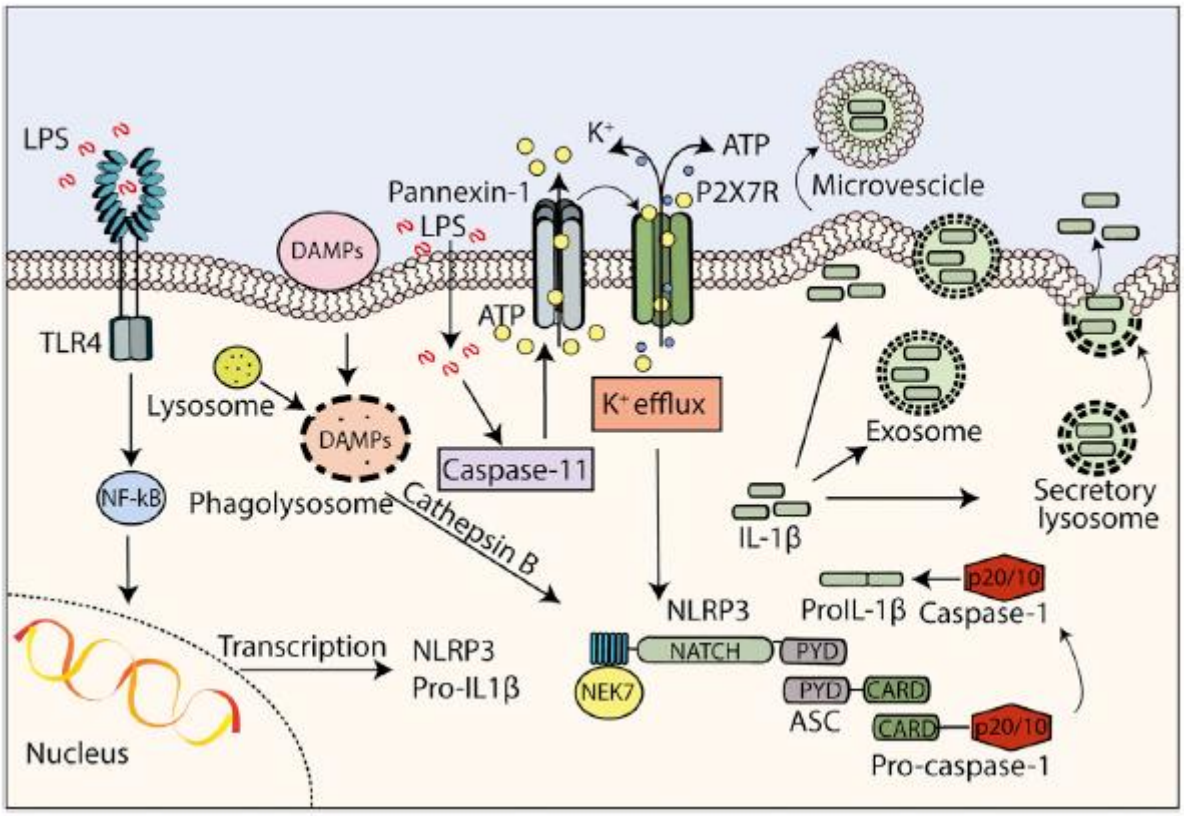


Figure 5: Central role of P2X7R in IL-1β processing and release

Radiotherapy and the immune system

Radiotherapy (RT) is an important modality used in the treatment of more than 50% of cancer patients. However, despite sophisticated techniques for radiation delivery as well as the combination of radiation with chemotherapy, tumors can recur. Thus, any method of improving the local control of the primary tumor by radiotherapy would produce a major improvement in the curability of cancer patients. The central dogma of traditional radiobiology states that the cytotoxic effects of radiation on tumor cells are primarily due to the production of DNA double-strand breaks followed by some form of cell death, including apoptosis, necrosis, autophagy, mitotic catastrophe, or replicative senescence. In accordance, DNA damage and subsequent tumor cell kill has been ascribed to 4 basic principles (known as the 4 “Rs” of radiobiology): reassortment of tumor cells into radiosensitive phases of the cell cycle (G2/M), reoxygenation of hypoxic areas within a tumor, repair of sublethal DNA damage, and repopulation of surviving tumor cells; whereby, the manipulation of each factor alters tumor cell radiosensitivity. However, in the context of the tumor

microenvironment and host antitumor immunity, the scope of this aforementioned traditional view is limited.

RT uses high dose radiation to treat cancer. It works by destroying malignant cells in the area that is treated. The main aim of RT is to increase local control of the tumor, minimizing damage to the patient's normal tissue. Exposure to ionizing radiation of normal tissue and tumors has immediate and persisting consequences that span from modest inflammatory changes to distinct forms of programmed cell death. It's known that in cancer management, natural antitumor immune responses are detectable often only in a fraction of patients across multiple malignant neoplasms. In most patients, however, radiation therapy can convert the tumor into an in situ vaccine able to induce a de novo anticancer immune response (Schumacher and Schreiber 2015). The dose and fractionation applied determines the degree and type of cell death in a tissue-specific fashion (Golden, Pellicciotta et al. 2012). The radiation-induced cell death is followed by the release of tumor antigens together with pro-inflammatory signals. First, it was demonstrated that cell death is an efficient process to transfer antigens from tumor cells to DCs and that DCs are required to activate tumor-specific T cells (Schuler and Steinman 1997, Albert, Sauter et al. 1998). Moreover, during the past 5 years, a functional redefinition of cell death, based on its effects on immune cells (ie, tolerance or activation) has emerged. Molecular signals required to achieve an "immunogenic cell death" have been established (Galluzzi, Maiuri et al. 2007, Ma, Kepp et al. 2010). To date, they include: 1) cell surface translocation of calreticulin (an endoplasmic reticulum resident protein); 2) extracellular release of high-mobility group protein B1 (HMGB1, a non histone nuclear protein), and 3) release of ATP (the primary unit of cellular energy transfer) (Apetoh, Ghiringhelli et al. 2007, Obeid, Tesniere et al. 2007).

Current evidence indicates that ionizing radiation and some, but not all, commonly used chemotherapy agents successfully induce each of these steps and culminate in immunogenic cell death. Additional or alternative signals and pathways remain an area of active investigation. However, the exact contribution of immunogenic cell death to the successful results of standard anticancer therapy observed in the clinic remains to be quantified.

Anyway, radiotherapy strongly modulate the immunity response (Ma, Kepp et al. 2010). Immunostimulatory activity can occur through release from RT-damaged cells of DNA (which enhances the activity of antigen-presenting cells) and ATP, which activates the inflammasome with release of IL-1ß (Formenti and Demaria 2013).

The tumor suppressor PML

PML structure

The promyelocytic leukemia (PML) protein is a tumor suppressor frequently lost or aberrant in hematopoietic malignancies and human solid tumors (Gurrieri, Capodieci et al. 2004, Salomoni, Bernardi et al. 2005). Its gene was originally identified at the break point of the t(15;17) chromosomal translocation of acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia. As a consequence of this translocation, PML fuses to the retinoic acid (RA) receptor alpha (RARα) gene. Two fusion genes are generated encoding PML-RARα and RARα-PML fusion proteins, which coexist in the leukemic cells, blocking hematopoietic differentiation (Pandolfi, Grignani et al. 1991). PML has therefore, become the object of intense research on the basis of this premise. Since then, PML has been shown to regulate diverse cellular functions, such as transcriptional regulation, DNA-damage response, sumoylation process, cellular senescence, neoangiogenesis and apoptosis (Salomoni, Ferguson et al. 2008).

Located on the q arm of chromosome 15, the 53 kbp PMLgene is a single gene member of the tripartite motif (TRIM) family. It is organized into 9 exons, and through alternative splicing of its C-terminal exons, it can give rise up to 7 known PML protein isoforms (Fig. 6). All the isoforms share the same N terminal part that spans 418 amino acids, where the domains that characterize the PML protein are located. In particular, at the N-terminus of the protein the RBCC/TRIM motif is present, which, following a scheme quite common in the majority of TRIM proteins, includes three zinc-binding domains, a RING (really interesting new gene) finger, a B-box type 1 and a B-box type 2, and a coiled-coil region (Jensen, Shiels et al. 2001). The RBCC domain controls not only the capability of PML to dimerize and multimerize (Reymond, Meroni et al. 2001) but also its interaction with a huge number of partners. In addition to the N-terminus, which is common to all the isoforms, PML protein harbors other domains that can be present only in some of the isoforms. They include nuclear localization signal (NLS), nuclear export signal (NES) and several sites for post-translational modifications, such as phosphorylation, ubiquitination, and SUMO-interacting motifs (SIM) for SUMOylation (Schmitz and Grishina 2012). PML isoforms are distinguished into seven classes on the bases of their length, and numbered accordingly, where PML I is the longest and PML VII the shortest. Typically, NLS, located on exon 6, is expressed in all the isoforms from I to VI, (with the exception of PML IVc). These isoforms are also termed as nuclear isoforms and can localize to the nucleus and organize multiprotein complexes termed PML nuclear bodies (PML-

NB). Also, PML I is the only nuclear PML that contains a NES and consequently is expressed both at cytoplasmic and nuclear level. On the other hand, the cytoplasmic isoforms PML VII and PML IVc are devoid of NLS and expressed only at cytoplasmic level. It is to note that for each class up to three variants (a, b and c) may be described, each one missing specific parts of exons, with the potential generation of new cytoplasmic forms (Jul-Larsen, Grudic et al. 2010).

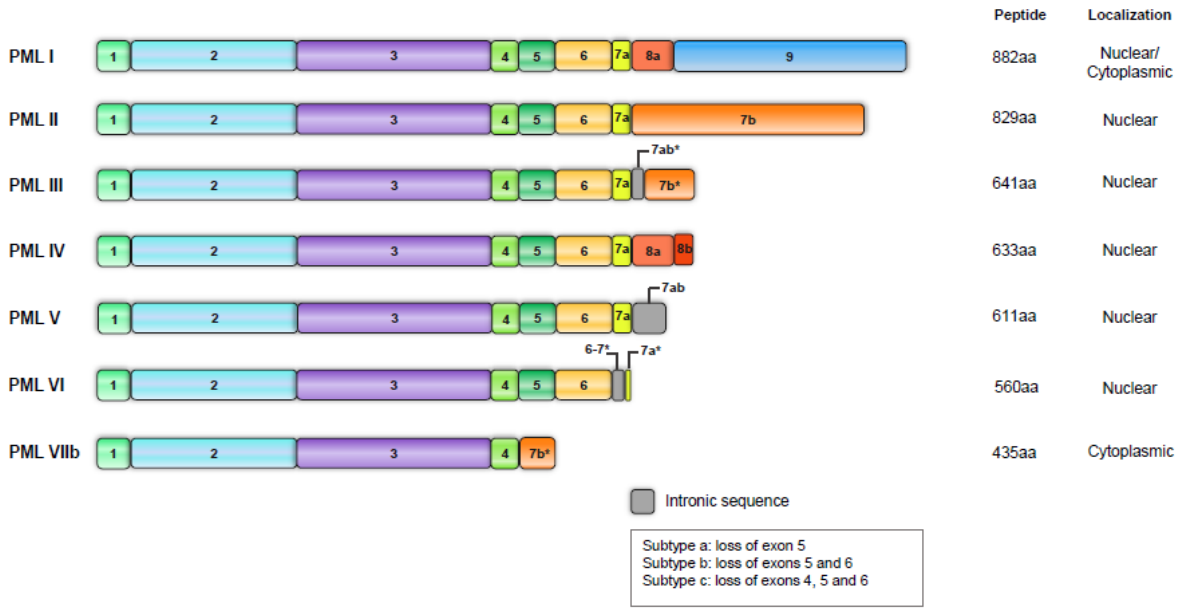


Figure 6: PML isoforms and their localizations

PML is typically concentrated in subnuclear macromolecular structures termed PML-nuclear bodies (PML-NBs), of which PML is the essential component, however an extranuclear localization of PML has been described (see Extra-nuclear PML function chapter). PML-NBs have a diameter of 0.2-1 μm and the shape of a doughnut. PML-NBs are multiprotein dynamic structures that undergo significant changes in number, size and position, particularly in response to cellular stress (Lallemand-Breitenbach and de The 2010). They critically depend on PML to be correctly assembled (Shen, Lin et al. 2006). PML functionally interacts with a large number of proteins within PML-NBs; some are in direct physical contact with PML, while others are not. PML SUMOylation and noncovalent binding of PML to SUMOylated PML through the SUMO-binding motif constitutes the nucleation event for subsequent recruitment of SUMOylated proteins and/or proteins containing SUMO-binding motifs to the PML-NBs.

PML and pro-apoptotic signaling

Many molecular pathways regulated by PML are involved in the apoptotic process. PML and PML-NB control the levels of nuclear p53 by interacting with and sequestering Mdm2, which is the major p53 E3 ubiquitin ligase and acts decreasing p53 levels by targeting it to proteasomal degradation. Also, DAXX, which is a principal PML interactor (Khelifi, D'Alcontres et al. 2005), has been shown to control p53 ubiquitylation by inhibiting MDM2 degradation (Tang, Qu et al. 2006, Song, Song et al. 2008). Furthermore, PML controls genotoxic-induced cell death via ataxia-telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2). ATM is a serine-threonine kinase, activated by DNA double-strand breaks, which has among its targets Chk2, while Chk2 is a DNA damage-induced kinase which, recruited at PML-NB, phosphorylates p53 preventing its Mdm2-mediated proteolysis (Yang, Kuo et al. 2002). After UV treatment, and more in general after DNA damage, several proteins like the acetyltransferases CBP/p300 and the homeodomain-interacting protein kinase-2 (HIPK2), along with the tumor suppressor AXIN, relocate into PMLNB where they can acetylate or phosphorylate p53. These post-translational modifications prevent p53 degradation,induce its transcriptional activity, leading to apoptosis or senescence. On the other hand, variation in the amount of PML present into NB remodels the PML-NB composition and can also recruit p53-inactivating enzymes like as then NAD-dependent deacetylase sirtuin-1 (Langley, Pearson et al. 2002), indicating that the regulative activities of NBs are strongly dependent on their structure and composition, which further strengthen the importance of PML downregulation observed in a wide number of tumors. PML affects cell death induction also trough p53-independent pathways. The specific nuclear PML IV sensitizes cells to TNFα-induced apoptosis. This also occurs in the p53-negative Saos-2 cell line like in other cell models, triggering the death receptor-dependent apoptotic pathway, which recruits caspase-8, -7, and -3.

Consistently, the loss of PML function, which occurs in *Pml* KO mouse embryonic fibroblasts renders cells resistant to TNFα-induced apoptosis (Wu, Xu et al. 2003). Furthermore, PML IV acts as a repressor of the transactivation function of NF-kB by interacting with p65/RelA and preventing its binding to the NF-kB target sequence (Kuwayama, Matsuzaki et al. 2009).

Extra-nuclear PML function

Cytoplasmic localization of PML has been described to occur in two diferent manners, one implying a constitutive association of cytoplasmic isoforms (cPML) to ER or mitochondria, the other seeing a temporary cytoplasmic relocation of nuclear isoforms, due to cell cycle dynamics or

viral infection. During the M-phase of the cell cycle, together with the dissolution of nuclear structure, nuclear PML involved in the structure of PML-NB can induce an alternative and temporary localization into specific cytoplasmic complexes, where it can be transiently stocked. During the G1 phase, PML moves to the reassembled nuclear structure where it participates in the reconstitution of NB (Dellaire, Eskiw et al. 2006). Up to now, it is not clear if this temporary cytoplasmic distribution of PML isoforms entails active participation in cell cycle dynamics. Also, during viral infection, nuclear PML can redistribute to the cytoplasm to express its antiviral proficiency binding to viral components at cytoplasm level, to reduce viral protein transduction and translation (Kentsis, Dwyer et al. 2001, Turelli, Doucas et al. 2001).

In addition to the redistribution of nuclear isoforms, cytoplasmic localization of PML can be supported by specific isoforms, expressing NES or lacking NLS. In the cytoplasm, PML can carry on its tumoral suppressive action operating in association with different subcellular organs. Several reports highlighted as PML can control TGF-β signaling, which can induce cell cycle arrest and senescence, and is a known up-regulator of the two cyclin inhibitors p15 and p21. TGF-β signaling is strongly impaired in Pml KO mice (Lin, Bergmann et al. 2004), but this deficiency can be restored only upon transfection of cytosolic and not with nuclear isoforms of PML, suggesting that cytoplasmic PML can carry on specific antitumor activity. This view is further reinforced by supporting evidence which shows that nuclear sequestration of cPML, operated by TG-interacting factor and c-Jun, inhibits TGF-β signaling (Seo, Ferrand et al. 2006). This interferes with the formation of the complex formed by TGF-β, its receptors, Smad protein and the adaptor protein, SARA, the Smad anchor for receptor activation, whose phosphorylation is regulated by cPML.

Paradoxically, in the presence of dysfunctional TGF-signaling like in prostate cancer, the cytoplasmic localization of PML I and its nuclear export carrier, exportin 1, promotes epithelial to mesenchymal transition. Cytoplasmic PML I localization was found in patients with poor prognosis, strengthening the activator role of PML on TGF-β signaling, up to constituting a pro-tumoral attitude in a pathological environment (Buczek, Miles et al. 2016). Presence of cytoplasmic mutants of PML has been reported (Gurrieri, Capodieci et al. 2004). cPML can have additional regulative roles. cPML was shown to be associated to and to be enriched at ER and mitochondria-associated membranes (MAMs) (Pinton, Giorgi et al. 2011). The latter are specialized contact sites between ER and mitochondria (Giorgi, Missiroli et al. 2015), which organize an integrated scaffold of proteins able to warrant not only structural tethering among the vicinal structures, but also integrate mitochondrial and reticular physiology, regulating phospholipids and cholesterol

exchange, and being involved in autophagy and mitophagy regulation (Giorgi, Missiroli et al. 2015).

Furthermore, MAMs represent a special regulatory site in calcium transfer from ER and cytosol (Marchi, Bittremieux et al. 2017). In fact, MAMs can fulfill the role of specialized buffering sites, relying on an enriched presence of the low-affinity VDAC1 channel (Danese, Patergnani et al. 2017), which can create a calcium microdomain at MAM level, which stimulates the function of the MCU transporter and other related proteins, allowing for fast calcium transfer into mitochondria (Missiroli, Danese et al. 2017).

Far from being just a mere buffering activity, the Ca^{2+} transfer operating at MAMs can influence and shape the calcium concentration variations occurring at the perireticulum space, regulating the action of IP3Rs and SERCA pumps, whose activity is influenced by vicinal calcium concentrations. A complex equilibrium, based on the Ca^{2+} -mediated interplay and feedback loops, occurs between ER and MAMs, which is further tightened by the fact that Ca^{2+} entry in the mitochondria affects oxidative phosphorylation, which in turn provides ATP to fuel SERCA activity. Notably, the Ca^{2+} entry in the mitochondria regulates mitochondrial activity only inside a defined physiological range of Ca^{2+} concentrations. Excess of Ca^{2+} entry triggers the dissipation of mitochondrial potential, the activation of mitochondrial transition pore and, at the end, the intrinsic pathway of apoptosis (Pedriali, Rimessi et al. 2017). ER calcium pumps assume a central role in programmed cell death, in particular, IP3R3 whose activity results not to be inhibited in a feedback loop by high calcium concentrations (Kuchay, Giorgi et al. 2017).

Some recent papers of Giorgi's group highlighted the role of PML in the regulation of cell death acting at MAMs level. In particular, Giorgi et al. (2010) identified cPML as a part of a macromolecular complex also including IP3Rs, the pivotal protein kinase Akt and the protein phosphatase PP2a. Here, PML regulates the Akt- and PP2a-dependent IP3R phosphorylation (Giorgi, Ito et al. 2010). Tumor-dependent downregulation of PML, or cPML somatic mutations can behave as a brake on calcium release from ER, strongly reducing the cell ability to undergo apoptosis.

PML and autophagy

Autophagy is a catabolic process that allows lysosomal-mediated degradation of unnecessary or dysfunctional cellular components. Autophagy functions as an adaptive mechanism, which grants

cell survival under several stress conditions (e.g., starvation, hypoxia) and maintains cellular integrity by clearing of subcellular debris and regeneration of metabolic precursors (Mizushima, Levine et al. 2008, Boe and Simonsen 2010).

In cells growing in a nutrient-rich environment (Meijer and Codogno 2004, He and Klionsky 2009) autophagy is negatively regulated by mTOR and AKT signaling. PML inhibits the AKT-mTOR signaling pathway, thus suggesting an involvement in the regulation of autophagy. A study from Laane and colleagues (Laane, Tamm et al. 2009) shows that impairment of autophagy by siRNA-mediated repression of the autophagy regulator BECN1 interferes with glucocorticoid dexamethasone-mediated lymphoblastic leukemia cell death. Dexamethasone treatment leads to PML up-regulation, its interaction with AKT, and PML-dependent AKT dephosphorylation, which was required for dexamethasone-dependent cytotoxicity effects on thymocytes. Conversely, autophagy was shown to contribute to ATRA mediated differentiation and PML-RARα degradation in NB4 cells, where PML-RARα is targeted to lysosomes by the ubiquitin binding adaptor protein p62/SQSTM1 (Isakson, Bjoras et al. 2010, Wang, Cao et al. 2011). These studies coherently associate PML activity with autophagy induction and tumor suppression in acute promyelocytic leukemia.

In addition, Missiroli et al. (2016) showed that cytoplasmic p53 is also enriched in the MAM fraction and that PML needs the p53 presence to localize at MAMs (Missiroli, Bonora et al. 2016). In fact, PML was not found at MAMs in p53 KO murine fibroblasts (MEFs), whereas p53 exhibited a normal subcellular distribution in *Pml*$^{-/-}$ MEFs. Moreover, loss of p53 or PML was associated with an increased autophagy, which greatly increased tumor fitness, providing essential metabolic precursors to sustain tumor growth, also in the presence of stress and growth-limiting conditions. Notably, autophagy was turned to basal levels also in p53 KO MEFs by overexpressing an ER-targeted PML version. These results indicate that PML can have a tumor suppressive function at MAMs acting as a calcium and autophagy modulator, at least in the latter downstream of p53 (Fig. 7).

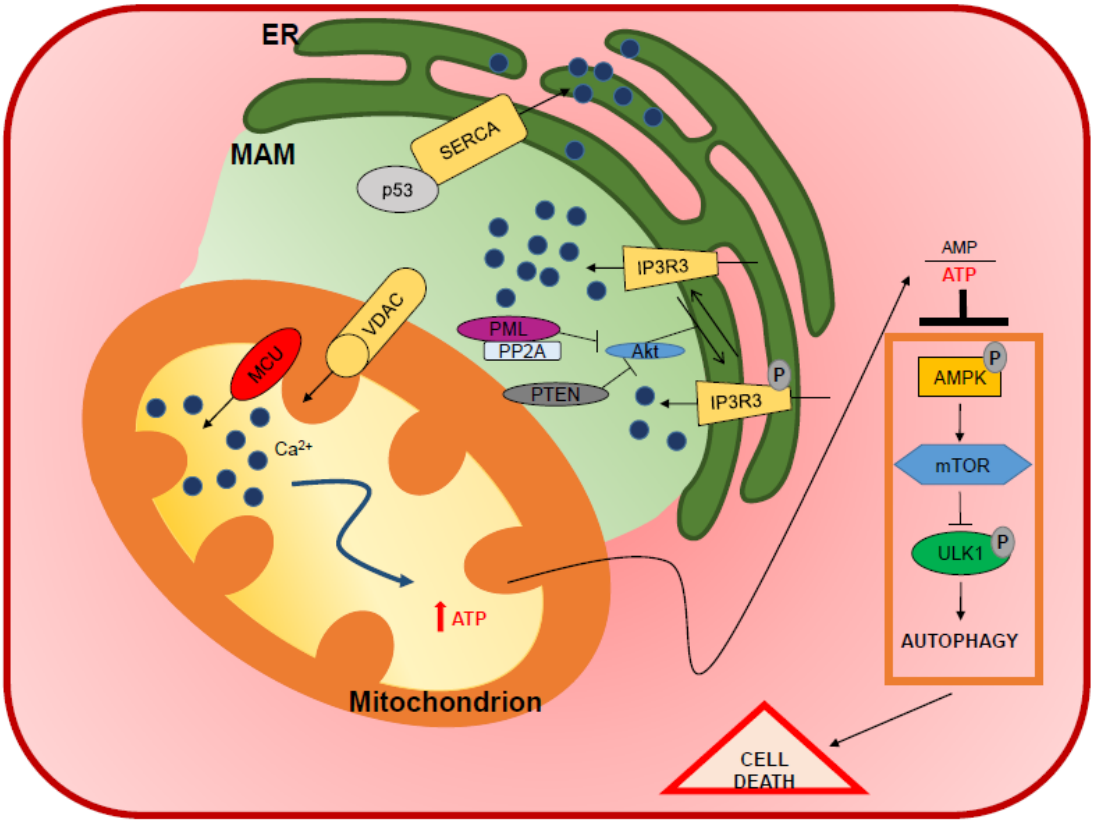


Figure 7: PML function at MAM level

PML and Immunity

The TRIM family has been involved in the control of viral infection, and consistently, the PML gene promoter contains target sequences for interferon regulatory factors (El Bougrini, Dianoux et al. 2011, Scherer and Stamminger 2016). After viral aggression, cells produce and release interferons, which trigger cellular antiviral response upon binding to specific receptors and activation of DNA transcription. Interferons type I and II strongly increase transcription of PML and SUMO proteins and also of several NB-associated proteins, like Sp100 (Speckled protein of 100 kDa), Sp140, Sp110, ISG20, PA28, and death-associated protein (DAXX). This allows an interferon-dependent increase of NB number, which in turn consents sequestration of viral proteins with diminution of their activity at the nuclear level, followed by increased SUMOylation and degradation.

PML IV can interact with the 3D polymerase of encephalomyocarditis virus (EMCV) and to sequester it into PML-NB, with the ultimate effect of reducing the viral replication effort (Xu and Roizman 2017), (El McHichi, Regad et al. 2010). This suggests that PML-NB complex can behave

as defense station able to slow down and adsorb the viral assault to nuclear structures, with the goal of reducing the viral particles production.

Consistently, Pml KO mice, which not express PML protein, show a reduced level of apoptosis induced by interferon I and II, and by cytokines like as interleukin 6 (Maroui, Pampin et al. 2011). Also,several viruses, such as the herpes simplex virus type 1 (HSV-1), oppose the PML-NB sequestration: the immediate-early protein of HSV-1 can induce the disassembly of NBs. In turn, a cytoplasmic variant of PML lacking exons 5 and 6 (PML Ib) can bind the infected cell protein directly in the cytoplasm, preventing its nuclear action and greatly reducing HSV-1 replication (Lunardi, Gaboli et al. 2011).

The preventive block of viral proteins at cytoplasmic level seems to be a more general strategy for PML. Given the relocation of nuclear PML isoforms in the cytoplasm operated by the respiratory syncytial virus, the lymphocytic choriomeningitis virus (LCMV) and the human immunodeficiency virus type 1 (HIV-1), PML can inhibit the activity of viral proteins also in the cytoplasm. As an example, PML and the integrase interactor 1 of HIV-1 redistribute from nucleus to cytoplasm, in a manner dependent upon exportin-1. At cytoplasm level, PML is again able to show an antiviral activity, binding and interfering with the HIV-1 preintegration complex (McNally, Trgovcich et al. 2008). PML also cooperates with p53 during viral infections. Infact, poliovirus infection induces PML phosphorylation through a mitogen-activated protein kinase (MAPK) pathway and this triggers an increased PML SUMOylation and p53 recruitment within the NBs, leading to p53 activation and induction of p53 target genes, Mdm2 and Nox. This event induces the process of apoptosis in poliovirus-infected cells and the inhibition of viral replication (McNally, Trgovcich et al. 2008).

Recently, two different studies describe the involvement of PML in the NLRP3 activation. Dowling et al. demonstrate the interaction between PML and ASC (Turelli, Doucas et al. 2001). PML deficient macrophages display enhanced levels of ASC in the cytosol and a high IL-1 production in response to both NLRP3 and AIM2 inflammasome activation and following bone marrow-derived macrophage infection with herpes simplex virus-1 (HSV-1) and Salmonella typhimurium. Collectively, their data indicate that PML limits ASC function, retaining ASC in the nucleus. On the other side, Lo et al. show that in *Pml−/−* macrophages the expression of pro–IL-1β, NLRP3, ASC, and procaspase-1 is not affected (Pampin, Simonin et al. 2006). All PML isoforms are capable of stimulating NLRP3 inflammasome activation. In addition, downregulation of PML by arsenic trioxide suppressed monosodium urate (MSU)-induced IL-1β production, suggesting that targeting to PML could be used to treat NLRP3 inflammasome–associated diseases. Two different

studies that report conflicting roles of the PML protein in the activation of the NLRP3 inflammasome, one inhibitory and the other activator, with a molecular mechanism still unclear, which allows for further considerations in this field of research.

Aims

This thesis is the result of my PhD in “Biomedical and Biotechnological Science” attended in “Signal Trasduction Lab” leaded by Prof. Paolo Pinton.

Our lab is aimed in studying of signal transduction, the process by which a cell receives and responds to stimuli, which lies at the heart of many interesting and important processes, including cell growth, cell death, immune function and differentiation process.

I focused my studies on tumor development and immune response.

The first work is focus on a new role of promyelocytic leukemia protein (PML), a well-known oncosuppressor directly involved in human tumor development. While PML has been widely studied in cancer cells, far less is known about PML function in tissue-resident primary immune cells that serve as front-line sentinels in response to stress signals. The aim of this research is to investigate the crosstalk between PML and the NLRP3 inflammasome at the ER/MAM compartments and how this molecular pathway in the host can impact cancer development.

In the second work, we decided to deeply investigate the contribution of radiotherapy to NLRP3-mediated inflammation in a PML-dependent manner. Our aim is to determine how to properly modulate the impact of anticancer therapies on tumor growth and the immune system, defining the role of IL-1β as a predictive marker of tumor behavior in order to better profile the use of radiotherapy.

Loss of PML promotes NLRP3 inflammation-associated cancer in a P2X7 dependent manner

Introduction

Promyelocytic Leukemia Protein (PML) is a multi-faceted protein, that can be present at nuclear level, where it plays pivotal roles in tumor growth, modulating a wide number of cell activities, including tumor suppression, anti-viral and anti-bacterial responses and inflammatory responses.

Among its very numerous functions - it has been reported to interact with more than 160 different proteins - PML can also localize to the ER-mitochondria contact sites (also called Mitochondria-Associated Membranes, MAMs) where it can control several functions such as the transfer of calcium into the mitochondria, ROS production, apoptosis and autophagy (Guan and Kao 2015)(Missiroli, Bonora et al. 2016).

Recently, MAMs have been shown to function as a platform for inflammatory signaling regulated by the most widely characterized inflammasome, so-called NLRP3. At rest, the NLRP3 protein resides at the ER, whereas upon inflammation activation, both NLRP3 and its adaptor ASC redistribute to MAMs to permit inflammasome formation (Zhou, Yazdi et al. 2011, Missiroli, Patergnani et al. 2018).

While PML is widely studied in the context of cancer cells, far less is known about PML function in tissue-resident primary immune cells that serve as front-line sentinels in response to stress signals and its role in regulating tumor microenvironment (TME) has not been fully clarified.

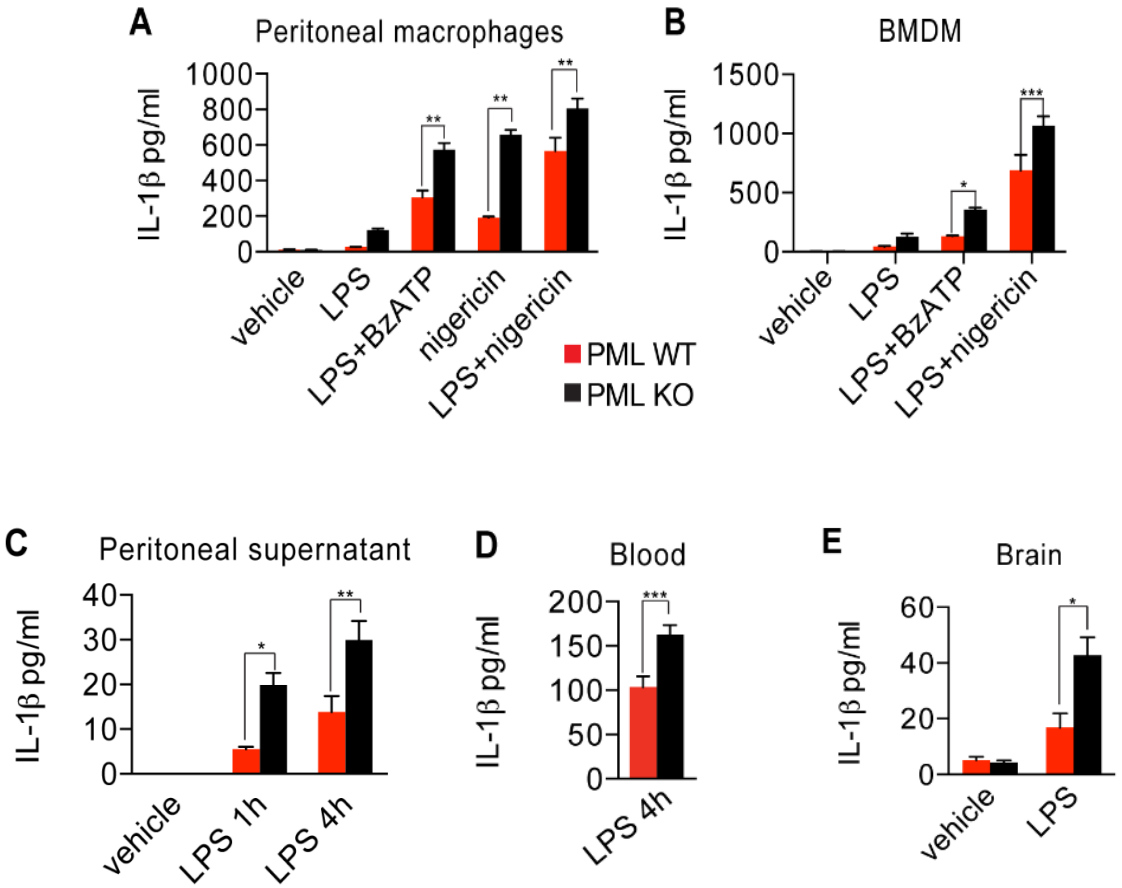
Here we discover that the down-regulation of the tumor suppressor PML at the host level can drive an immune response that is able to worsen cancer instead of ameliorate it, enhancing the process of IL-1β release. In particular we found that crosstalk between PML and the NLRP3 inflammasome at the ER/MAMs compartments exist and we demonstrated how this molecular pathway in the host could impact cancer development. Furthermore, we revealed that genetic or pharmacological manipulation of NLRP3-inflammasome and of one of its activator, the P2X7 receptor, have the benefit of blocking the release of IL-1β in a PML KO background, reducing tumor growth.

Taken together our results provide a mechanistic rationale for the powerful ability of the PML-NLRP3 axis to regulate TME in human carcinogenesis.

Results

Loss of PML increases IL-1β release.

To understand the impact of PML on NLRP3 inflammasome activation, we evaluated IL-1β release *in vitro*, *ex-vivo* and *in vivo* from peritoneal macrophages, bone marrow derived macrophages (BMDM), peritoneal exudate, blood and brain derived from WT and *Pml*$^{-/-}$ mice. The absence of PML displays a higher inflammatory response compared to that observed in WT (Fig. 8A-E) as confirmed also checking the cleaved (active) form of caspase-1 and IL-1β by WB (Fig 8F-G). Consistently, in the presence of down-regulated PML, IL-1β was found to be strongly increased in liver tissue (Fig. 8H) and also caspase-1 release was heightened in response to treatment in *Pml*$^{-/-}$ mice (Fig. 8I). Accordingly, also IL-18 release was altered in absence of PML (Fig. 8J).



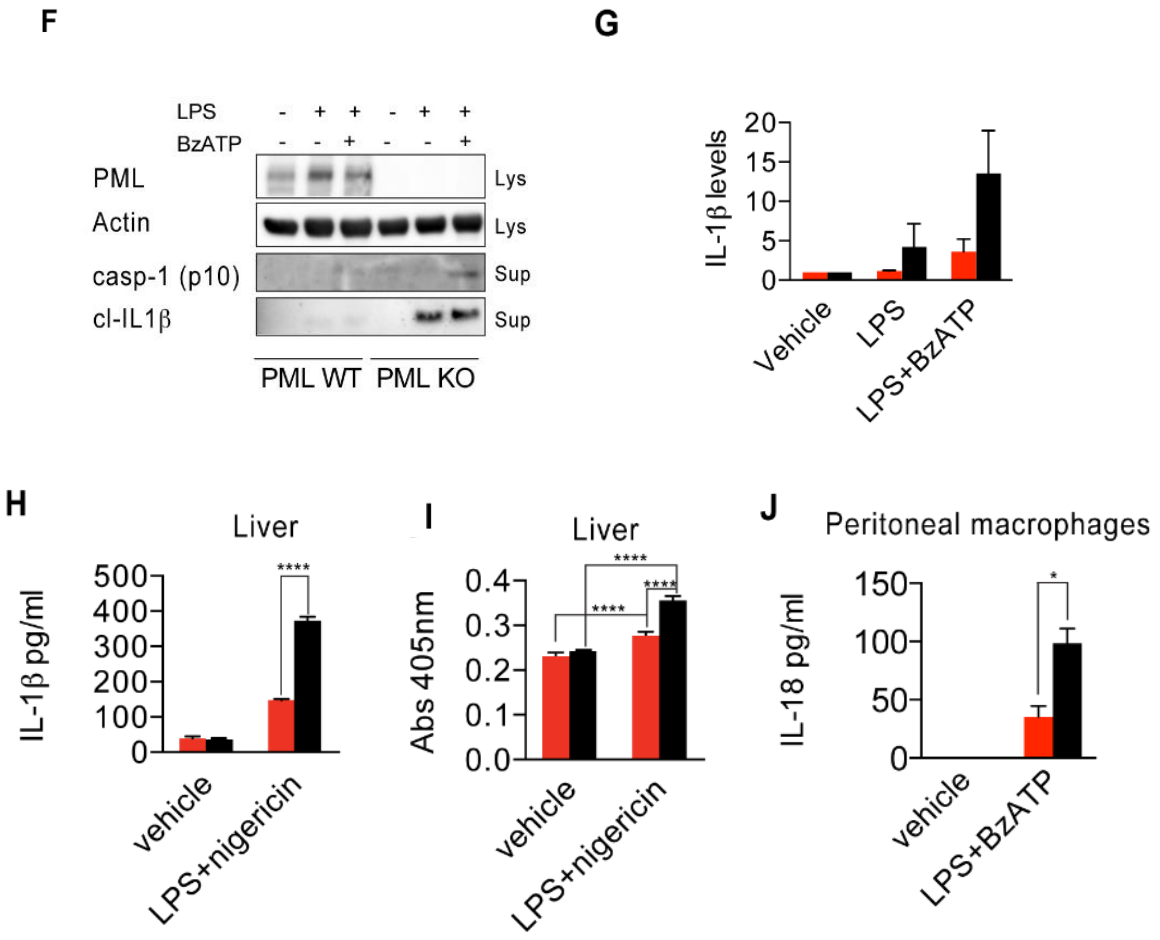


Figure 8: PML deficiency in the host favors higher levels of IL-1β in response to inflammasome activation. Peritoneal-derived macrophages from wild type (WT) and *Pml*$^{-/-}$ mice were left unstimulated or stimulated with: LPS (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes) or nigericin (10µM for 1h). Supernatants were analyzed by ELISA for IL-1β (A) and IL-18 (J). Error bars indicate s.e.m., n=3.(B) Production of IL-1β from BMDM stimulated with LPS (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes) or nigericin (10µM for 1h) as measured by ELISA. Production of IL-1β from peritoneal supernatants (C), from blood (D) and from brain (E) from WT and *Pml*$^{-/-}$ mice after treatment with 250ng\gr LPS as measured by ELISA. Error bars indicate s.e.m., n=3. (F-G) Culture supernatants and peritoneal macrophages from WT and *Pml*$^{-/-}$ mice were collected before and after LPS activation (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes). Total cell lysates (Lys) and supernatants (Sup) were resolved by SDS-PAGE. Results shown were representative of three independent experiments. Production of IL-1β analyzed by ELISA (H) and caspase 1 activity assay (I) from WT and Pml-/- liver after treatment with 250ng\gr LPS for 1h with the addition of 2,5mg\Kg nigericin for 1h. Error bars indicate s.e.m., n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$**** $p < 0.0001$.

To verify specificity for NLRP3 inflammasome activation we checked for other pro-inflammatory cytokines, such as IL-6 (Fig. 9A) and TNFα (data not shown) and we tested siRNA for NLRP3 and NLRC4 demonstrating that tumorsuppressor-linked inflammation is only NLRP3 dependent (Fig. 9B-C).

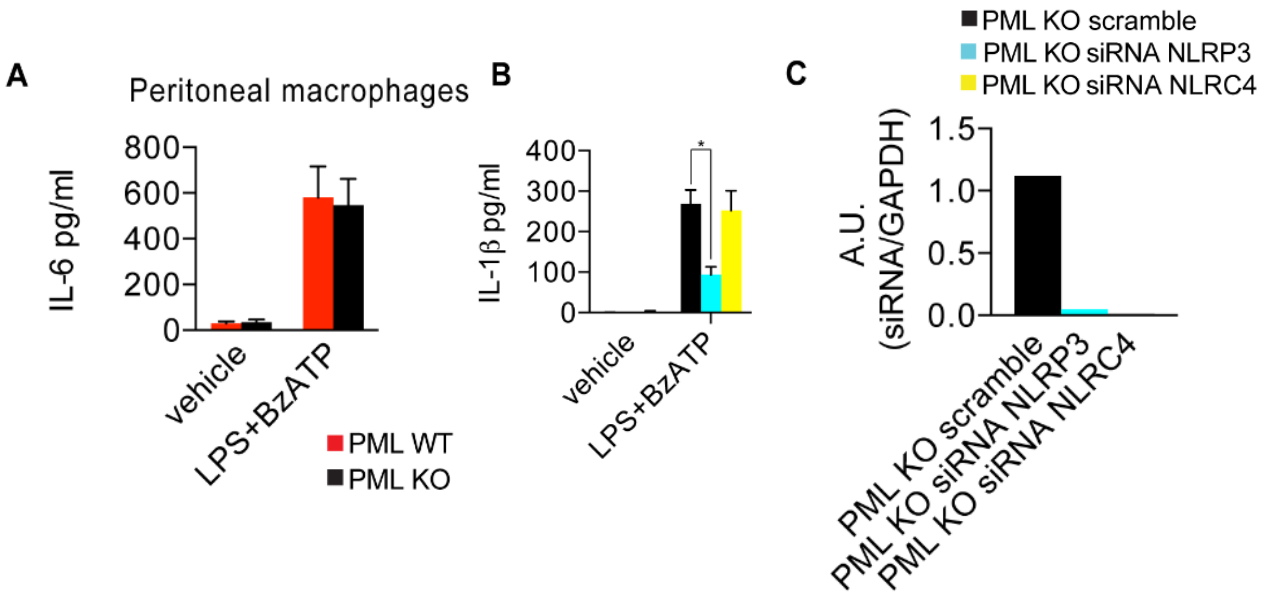
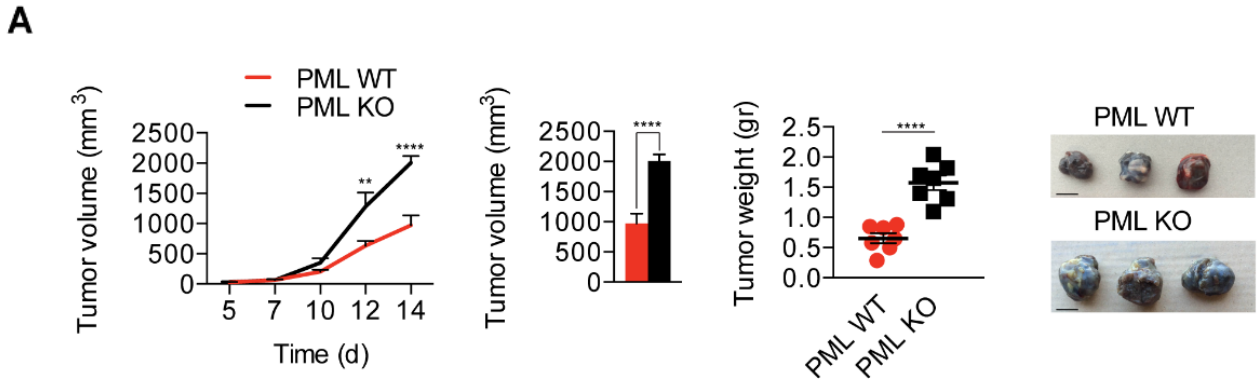


Figure 9: PML deficiency favors NLRP3 inflammasome activation exclusively. (A) Peritoneal-derived macrophages from wild type (WT) and *Pml*$^{-/-}$ mice were left unstimulated or stimulated with: LPS (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes). Supernatants were analyzed by ELISA for IL-6. Error bars indicate s.e.m., n=3. (B) IL-1β production (as measured by ELISA) from Pml-/- peritoneal macrophages stimulated with LPS and BzATP after siRNA transfection. Error bars indicate s.e.m., n=3. (C) RNA expression of NLRP3, NLRC4 and G3PDH as a housekeeping gene by RT-PCR. * $p < 0.05$

IL-1β is a pleiotropic pro-inflammatory cytokine, and its up-regulation is closely associated with carcinogenesis and tumor progression. To investigate whether PML null-mediated inflammation promotes tumor growth, we subcutaneously injected B16-F10 melanoma cancer-derived syngeneic cell lines in two different hosts: *Pml* WT and *Pml*$^{-/-}$ C57BL/6 mice. Our results clearly demonstrate an accelerated tumor growth in *Pml*$^{-/-}$ mice (Fig. 10A-B) associated with hyper-inflammation and exacerbated secretion of IL-1β in the peritumoral tissue (Fig. 10C).



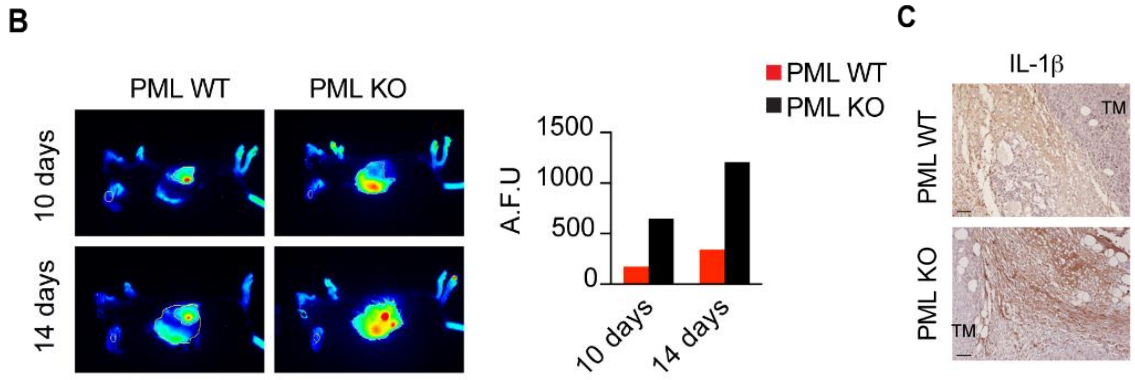
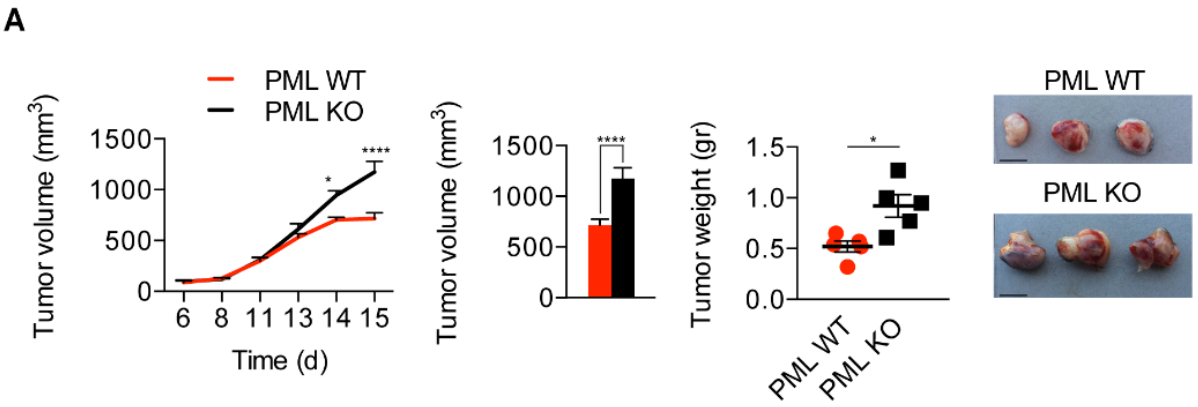


Figure 10: Hyperactivation of NLRP3 in PML -/- host promotes tumor growth. (A) WT and *Pml*$^{-/-}$ C57BL/6 mice (7 for each condition) were subcutaneously inoculated with B16-F10 melanoma cells (1 x 10^6). Left panel, graphs show tumor growth kinetics for the indicated time points. Middle panels show quantification of tumor volumes and tumor weights. Right panel shows representative excised tumors imaged 14 days post-injection. Error bars indicate s.e.m. Scale bars, 1cm. (B) Representative tumours imaged with IRDye 2-DG and quantification of fluorescence at 10 e 14 days after inoculation.(C) Intratumor IL-1β levels in melanoma-bearing WT and *Pml*$^{-/-}$ mice. Nuclei are counterstained with Harris' haematoxylin. TM: tumor mass. Scale bars, 50 μm. **** $p < 0.0001$

To strengthen the results reported above and avoid differences linked to the cell type, we explore the effect of PML deletion with a different cancer cell model. *Pml* WT and *Pml*$^{-/-}$ C57BL/6 mice were inoculated with the syngeneic LL/2 Lewis lung carcinoma cell line and tumor growth was monitored. In line with our results reported above, PML deletion caused an acceleration of tumor growth (Fig. 11A) associated to higher levels of IL-1β in the peritumoral tissue (Fig. 1B). Interestingly both B16-F10 melanoma and LL/2 cells display PML levels (Fig. 11C), to indicate that the effects that we saw are dependent to loss of PML in the host.



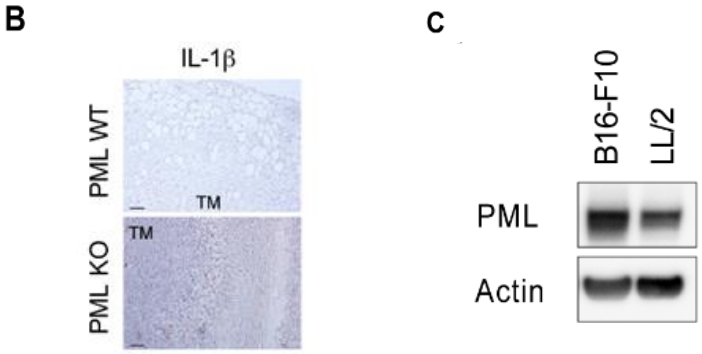
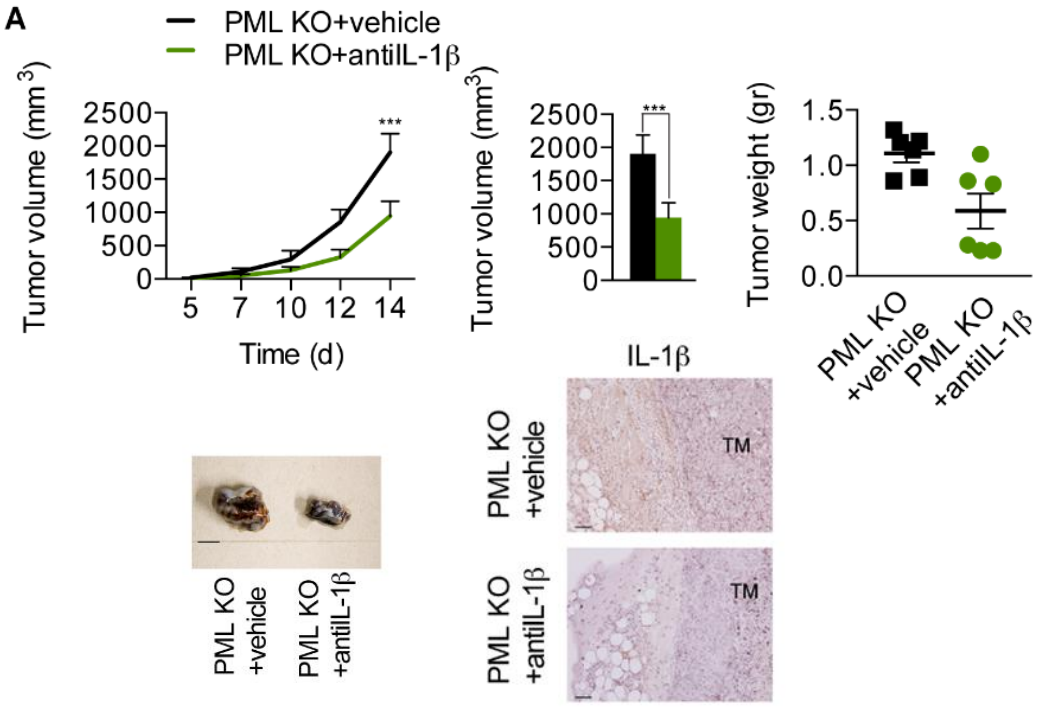


Figure 11: Hyperactivation of NLRP3 in PML -/- host promotes different tumor growth. (A) WT and *Pml*$^{-/-}$ C57BL/6 mice (7 for each condition) were subcutaneously inoculated with LL/2 Lewis lung carcinoma cells (1 x 10^6). Left panel, graphs show tumor growth kinetics for the indicated time points. Middle panels show quantification of tumor volumes and tumor weights. Right panel shows representative excised tumors imaged 15 days post-injection. Error bars indicate s.e.m. Scale bars, 1cm. (B) Intratumor IL-1β levels in melanoma-bearing WT and *Pml*$^{-/-}$ mice. Nuclei are counterstained with Harris' haematoxylin. TM: tumor mass. Scale bars, 50 μm. (C) PML expression in syngeneic tumor cells by western blot. * $p < 0.05$, ** $p < 0.01$**** $p < 0.0001$.

To ascertain whether the alterations in PML-mediated tumor progression are dependent on secreted IL-1β, we used an anti- IL-1β antibody to deplete IL-1β in the TME (Fig. 12A). Our data demonstrated that blockade of IL-1β in the TME slowed down the tumor growth in a *Pml*$^{-/-}$ host, without affecting tumor development in a WT host (Fig. 12B)



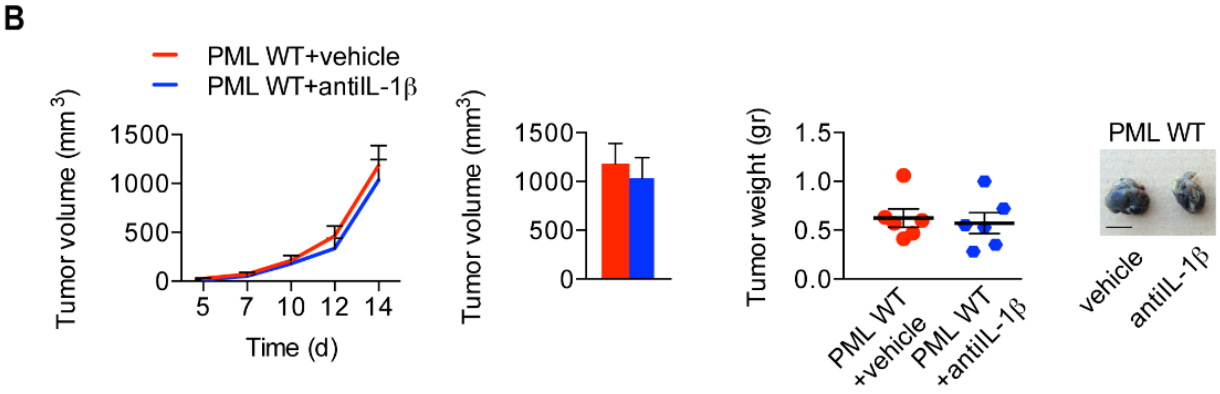


Figure 12: Anti-IL1β treatment reduces tumor growth in PML KO mice.. Graphs show primary tumor growth kinetics for indicated time points from (A) *Pml*$^{-/-}$ C57BL/6 or WT mice (B) (6 for each condition) inoculated subcutaneously with B16-F10 cells (1 x 10^6). Mice were injected intraperitoneally with 50µg twice weekly anti IL-1β (clone B122; BioXCell); control mice received equal amounts of isotope control antibodies. Middle panels, quantification of tumor volumes and tumor weights of primary tumors from *Pml*$^{-/-}$ or WT mice at 14 days post-injection. Right panel shows representative excised tumors imaged 14 days post-injection. Error bars indicate s.e.m. The strong IL1β immunoreactivity detected in the peritumoral tissue of *Pml*$^{-/-}$ mice is almost depleted by in vivo treatment with anti-IL1β serum. TM: tumor mass. Scale bars, 50 µm. *** $p < 0.001$

In addition, to verify if the effect of IL-1β released from macrophages is direct on tumor cells, we established a co-culture model. Peritoneal macrophages obtained from WT and Pml-/- mice have been cultured into removable inserts on the top of a plate, activated by Lipopolysaccharides (LPS) and Benzoil-ATP (BzATP) as inflammasome activators, and incubated with B16-F10 melanoma cancer cells seeded on the bottom of the plate separated by a porous membrane that allows free exchange of cytokines (Fig. 13A). Co-culture with *Pml*$^{-/-}$ peritoneal macrophages enhanced cell growth of tumor target cells, while treatment with anti- IL-1β antibody inhibits cancer cell growth (Fig. 13B). Together these results were compatible with a scenario in which PML loss in the TME favors tumor establishment and progression.

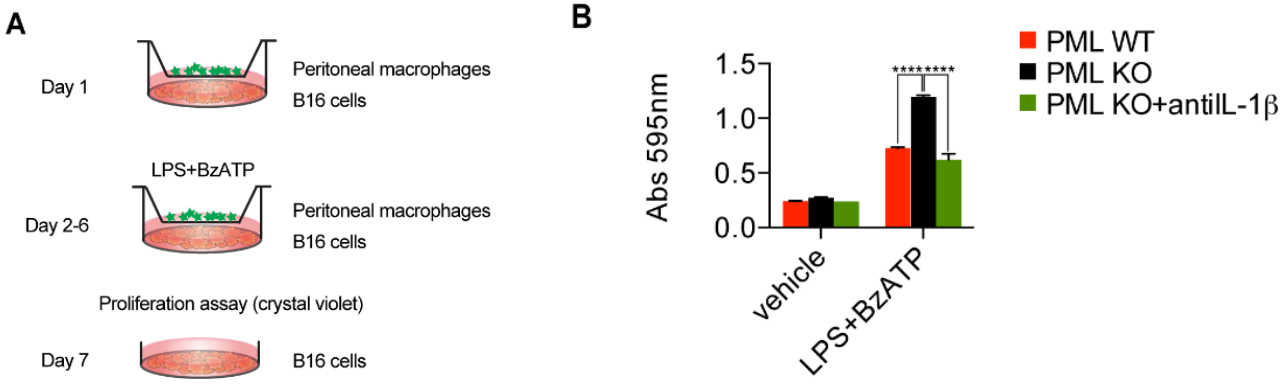


Figure 13: IL-1β released from macrophages affects tumor growth. (A) Schematic representation of co-colture system. (B) B16-F10 cells were co-cultured with macrophages from Pml WT and *Pml*$^{-/-}$ mice, treated with LPS (1ug\ml) and BzATP (100µM) and the anti-IL1β (50 pg/ml). After one week, proliferation of B16-F10 cells was analyzed by crystal violet staining (absorbance at 595 nm). Experiments were performed twice. Error bars indicate s.e.m. ****$p < 0.0001$.

PML inactivation in the TME provides a permissive environment for tumor growth

Since it is known that the ratio between M2-like (pro-tumoral phenotype) and M1-like (anti-tumoral phenotype) tumor-associated macrophages (TAMs) has prognostic value in cancer (Mantovani and Locati 2013, Zhang, He et al. 2014), we determined if different amounts of IL-1β released in WT and *Pml*$^{-/-}$ mice are associated with different types of macrophage polarization (M1, M2). We examined the phenotype of the TAMs by performing immunohistochemical analyses and FACS analysis on tumor extracts and we revealed a statistically significant increase in number of M2 polarized macrophages in *Pml*$^{-/-}$ mice, compared with WT group (Fig. 14A-C). The peri-tumoral inflammatory infiltrate reveals that the number of free cells in peritumoral environment was distinctly more pronounced in Pml KO compared with WT group. IHC detected very numerous F4/80-immunoreactive (-IR) free cells, with cytological features of DCs, also occurring intra-tumor. In WT host, scattered IgG-IR cells localized peri-tumoral and also infiltrated the tumor mass, concentrating in necrotic zones, while in *Pml*$^{-/-}$ host IgG-IR cells were hardly detectable in the oedematous peri-tumoral environment, and poorly represented intra-tumor.

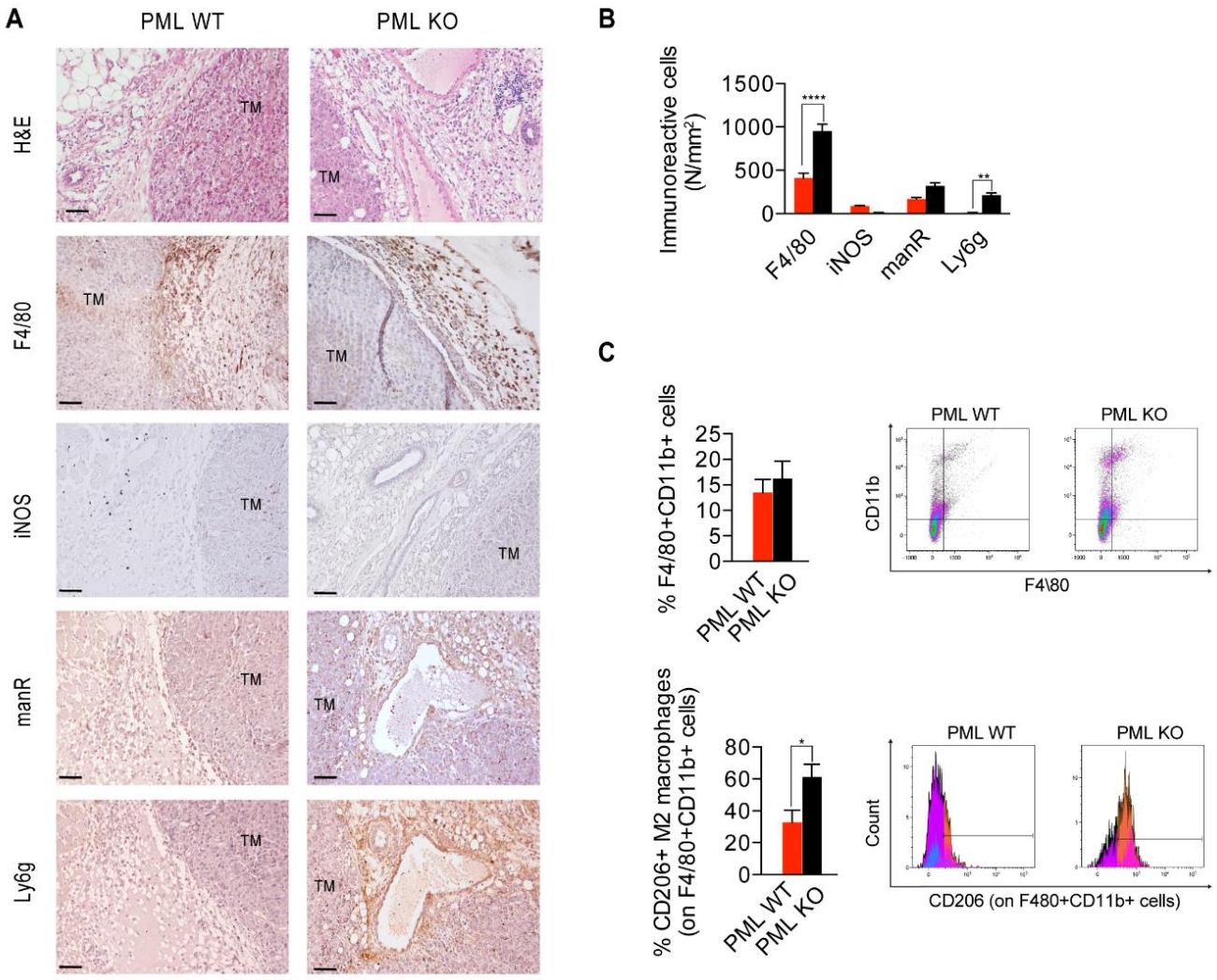
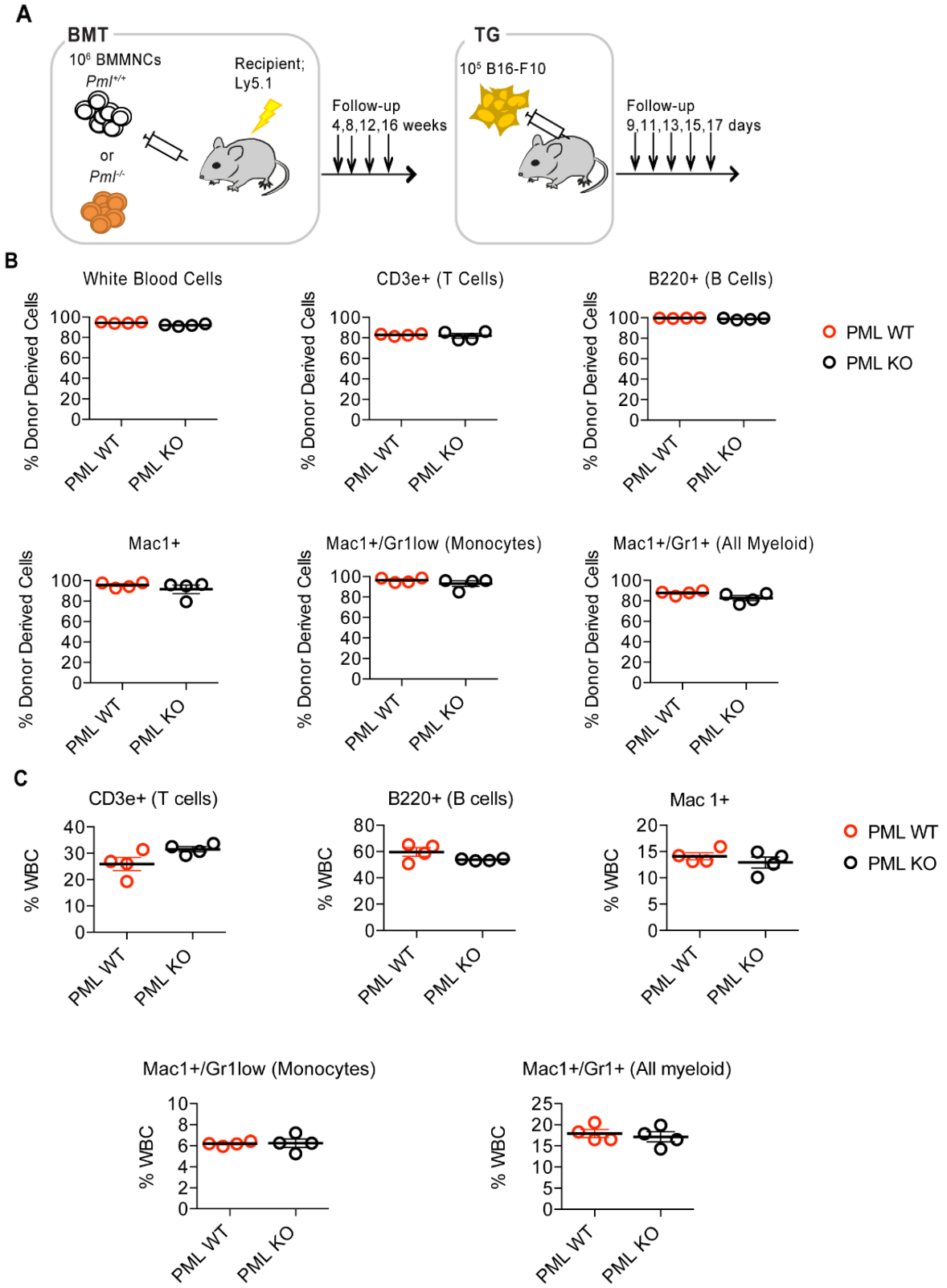


Figure 14: The accelerated tumor progression in Pml-/- hosts is associated with misdirected TAMs. (A) Representative sections of tumors from specimens of *Pml* WT (left panels) and *Pml*$^{-/-}$ groups (middle panels), stained with H/E or immunostained for F4/80, iNOS, mannose receptor (manR), and Ly6g. Nuclei are counterstained with Harris' haematoxylin. TM: tumor mass. Scale bars: 50 µm.(B) The peritumoral tissue of *Pml*$^{-/-}$ mice is distinctly enriched with cells expressing F4/80, mannose receptor and Ly6g immunoreactivity, and almost devoid of iNOS immunoreactive cells, compared with WT mice. Error bars indicate s.e.m. (C) Tumor-infiltrating macrophages from WT and *Pml*$^{-/-}$ mice were analyzed (n=11) as F4/80^{+}CD11b^{+} and subpopulations M2 macrophages were analyzed as F4/80^{+}CD11b^{+}CD11c^{-} CD206^{+}. Error bars indicate s.e.m. * $p < 0.05$. **** $p < 0.0001$

Finally, to dissect the relevance of tissue-resident macrophages, we used bone marrow chimeras. Eight-week-old male congenic C57BL/6J Pml WT have been lethally irradiated with 9 Gy RT. Bone marrow mononuclear cells (BMMNCs) have been obtained from the femur and tibia of male C57BL/6J *Pml* WT and *Pml*$^{-/-}$ donor mice and injected into the tail vein at 4x10^{5} cells/recipient according to the Ly5.1/Ly5.2 mismatch (Fig. 15A). Engraftment of donor-derived cells have been monitored via flow analysis of the myeloid and lymphoid populations in the peripheral blood every 4 weeks up to complete reconstitution (Ito, Turcotte et al. 2016) (Fig. 15B-C). Accordingly we subcutaneously injected B16-F10 melanoma cancer-derived syngeneic cell lines in *Pml*$^{+/+}$ mice transplanted with PML KO BMMNCs and *Pml*$^{+/+}$ mice transplanted with *Pml* WT BMMNCs (Fig.

15D). Our data revealed that after seventeen days tumor growth was significantly higher in *Pml*$^{+/+}$ mice transplanted with PML KO BMMNCs compared to that transplanted with PML WT BMMNCs (Fig. 15D). These data supported the notion that PML deficiency in the TME favors tumor establishment and progression by enhancing IL-1β secretion from tissue-resident macrophages.



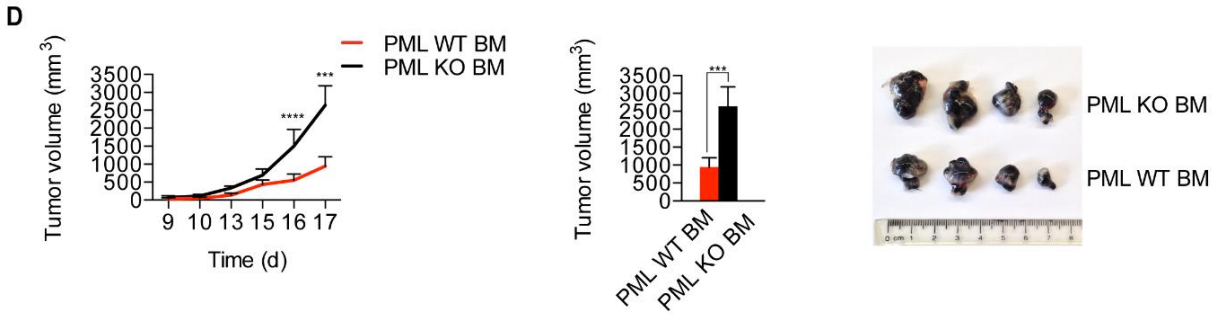


Figure 15: Bone Marrow Replacement Experiment. (A) – (C) Reconstitution of donor cells and repopulation of donor myeloid and lymphoid cells were monitored by staining peripheral blood cells with antibodies against CD45.1, CD45.2, CD3 (T cell), B220 (B cell), CD11b and Gr-1 (myeloid) every 4 weeks starting at 4 weeks after transplantation. At 16 week post-transplantation the engraftment was considered stable and mice were subjected to tumor engraftment. (D) Tumor growth in PML-wt>PML-wt and PML-wt>PML-KO chimeric mice. Error bars indicate s.e.m. (n = 4). *** $p < 0.001$, **** $p < 0.0001$.

Genetic and/or pharmacological approaches reduce NLRP3-mediated inflammation and thus cancer development.

To investigate whether pharmacological strategies could directly limit NLRP3-dependent pro-inflammatory and pro-tumoral signals, we propose Nimodipine as a drug to inhibit NLRP3 activation.

Nimodipine is a calcium channel antagonist originally developed for the treatment of high blood pressure. Recently, nimodipine has been found to protect microglia from Aβ-dependent cytotoxicity due to its anti-inflammatory effect by inhibiting IL-1β release, which points to a novel role for this drug as a centrally acting anti-inflammatory compound (Sanz, Chiozzi et al. 2012). Here we tested the anti inflammatory ability of Nimodipine evaluating IL-1β release *in vitro* and *in vivo* from peritoneal macrophages and blood derived from WT and *Pml*$^{-/-}$ mice (Fig. 16A-B) and we observed that nimodipine reduced the undesired host-dependent phlogosis. Intraperitoneally injection of nimodipine caused a strong inhibition of tumor growth in B16-inoculated *Pml*$^{-/-}$ C57Bl/6 mice (Fig. 16D), without affecting in vitro B16-F10 cell growth (Fig. 16C). Nimodipine treatment didn't affect tumor growth in a *Pml* WT host (Fig. 16 G), indicating that its action is direct on hyper-production of IL-1β.

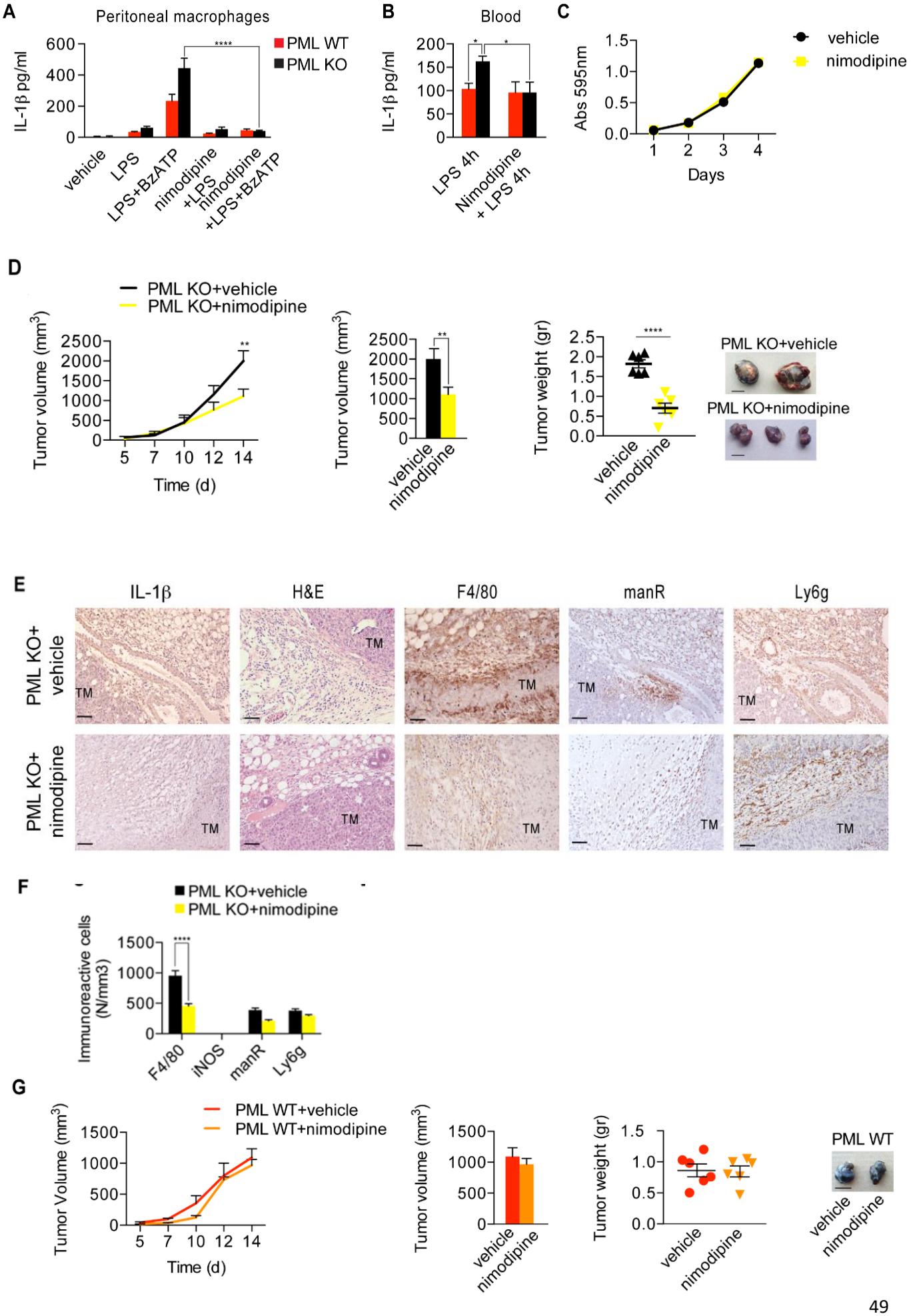

A
Peritoneal macrophages
IL-1β pg/ml
PML WT
PML KO
vehicle
LPS
LPS+BzATP
nimodipine +LPS
nimodipine +LPS+BzATP
B
Blood
IL-1β pg/ml
LPS 4h
Nimodipine + LPS 4h
C
Abs 595nm
Days
vehicle
nimodipine
D
PML KO+vehicle
PML KO+nimodipine
Tumor volume (mm3)
Time (d)
Tumor weight (gr)
E
IL-1β
H&E
F4/80
manR
Ly6g
PML KO+ vehicle
PML KO+ nimodipine
TM
F
Immunoreactive cells (N/mm3)
F4/80
iNOS
manR
Ly6g
G
PML WT+vehicle
PML WT+nimodipine
Tumor Volume (mm3)
Time (d)
PML WT

Figure 16: Nimodipine treatment as NLRP3 inflammasome inhibitor. (A) Peritoneal-derived macrophages from wild type (WT) and *Pml*$^{-/-}$ mice were left unstimulated or stimulated with: LPS (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes) or with pretreatment of Nimodipine (100nM for 30 minutes).Supernatants were analyzed by ELISA for IL1β. Error bars indicate s.e.m., n=3. (B) Production of IL-1β from blood from WT and Pml$^{-/-}$ mice after treatment with 250ng\gr LPS and Nimodipine 100nM as measured by ELISA. Error bars indicate s.e.m., n=3. (C) Proliferation of B16-F10 cells untreated or treated with nimodipine analyzed by crystal violet staining. (D) Pml$^{-/-}$ or WT (G) C57BL/6 mice (6 for each condition) were subcutaneously inoculated with B16-F10 cells (1 x 10^6). Mice, chosen randomly, received nimodipine intraperitoneally thrice weekly. Left panel, tumor growth kinetics at the indicated time points. Middle panels, quantification of tumor volumes and tumor weights. Right panel, representative excised tumors imaged 14 days post-injection. Error bars indicate s.e.m. Scale bars, 1cm. (E) Representative sections of tumors from specimens of *Pml* KO and *Pml* KO with nimodipine treatment groups, stained with H/E or immunostained for IL1β, F4/80, mannose receptor (manR), and Ly6g. Nuclei are counterstained with Harris' haematoxylin. TM: tumor mass. Scale bars: 50 µm. (F) The hyper-inflamed state induced by PML genetic deletion was counteracted by nimodipine treatment, that led to significant reduction in number of F4/80+, CD8+ and CD3+ cells compared with *Pml* KO group. Error bars indicate s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$**** $p < 0.0001$.

To determine a model of genetic inflammasome inhibition, we generated PML/P2X7 double knockout mice (*Pml*$^{-/-}$*/P2x7*$^{-/-}$) and PML/NLRP3 double knockout mice (*Pml*$^{-/-}$*/Nlrp3*$^{-/-}$). Mice were crossed together, and after four generations, we obtained a pure colony, confirmed with PCR and biochemical analysis of mouse tissue (Fig. 17A-B). We evaluated IL-1β release inflammasome activation *in vitro*, *ex-vivo* and *in vivo* from macrophages, peritoneal exudate and blood after derived from *Pml*$^{-/-}$*/P2x7*$^{-/-}$, *Pml*$^{-/-}$*/Nlrp3*$^{-/-}$ mice compared to WT and *Pml*$^{-/-}$ mice and we found that genetic inhibition of P2X7 and NLRP3 drastically abolished IL-1β response (Fig. 17C-E).

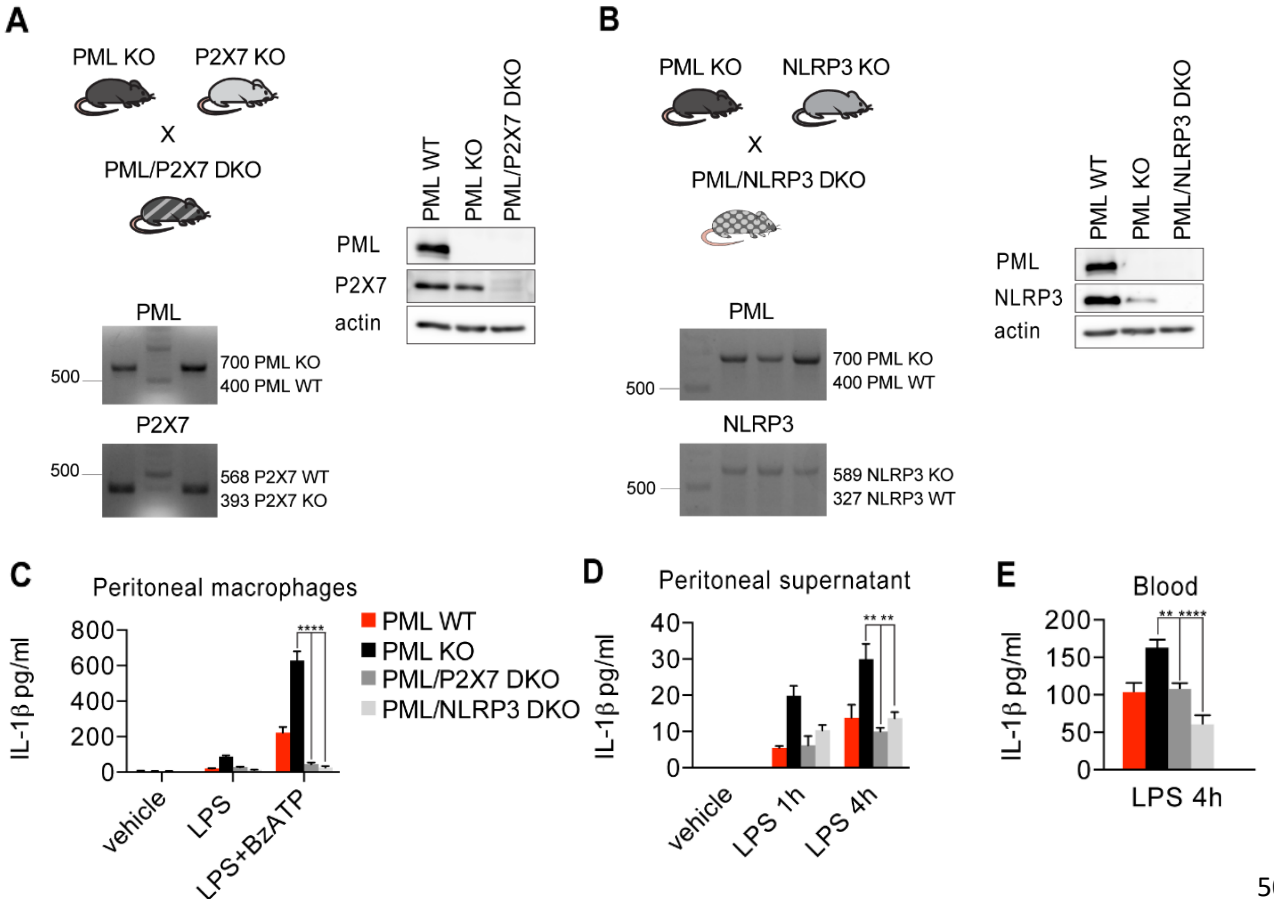
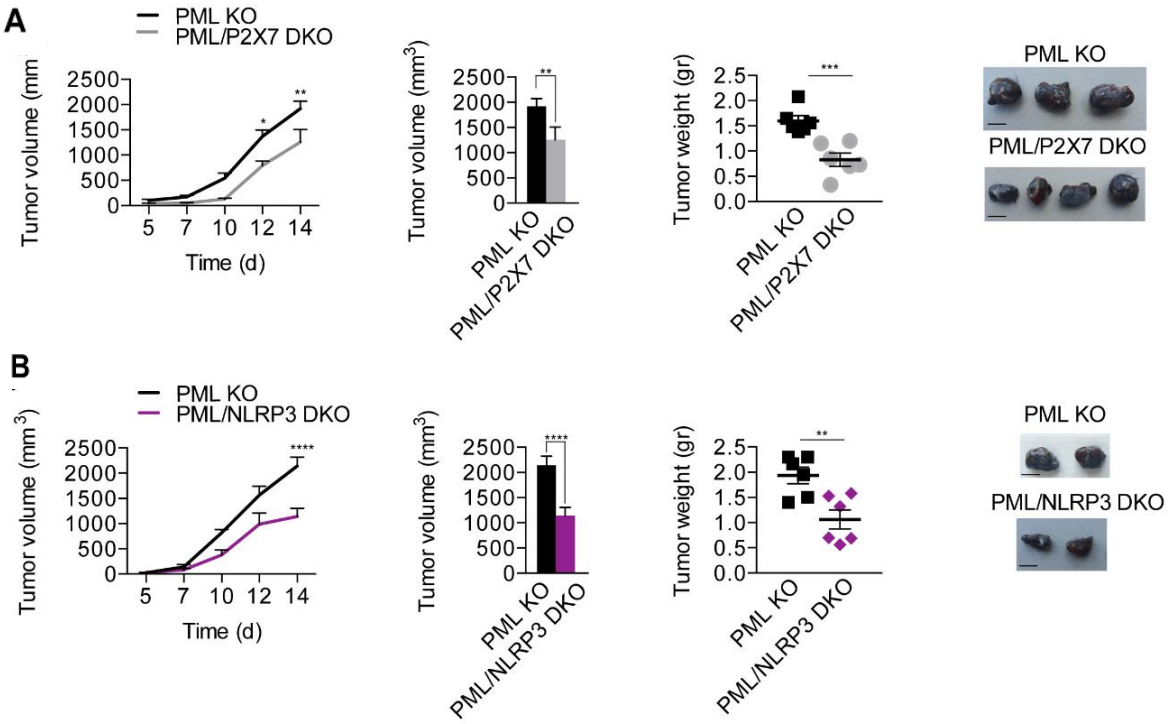


Figure 17: Genetic inhibition of P2X7 and NLRP3 reduces IL1β release in the PML deficient host. (A)-(B) Generation of murine colony PML/P2X7 and PML/NLRP3 DKO. (C) Peritoneal-derived macrophages from wild type (WT), *Pml*-/-, *Pml*/P2X7 -/- and *Pml*/NLRP3 -/- mice were left unstimulated or stimulated with: LPS (1µg\ml for 2h) alone or with the addition of BzATP (100µM for 30 minutes. Supernatants were analyzed by ELISA for IL1β. Error bars indicate s.e.m., n=3. Production of IL-1β from peritoneal supernatant (D) and blood (E) from WT, *Pml*-/-, *Pml*/P2X7 -/- and *Pml*/NLRP3 -/- mice after treatment with 250ng\gr LPS as measured by ELISA. Error bars indicate s.e.m., n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,****$p<0.0001$

To test the *in vivo* relevance of our observations about IL-1β release in genetic inhibition of NLRP3 components, we determined tumorigenesis by B16-F10 cells inoculation and we found that inhibition of P2X7 and NLRP3 was sufficient to reduce tumor growth (Fig. 18A-B).



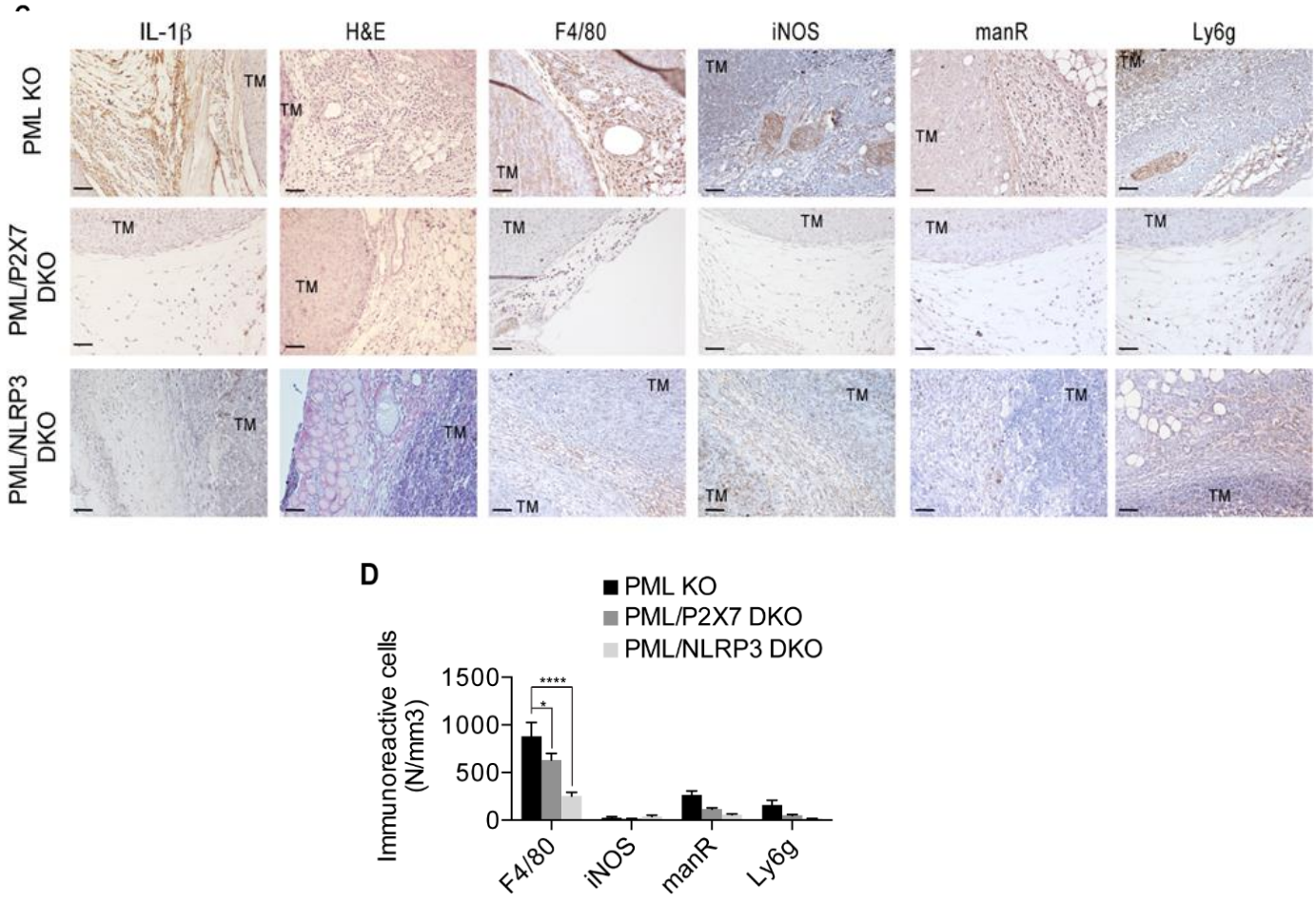


Figure 18: Genetic inhibition of NLRP3 inflammasome reduces cancer development. (A)-(B) *Pml*-/-, *Pml*-/-/P2X7-/- and *Pml* -/-/NLRP3 -/- C57BL/6 mice (6 for each condition) were inoculated subcutaneously with B16-F10 cells (1 x 10^6). Left panel, tumor growth kinetics for the indicated time points. Middle panels, quantification of tumor volumes and weights. Right panel, representative excised tumors imaged 14 days post-injection. Error bars indicate s.e.m. Scale bars, 1cm. (C) Representative sections of tumors from specimens of *Pml* KO, *Pml*/P2X7 DKO and *Pml*/NLRP3 DKO groups, stained with H/E or immunostained for F4/80, iNOS, mannose receptor (manR) and Ly6g. Nuclei are counterstained with Harris' haematoxylin. TM: tumor mass. Scale bars: 50 μm. (D) The peritumoral tissue of *Pml*$^{-/-}$ mice is distinctly enriched with cells expressing F4/80, mannose receptor and Ly6g immunoreactivity, and almost devoid of iNOS immunoreactive cells, compared with *Pml*/P2X7 DKO and *Pml*/NLRP3 DKO mice. Data are expressed as the mean ±s.e.m. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, ****$p<0.0001$

Regulation of the NLRP3 inflammasome at ER/MAM compartments by PML

Finally, to clarify the molecular mechanism through which PML modulates NLRP3 and IL-1β release, we investigated the intracellular localization of NLRP3 and P2X7 in PML-depleted model either in resting conditions or after treatment with different inflammasome inducers (LPS and nigericin). Through immunofluorescence (Fig. 19A-B) we discovered that in PML-deficient cells there was enhanced NLRP3 localization at the ER in response to NLRP3 activation.

As recently reported, the P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein 25690658(Franceschini, Capece et al. 2015) and mediates NLRP3 inflammasome-dependent IL-1β secretion in response to ATP 26877061(Karmakar, Katsnelson et al. 2016). Using the proximity ligation assay (PLA) we confirm that NLRP3 interacts with P2X7 in large extent in the ER in *Pml*$^{-/-}$ model (Fig. 19C) compared to WT.

To support our findings we performed subcellular fractionation of WT and *Pml*$^{-/-}$ mouse BMDMs (Fig. 19D) and we revealed that in *Pml*$^{-/-}$ model, NLRP3 and P2X7 redistribute to MAMs upon NLRP3 inflammasome activation.

Interestingly, we first demonstrated that P2X7 receptor, one of the most well-known activators of the NLRP3 inflammasome, in addition to its localization to the cell membrane, is present at the ER and MAM.

Furthermore, we performed co-immunoprecipitation experiments of proteins extracted from the ER fraction in resting conditions or after treatment with inflammation inducers finding that P2X7 and NLRP3 interaction is stronger when PML is lost (Fig. 19E).

Moreover, taken together these results showed that PML at MAMs can orchestrate the interaction between P2X7 and NLRP3 in order to control inflammasome activation (Fig. 19F).

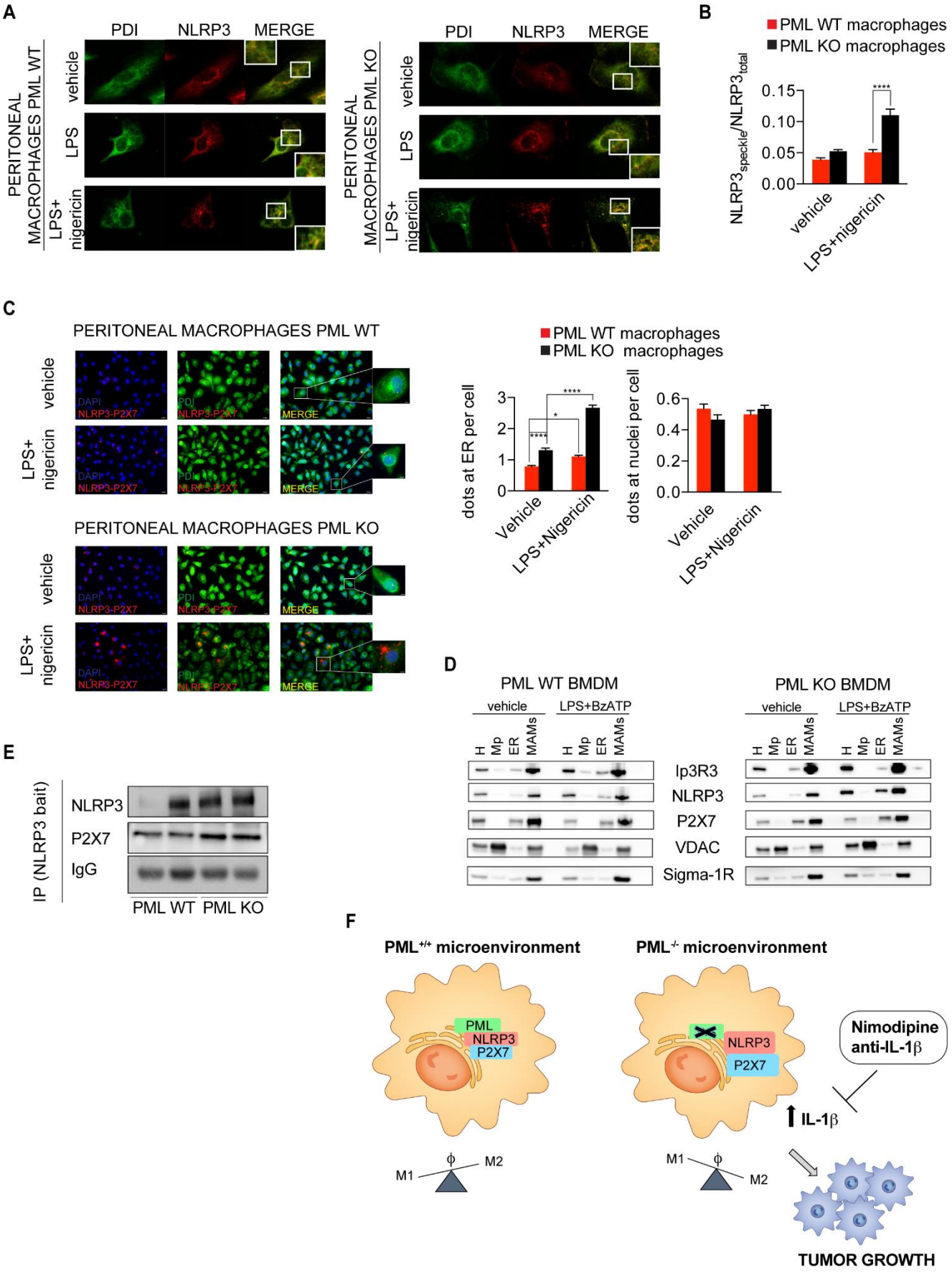

A
PDI
NLRP3
MERGE
PERITONEAL MACROPHAGES PML WT
PERITONEAL MACROPHAGES PML KO
vehicle
LPS
LPS+ nigericin
B
PML WT macrophages
PML KO macrophages
NLRP3speckle/NLRP3total
0.15
0.10
0.05
0.00
****
vehicle
LPS+nigericin
C
PERITONEAL MACROPHAGES PML WT
PERITONEAL MACROPHAGES PML KO
DAPI
NLRP3-P2X7
PDI
MERGE
PML WT macrophages
PML KO macrophages
dots at ER per cell
dots at nuclei per cell
Vehicle
LPS+Nigericin
D
PML WT BMDM
PML KO BMDM
vehicle
LPS+BzATP
H
Mp
ER
MAMs
Ip3R3
NLRP3
P2X7
VDAC
Sigma-1R
E
IP (NLRP3 bait)
NLRP3
P2X7
IgG
PML WT
PML KO
F
PML+/+ microenvironment
PML-/- microenvironment
PML
NLRP3
P2X7
M1
M2
Nimodipine
anti-IL-1β
IL-1β
TUMOR GROWTH

Figure 19: PML at MAMs orchestrates the interaction between P2X7 and NLRP3 to control inflammasome activation. (A) The co-localization of NLRP3 (red) and PDI (used as a ER marker, green) in WT and *Pml*$^{-/-}$ peritoneal macrophages untreated and after treatment with LPS alone and LPS with the addition of nigericin, was analyzed based on immunofluorescence using confocal images. The lower right panels display the merged image of the two stains. (B) Manders coefficient for NLRP3 staining was calculated according to Manders coefficient method as the proportion of NLRP3 speckle signal overlapping with the NLRP3 total signal. (C) Representative images of PLA (red signal) from WT and *Pml*$^{-/-}$ peritoneal macrophages untreated or treated with LPS and nigericin. PDI (green) was used as an ER marker. Scale bar, 10 μm. On the bottom right panel zoomed regions display interaction sites at ER (scale bar, 2 μm). On upper right panel, quantitative analysis of PLA signal between NLRP3 and P2X7. Bars: S.E.M. n=3. (D) Immunoblot of subcellular fractions isolated from WT and *Pml*$^{-/-}$ mice livers, where IP3R is used as an ER marker, Sigma 1-R is used as a MAM marker, and β-tubulin as a cytosolic marker. H: homogenate; ER: endoplasmic reticulum; MAMs: mitochondria-associated membranes. (E) ER fractions prepared as in (D) were used for co-immunoprecipitation of endogenous NLRP3 with P2X7. Using NLRP3 as bait, the levels of P2X7 can be detected showing that interaction between NLRP3 and P2X7 changes in the absence of PML or after inflammasome activation. (F) A model of how the PML-dependent regulation of macrophage phenotype contributes to oncogenesis and how the PML-NLRP3 axis might be a suitable target for pharmacological intervention. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, ****$p<0.0001$

Discussion

PML is a tumor suppressor that is present in the nucleus, where it facilitates targeting of transcription factors and co-regulators to specific regions of the genome to control gene expression. PML exists not only at the nuclear level but also in MAMs, where it controls several key cellular functions, such as Ca^{2+} transfer to mitochondria, cell death and autophagy (Pinton, Giorgi et al. 2011). PML represses autophagy, controls autophagosome formation at MAMs and PML loss promotes tumor development (Guan and Kao 2015). Interestingly, MAMs have been shown to function as a platform for inflammatory signaling regulated by the most widely characterized inflammasome, so-called NLRP3. An involvement of PML in NLRP3 activation has been recently described by two studies that both confirm an interaction between PML and NLRP3 inflammasome (Dowling, Becker et al. 2014) (Lo, Huang et al. 2013). However, the study by LO et al demonstrated that PML could promote NLRP3 inflammasome activation, while data from Dowling and colleagues identified PML as a novel regulator of ASC localization, limiting NLRP3 inflammasome activation. To date the precise role played by PML in NLRP3 inflammasome formation and activation remains largely obscure.

While PML has been widely studied in cancer cells, far less is known about PML function in tissue-resident primary immune cells that serve as front-line sentinels in response to stress signals.

In this study we demonstrated that loss of PML activates NLRP3 inflammasome, investigated by assessing the cleavage of caspase-1 and the secretion of mature IL-1β and IL-18. Through silencing we revealed that selective inhibition of NLRP3, in a PML KO model, reduces IL-1β release suggesting that PML targets to NLRP3 inflammasome but not to a common mechanism downstream of different inflammasomes.

Furthermore, we have addressed some of the issues relevant to the comprehension of the possible role of PML in tumorigenesis *in vivo*. We took advantage of the B16-F10 melanoma cancer-derived syngeneic cell lines and we compared their growth in two different hosts: *Pml* WT and *Pml*$^{-/-}$ C57BL/6 mice. Under the experimental conditions used here, tumors generated in *Pml*$^{-/-}$ mice was two folder compared to those generated in Pml WT one. Using LL/2 cells also showed faster growth rate of tumors in *Pml*$^{-/-}$ C57BL/6 mice. These effects are consistent with the finding that there is IL-1β increase that promotes a pro-tumor TME.

This phenomenon represents an important finding because it indicates that, in the absence of the PML drive, not only can the tumor more easily escape from programmed cell death, but can also paradoxically be sustained by elevated levels of inflammation.

Overall, these data demonstrated that PML deficiency in the TME favors tumor establishment and progression by enhancing IL-1β secretion, which in turn promotes tumor growth with a direct effect on cancer cells.

In particular, since macrophages are the main actors in the innate immune NLRP3 inflammasome-mediated response, and their function depends on the environment in which they reside, we focused on how PML-dependent regulation of macrophage phenotype contributes to oncogenesis demonstrating that in a PML null host, under certain stress conditions, misdirecting TAMs promotes a hyper-secretion of IL-1β, which in turn facilitates tumor immune escape. Indeed, by examining tumor-infiltrating leukocytes in the syngeneic model, we demonstrated that loss of PML increases the number of M2-like macrophages in TME.

The crucial role of PML expression on host hematopoietic cells in antitumor response was further confirmed by generating PML chimeric mice. PML-wt>PML-KO chimeras allowed a much faster tumor growth than PML-wt>PML-wt chimeras.

Manipulating the inflammasome therapeutically prior to activation would have the benefit of blocking the release of IL-1β. For this reason, drugs targeting the inflammasome constituents are increasingly being tested. Despite their promising efficacy, anti-IL-1 therapies do not resolve the disease, probably due to their inability to access the site of inflammation or to problems linked to other caspase-1 targets such as IL-18, which still maintains the ability to promote diseases (Missiroli, Patergnani et al. 2018).

An additional novel observation of great potential impact stemmed from our study is that pharmacologic blockade of NLRP3 inflammasome through nimodipine reduces the undesired host-dependent pro-tumoral phlogosis, due to its anti-inflammatory effect by inhibiting IL-1β release.

Nimodipine is a calcium channel antagonist originally developed for the treatment of high blood pressure. Recently, nimodipine has been found to protect microglia from Aβ-dependent cytotoxicity due to its anti-inflammatory effect by inhibiting IL-1β release, which points to a novel role for this drug as a centrally acting anti-inflammatory compound (Sanz, Chiozzi et al. 2012, Guo, Callaway et al. 2015). In the present study, we demonstrated the anti-inflammatory effects of nimodipine in reducing IL-1β release and consequently tumor growth in a PML KO model.

It is known that ATP represents one of the most central danger signals that leads to the activation of the NLRP3 pathway and the secretion of pro-inflammatory cytokines by innate immune cells through activation of the purinergic P2X7 receptor (Guo, Callaway et al. 2015). It has recently been demonstrated that P2X7 can also directly interact with the NLRP3 inflammasome scaffold protein

and mediate NLRP3 inflammasome-dependent IL-1β secretion in response to ATP (Karmakar, Katsnelson et al. 2016). In addition pharmacologic P2X7R blockade or silencing has a strong antitumor effect, in fact administration of two selective P2X7R blockers to P2X7R-wt tumor-bearing mice had a strong inhibitory effect on tumor growth even when systemically administered, (Adinolfi, Raffaghello et al. 2012, Adinolfi, Capece et al. 2015). Of further interest, we pinpointed that P2X7 silencing caused a large growth decrease of mouse melanoma B16-F10 tumor in *Pml*$^{-/-}$ limiting NLRP3-dependent pro-inflammatory and pro-tumoral signals.

The recent demonstration that WD repeat 4-containing cullin-RING ubiquitin R4 (WDR4) negatively regulates PML via ubiquitination to promote lung cancer progression by fostering an immunosuppressive and prometastatic tumor microenvironment (Wang, Chen et al. 2017), supports our findings that PML at the host level drives an immune response that is able to worsen cancer instead of ameliorate it, enhancing the process of IL-1β release.

To clarify the molecular mechanism through which PML modulates NLRP3 and IL-1β release we took advantage of immunofluorescence and subcellular fractionation and we revealed that in the absence of PML, NLRP3 is redistributed to MAMs. We therefore investigated the P2X7 receptor localization finding that, in addition to its localization to the cell membrane, is present at the ER and MAM. By co-immunoprecipitation and PLA assay we verified that NLRP3-P2X7 interaction is more stronger in the absence of PML or after inflammasome activation.

Taken together these data determine that PML at MAMs can orchestrate the interaction between P2X7 and NLRP3 in order to control inflammasome activation.

This finding not only points to a possible role of PML axis in regulating the efficacy of NLRP3 inflammasome activation, but also suggests a therapeutic strategy of targeting of NLRP3 inflammasome for treating patients with aberrant PML degradation.

Loss of PML reduces radiotherapy efficacy through NLRP3 inflammasome activation

Introduction

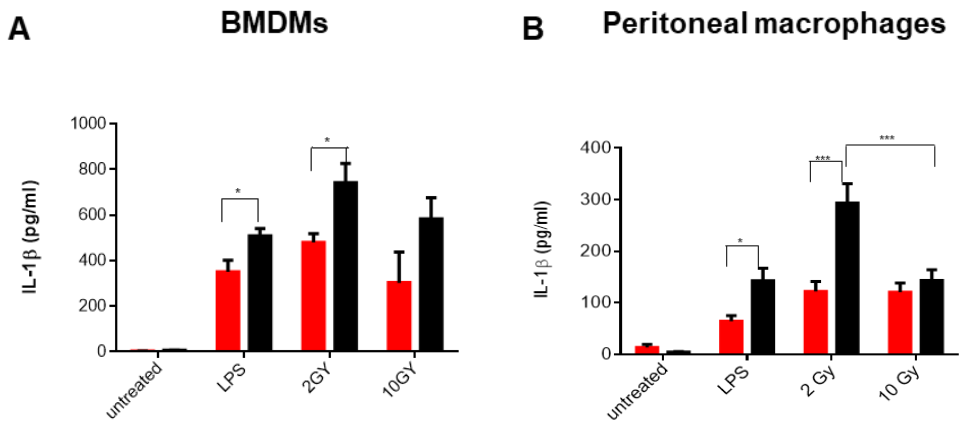
Radiation therapy (RT) is one of the most used treatments in cancer therapy, in fact more than 50% of patients with cancer are exposed to this medical treatment. This is based on the use of high doses of radiation that destroy the tumor cells in the treated area. The main goal is to increase local tumor control, minimizing damage to the patient's surrounding healthy tissue. Despite progress in research and technology, RT is not always effective.

It is known that radiotherapy induces the inflammatory process (Ratikan, Micewicz et al. 2015, Stoecklein, Osuka et al. 2015) and recently, Liu et al. have shown NLRP3 inflammasome activation by radiation at different doses (Liu, Chen et al. 2017). The immunostimulatory activity is due to the release of DNA and ATP from cells damaged by radiation, which activate the inflammasome with IL-1β release (Ohshima, Tsukimoto et al. 2010). This is due to the radiation-induced cell death, which is followed by the release of tumor antigens together with pro-inflammatory signals. In particular, IL-1β is a pleiotropic pro-inflammatory cytokine and its up-regulation is closely associated with various cancers, but it is still unclear how it could predispose or support malignant cell degeneration. However, activated inflammasome may influence carcinogenesis by modulating the amount of cytokines in the tumor microenvironment (TME) (Zitvogel, Kepp et al. 2012), due to its ability to drive immunosuppressive cell recruitment. These findings suggest that hyper-secretion of IL-1β by inflammasome into the tumor interstitium is damaging to the host and for this reason it has been proposed that IL-1β antagonists could be a therapy for primary and metastatic tumor. On the other hand, cell death due to radiations is followed by the release of tumor antigens and pro-inflammatory signals that together are able to transform the tumor into a sort of "in situ vaccine" capable of activating the anti-tumor immune response. Recently the involvement of the tumorsuppressor PML in NLRP3 inflammatory process has been demonstrated (Lo, Huang et al. 2013, Dowling, Becker et al. 2014). Nowadays, there are two different studies that report conflicting roles of the PML protein in the activation of the NLRP3 inflammasome with a molecular mechanism still unclear. Thus, the NLRP3 inflammasome is an important factor in the inflammatory process triggered by radiotherapy, but with a controversial role: understand when its activation can contribute in the anti-tumor immune response and when, instead, it can promote tumor progression, it will be fundamental in the development of personalized therapies.

Results

Loss of PML increases IL-1β release after low doses of RT

The first goal of this work is to deeply investigate the contribution of RT to NLRP3-mediated inflammation in a PML-dependent manner. We validate the effect of different gamma rays, 2Gy as conventional treatment and 10Gy as ablative treatment, either *in vitro*, by measuring IL-1β secretion in the cell supernatants of treated mouse bone marrow-derived macrophages (BMDMs) or peritoneal-derived macrophages using LPS stimulation as a positive control for NLRP3 activation, or *ex vivo*, into the peritoneal cavity of treated mice. We found a hyperactivation of NLRP3 inflammasome in absence of PML both *in vitro* and *ex vivo* after 2Gy (Fig. 20A-C). Low doses of radiation in absence of PML induce a higher inflammatory response compared to that observed in WT as confirmed also checking the cleaved (active) form of caspase-1 and IL-1β by WB (Fig 20D). In addition, we silenced the expression of PML in human peripheral blood mononuclear cells (PBMCs) from healthy donors. We tested the ability of radiotherapy to activate the NLRP3 inflammasome in the PBMCs and we found a high IL-1β release in PML silenced PBMCs, both with canonical stimulus such as LPS and 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP) both after 2Gy in PML silenced PBMCs (Fig. 20E-F).



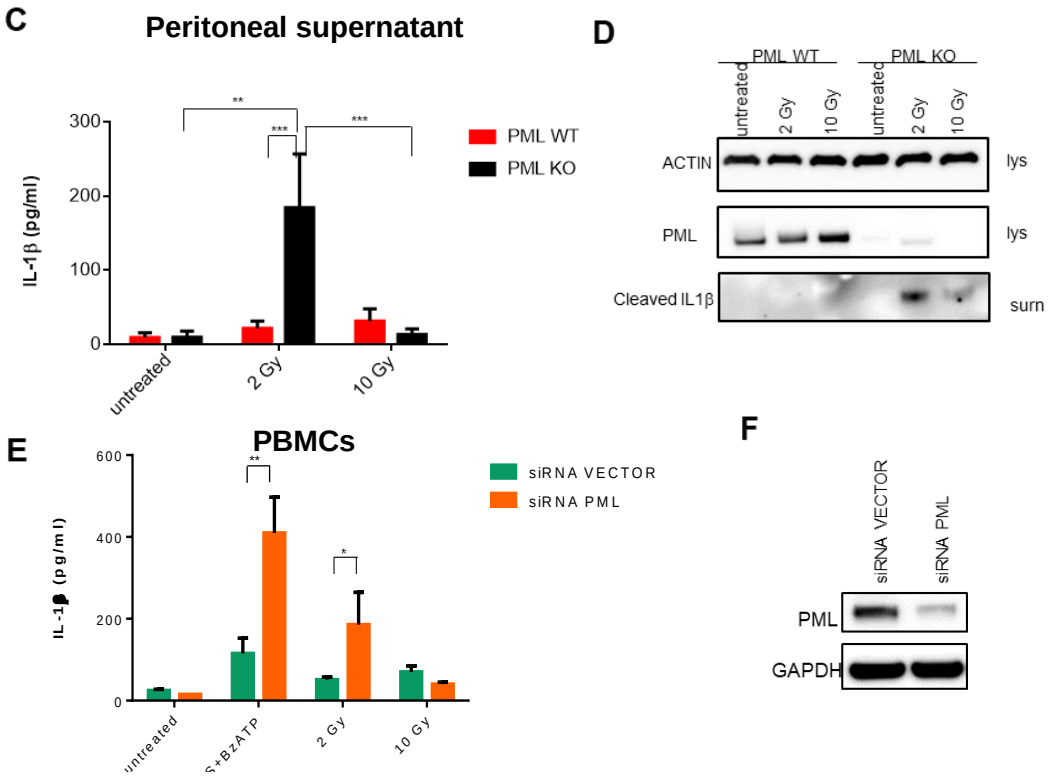


Figure 20: Loss of PML increases IL−1β release after low doses of radiotherapy. (A) BMDMs and peritoneal-derived macrophages (B) from wild type (WT) and *Pml*$^{-/-}$ mice were left untreated or stimulated with: LPS (1µg\ml for 2h), 2 Gy or 10 Gy. Supernatants were analyzed for IL-1β production by ELISA. Error bars indicate s.e.m. n=3 (C) Production of IL-1β from peritoneal supernatant from WT and *Pml*-/- mice after treatment with 2 Gy or 10 Gy as measured by ELISA. Error bars indicate s.e.m., n=3. (D) Culture supernatants and peritoneal macrophages from WT and *Pml*$^{-/-}$ mice were collected before and after radiotherapy treatments. Total cell lysates (Lys) and supernatants (Sup) were resolved by SDS-PAGE. Results shown were representative of three independent experiments. (E) IL-1β production (as measured by ELISA) from PBMCs stimulated with LPS and BzATP, 2 Gy or 10 Gy after siRNA transfection. Error bars indicate s.e.m., n=3. (F) PML expression by western blot after siRNA transfection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,****$p<0.0001$

To evaluate the relevance of NLRP3-mediated release of IL-1β after treatments we tested NLRP3 KO mice, either *in vitro* for peritoneal macrophages and *ex vivo* for peritoneal exudate. Radiotherapy is able to induce the IL-1β release also in NLRP3 KO mice suggesting a partially dependent activation (Fig. 21A-B). In fact, radiation can increase susceptibility to other inflammasomes such as NLRP1, NLRC4 or AIM2 that recognize PAMPs and also contribute to radiation-induced caspase-1 activation. Moreover, to determinate the pitoval role of PML in the NLRP3-mediated inflammation by RT we reintroduced PML *in vitro* in peritoneal macrophages derived from PML KO mice (Fig. 21C). The restoration of the tumorsuppressor drastically decreases IL-1β release demonstrating its essential function in the activation of the inflammasome (Fig. 21D).

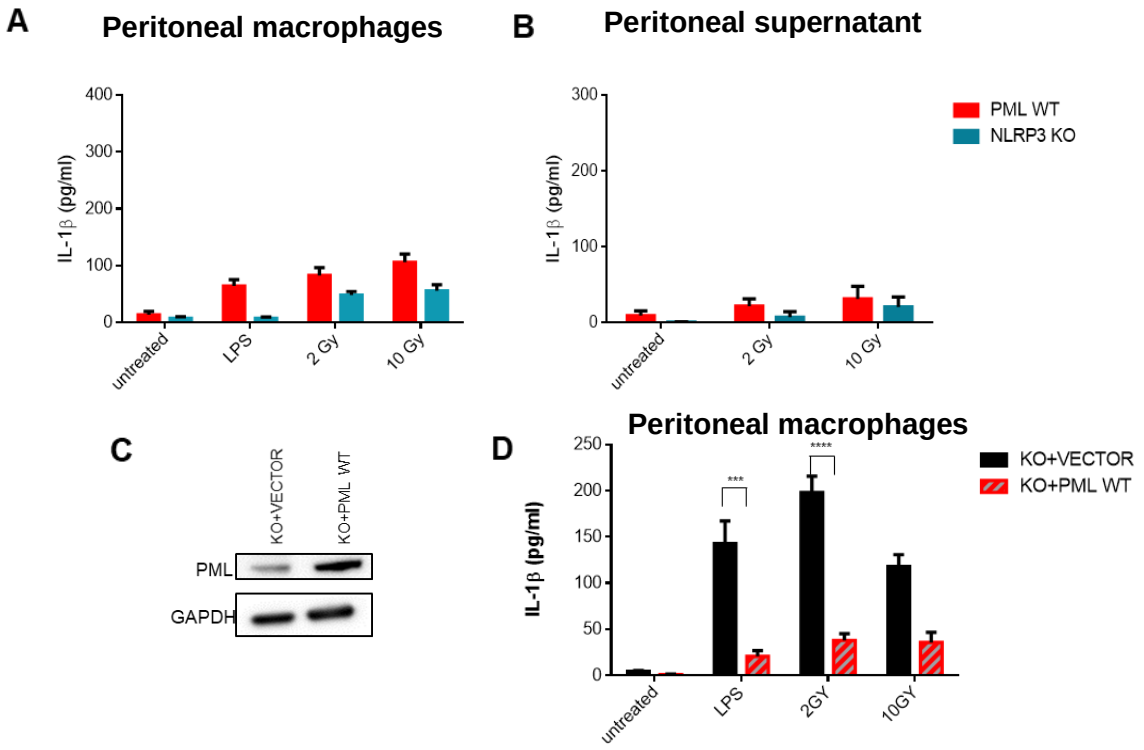


Figure 21: PML is essential for IL-1β release by radiation in partially NLRP3-dependent manner. (A) Peritoneal-derived macrophages from wild type (WT) and *Pml*$^{-/-}$ mice were left untreated or stimulated with: LPS (1µg\ml for 2h), 2 Gy or 10 Gy. Supernatants were analyzed for IL-1β production by ELISA. Error bars indicate s.e.m. n=3 (B) Production of IL-1β from peritoneal supernatant from WT and *Pml*-/- mice after treatment with 2 Gy or 10 Gy as measured by ELISA. Error bars indicate s.e.m., n=3. (C) PML expression after trasfetion by western blot in peritoneal macrophages lysate. (D) IL-1β release in peritoneal macrophages untreated or treated with LPS, 2 Gy or 10 Gy after transfection. * $p < 0.05$, *** $p < 0.001$,****$p<0.0001$

Correlation between IL-1β by radiotherapy and tumor growth

Although there is evidence that plasma IL-1β expression is elevated in patients with advanced cancers, it remains unclear how IL-1β may contribute to the initiation and development of these inflammation-associated cancers. Here, we hypothesize that PML deficiency in the Tumor Micro-Environment (TME) may favor tumor establishment and progression by enhancing IL-1β secretion, which in turn promotes tumor growth with a direct effect on cancer cells (see above).

We decided to investigate the biological effect of IL-1β, released as a consequence of the stress condition upon tumor cell inoculation and after RT on tumor development. We compare the growth of syngeneic tumor model, which maintain functional PML, in two different "recipients", WT and KO mice, as hosts. We subcutaneously injected melanoma cancer cells (B16-F10) and after one week we treated tumor mass on mice with conventional or ablative RT in a single dose (2Gy or 10Gy).

Tumor size was followed as markers of treatment outcome until day 14. Tumor growth rate was significantly reduced in WT mice (low inflammatory background) receiving both low and high doses of RT. On the contrary, low doses of conventional RT in a high inflammatory background (PML KO mice) had no significant effect on tumor growth rate, demonstrating that low doses of RT have harmful effects on tumors growth in PML KO host compared to WT (Fig. 22A-D).

In addition, to verify if the effect of IL-1β released from macrophages after RT is direct on tumor cells, we established a co-culture model. Peritoneal macrophages obtained from WT and *Pml*$^{-/-}$ mice were cultured into removable inserts on the top of a plate, activated by RT, and incubated with cancer cells seeded on the bottom of the plate separated by a porous membrane that allows free exchange of cytokines. The viability and cell growth of tumor target cells were analyzed with colorimetric assays (crystal violet). We found that cancer cells co-cultured with peritoneal macrophages from PML KO mice treated with 2Gy grow faster compared to cells co-cultured with peritoneal macrophages from PML KO mice left untreated. At the same time, cells co-cultered with peritoneal macrophages from WT mice grow less both after 2Gy and 10Gy (Fig. 22E) suggesting that the hyper production of IL-1β after 2Gy is responsible of the negative effect of RT on tumor growth in a *Pml* $^{-/-}$ host.

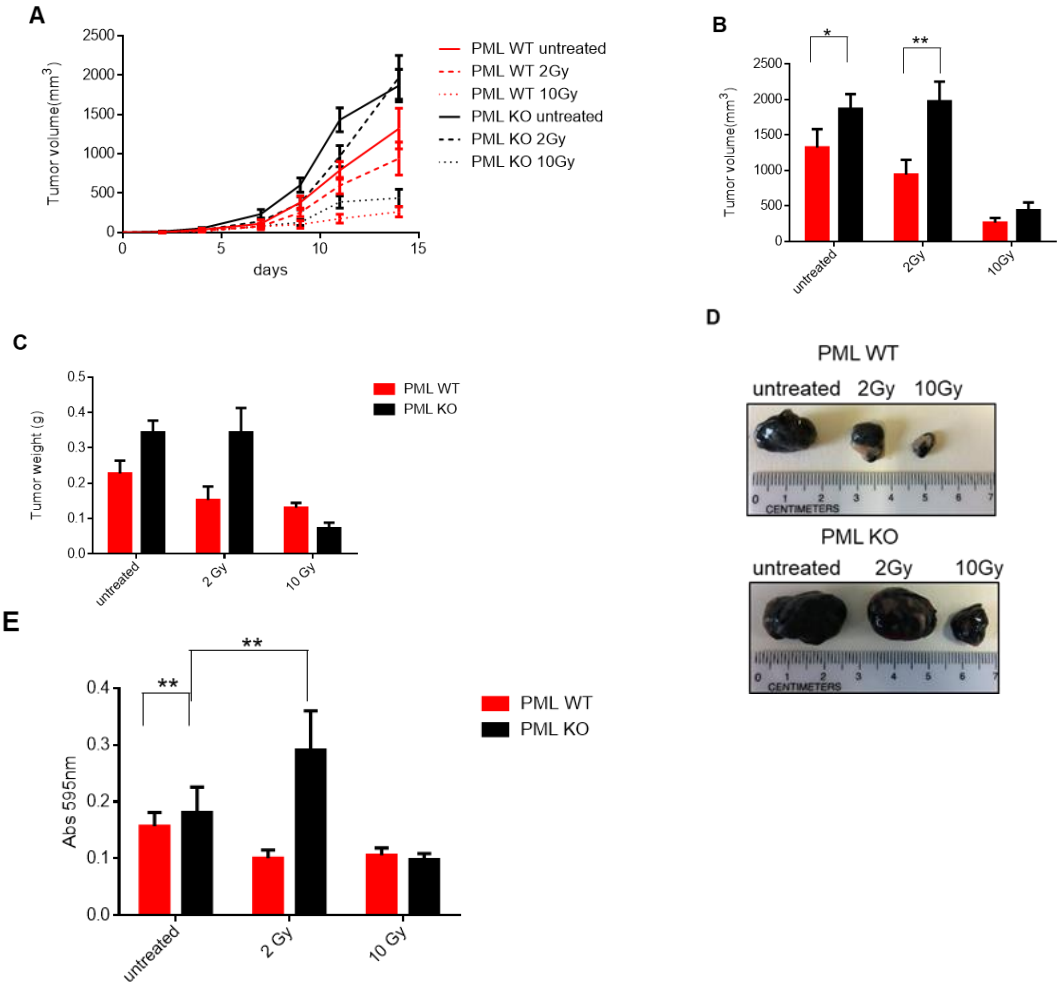
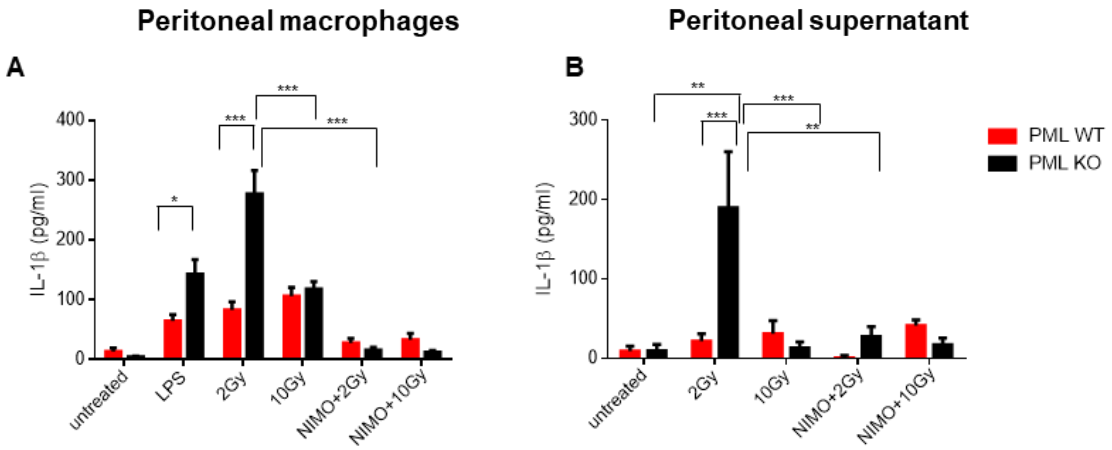


Figure 22: Relationship between IL-1β and tumor growth. (A) WT and Pml-/- C57BL/6 mice (7 for each condition) were subcutaneously inoculated with B16-F10 melanoma cells (1 x 10^6). After one week mice were divided in three groups: untreated, treated with 2Gy or 10Gy. Graphs show tumor growth kinetics for the indicated time points. Quantification of tumor volumes (B) and tumor weights (C) after 14 days post inoculation. (D) Representative excised tumors images. Error bars indicate s.e.m. Scale bars, 1cm. (E) B16-F10 cells were co-cultured with macrophages from Pml WT and Pml$^{-/-}$ mice, treated with 2Gy or 10Gy. After one week, proliferation of B16-F10 cells was analyzed by crystal violet staining (absorbance at 595 nm). Experiments were performed twice. Error bars indicate s.e.m. *p<0.05, **p<0.01.

NLRP3 inhibition reduces radiotherapy-induced IL-1β release

Despite their promising efficacy, anti-IL-1 therapies do not resolve the disease. This is probably due to their inability to access the site of inflammation or to problems linked to other caspase-1 targets such as IL-18, which still maintains the ability to promote diseases. Drugs that directly target inflammasome components or their modulators could offer greater therapeutic promise. Therefore, we developed pharmacological approaches to limit these NLRP3-dependent pro-inflammatory and pro-tumoral signals in order to reinforce the beneficial action of RT with a possible clinical application.

Nimodipine is a calcium channel antagonist originally developed for the treatment of high blood pressure. Recently, nimodipine has been found to protect microglia from Aβ-dependent cytotoxicity due to its anti-inflammatory effect by inhibiting IL-1β release, which points to a novel role for this drug as a centrally acting anti-inflammatory compound (Sanz, Chiozzi et al. 2012). In our study, we explored the anti-inflammatory effects of nimodipine in reducing the undesired host-dependent RT-induced pro-tumoral phlogosis, allowing for the better profiling of the use of RT. Our results show that pre-treatment with Nimodipine provokes a drastic reduction of IL-1β, both *in vitro* in peritoneal macrophages and in PBMCs, both *ex vivo* in peritoneal supernatant of mice (Fig. 23A-C).



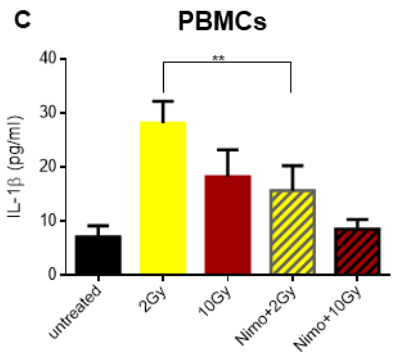


Figure 23: Nimodipine treatment inhibits IL-1β release. (A) Peritoneal-derived macrophages from wild type (WT) and Pml-/- mice were left untreated or stimulated with: LPS (1µg\ml for 2h), 2Gy or 10Gy and pre-treated with Nimodipine (100nM for 30 minutes). Supernatants were analyzed for IL-1β production by ELISA. Error bars indicate s.e.m. n=3 (B) Production of IL-1β from peritoneal supernatant from WT and Pml-/- mice after treatment with 2Gy or 10Gy and pre-treated with Nimodipine as measured by ELISA. Error bars indicate s.e.m., n=3. (C) Production of IL-1β from PBMCs after treatment with 2Gy or 10Gy and pre-treated with MCC950 as measured by ELISA. Error bars indicate s.e.m., n=3. *p<0.05, **p<0.01, ***p<0.001.

Recently, diarylsulfonylureas have been reported to be protective in a number of inflammatory diseases. In particular, MCC950 has been recognized as a potent NLRP3-specific inhibitor but with a suboptimal pharmacokinetic profile (Coll, Robertson et al. 2015). Here, we use MCC950 to reduce IL-1β production *in vitro* after radiotherapy in murine and human model (Fig. 24A-B).

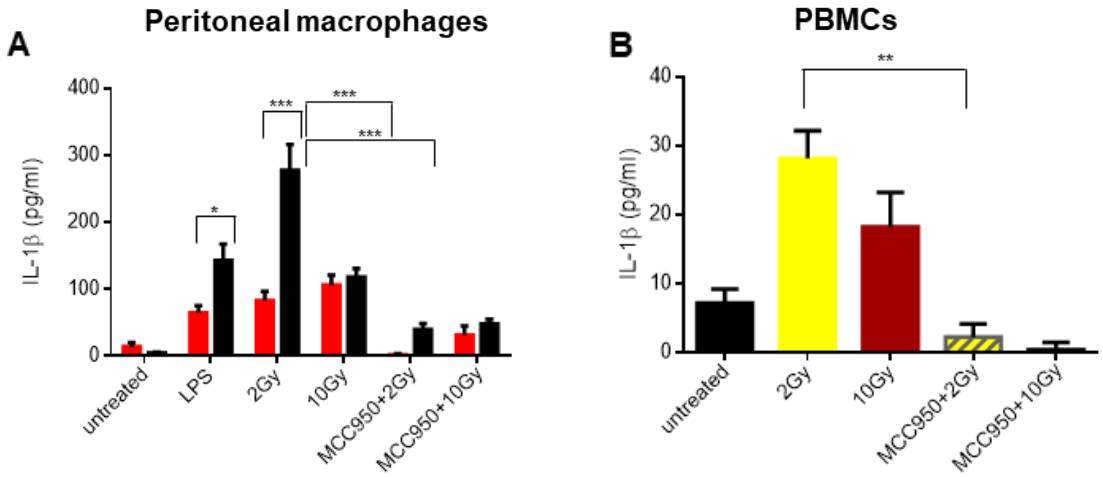


Figure 24: MCC950 inhibits IL-1β release. (A) Peritoneal-derived macrophages from wild type (WT) and *Pml*$^{-/-}$ mice were left untreated or stimulated with: LPS (1µg\ml for 2h), 2Gy or 10Gy and pre-treated with MCC950 (1 µM for 30 minutes). Supernatants were analyzed for IL-1β production by ELISA. Error bars indicate s.e.m. n=3 (B) Production of IL-1β from PBMCs after treatment with 2Gy or 10Gy and pre-treated with MCC950 as measured by ELISA. Error bars indicate s.e.m., n=3. *p<0.05, **p<0.01, ***p<0.001.

Considering the controversial role of NLRP3 inflammasome in tumor development, we evaluated the effect of systemic administration of inflammasome inhibitor on melanoma growth and progression. For this reason, we combined RT treatment (2Gy and 10Gy) with an inhibitor of NLRP3 inflammasome. We therefore hypothesized that Nimodipine, a NLRP3 inhibitor, provides protection in combination with RT in tumor growth in a mouse model. Thus, we subcutaneously injected syngeneic melanoma cancer cells (B16-F10) in mice WT and PML KO mice and as soon as the primary mass was visible and palpable the animals were subdivided randomly into further three groups: control group (untreated) and two distinct groups treated with Nimodipine and different doses of radiation. Tumor size was followed as markers of treatment outcome until day 14. Our results showed that in a condition of chronic and high inflammatory microenvironment, like PML KO mouse, low conventional doses of RT did notreduce tumor growth, while high doses of RT have some effect (reducing tumor comparable a WT). Nevertheless, inhibition of this hyper-inflammed microenviroment with nimodipine in combination with RT drastically reduced tumor growth increasing the citotoxic effect of the anti-cancer treatment (Fig. 25D-F). Meanwhile, nimodipine treatment did not affect tumor growth in a *Pml* WT host and not enhance radiotherapy efficacy, indicating that its action is direct selectively on hyper-production of IL-1β (Fig. 25A-B).

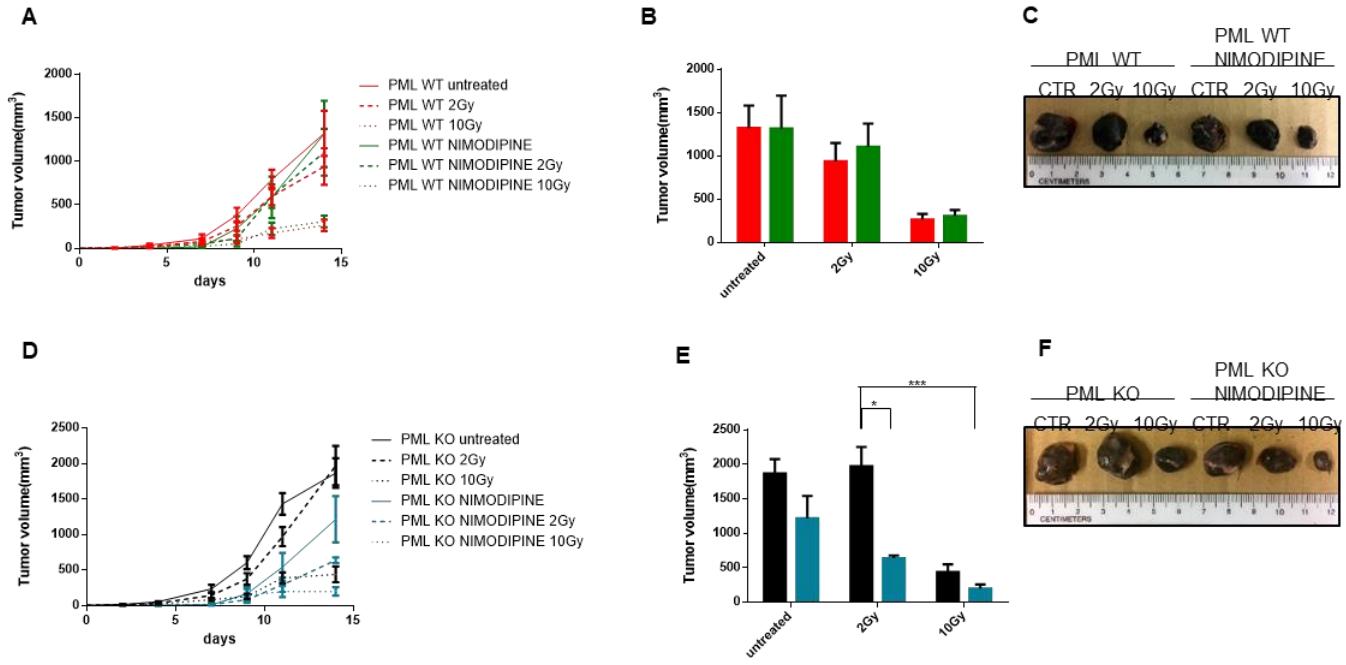


Figure 25: Nimodipine increases efficacy of radiotherapy in PML KO host. (A) WT or *Pml*$^{-/-}$ (B) C57BL/6 mice (6 for each condition) were subcutaneously inoculated with B16-F10 cells (1 x 10^6). Mice, chosen randomly, were treated with nimodipine (i.p.) thrice weekly and RT (2Gy or 10Gy) as single dose. Left panel, tumor growth kinetics at the indicated time points. Middle panels, quantification of tumor volumes. Right panel, representative excised tumors imaged 14 days post-injection. Error bars indicate s.e.m. Scale bars, 1cm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$**** $p < 0.0001$.

Discussion

Traditional research on RT is focused on increasing treatment doses to target volume and reducing treatment fields to spare healthy tissues and increase the direct tumoricidal effect. Advancements in RT technology have designed instrumentation that enables delivery of radiations with great precision to tumor reducing substantially injury to healthy tissues.

However, between patients with similar cancer characteristics (histology, grading, staging) there is evidence of a great unpredictable variability in radiation response. Different studies have investigated molecular mechanisms and have identified many cellular targets that can affect radiation sensitivity, like ROS, DNA-damaging response signals and tumor microenvironments.

Although several radiation sensitizers and protectors have been developed and clinically evaluated (Dolgin 2016), many of these turned out to be inconclusive, indicating that improvement remains needed. In the last decades, evidence has grown about the interaction of the immune system with radiations underlying an active role of RT in immune system activation and in tumor response (Frey, Rubner et al. 2014). Radiations can increase antigen presentation in dendritic cells and improve tumor response with immunotherapy drugs (Fiorica, Belluomini et al. 2018). A consequence of immunity stimulation guided by RT is the activation of an inflammatory response.

Inflammation obtained by RT has been extensively studied (Formenti and Demaria 2013), nevertheless the mechanisms under which RT causes inflammation, are not clearly understood. Recently, it has been shown that RT is able to activate “inflammasome”. In turn, inflammasome has also been shown to play an important role in modifying inflammation pathways. Among inflammasomes, the NLRP3 is implicated in inflammatory response induced by RT. The NLRP3 activation is characterized by the release of IL-1β, a pleiotropic pro-inflammatory cytokine whose up-regulation is closely associated with various cancers (Vitale, Rigante et al. 2016). Indeed, chronic inflammation (sustained by over-activation of IL-1β system) is a crucial event in carcinogenesis and tumor progression and there is evidence that plasma IL-1β level is higher in patients with advanced cancers (Lamkanfi and Dixit 2014).

How IL-1β may contribute to the initiation and development of these inflammation-associated cancers; whether this flogosis can sustain tumor growth and how would be possible to modulate it, are still open questions.

PML is a multi-faceted protein that plays pivotal roles in tumor growth. PML has been shown to modulate a wide number of cell activities, including tumor suppression, anti-viral and anti-bacterial

responses and inflammatory responses. We previously demonstrated that PML exists not only at the level of the nucleus but also at the endoplasmic reticulum (ER)-mitochondria contact sites (also called Mitochondria-Associated Membranes, MAMs) where it controls several key functions such as calcium transfer to mitochondria, cell death and autophagy.

Our previous data show that PML at MAMs can orchestrate the interaction between P2X7 and NLRP3 in order to control inflammasome activation in the host and determinate cancer development (see above). In particular, we found that a PML KO environment displays a higher inflammatory response that in turn favors tumor growth compared to that observed in the WT.

Thus, aim of this project is to demonstrate a clear correlation between NLRP3 inflammasome pathway activation during RT treatment and cancer outcomes.

Our results offer several interesting findings. First of all, radiotherapy is able to activate the NLRP3 inflammasome inducing release of IL-1β. We detected the IL-1β release either *in vitro*, from cultured murine BMDMs and peritoneal macrophages and human PBMCs after irradiation, or *ex vivo*, in peritoneal supernatant of irradiated mice. In particular, we demonstrate that low doses of radiation are able to iper-activate NLRP3 inflammasome in a PML KO background, a host with a high inflammatory environment. To assess NLRP3 inflammasome activation, we quantified either IL-1β secretion or the cleaved forms of caspase-1 after RT, using LPS or 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP) stimulation as a positive control for NLRP3 activation. Remarkably, in addition to the classical inflammation inducer, radiotherapy also strongly stimulated IL-1β secretion.

Moreover, we used NLRP3 deficient mice to evaluate if the effect of RT on IL-1β secretion is NLRP3 inflammasome-dependent. RT can induce the IL-1β release also in $NLRP3^{-/-}$ mice suggesting a partially dependent activation. However, other inflammasomes such as NLRP1, NLRC4 or AIM2 that recognize PAMPs could be activated by radiation and also contribute to radiation-induced caspase-1 activation (Coggle, Lambert et al. 1986, Francois, Milliat et al. 2013, Guo, Callaway et al. 2015).

There is evidence that higher doses of irradiation get better clinical effects on tumor growth compared to low dosage, even if up today the conventional RT is based on low doses of irradiation fractionated (Muller-Runkel and Vijayakumar 1991).

To verify the hypothesis that the RT-dependent inflammatory response due to the NLRP3 activation and IL-1β release promotes tumor growth, we tested the effect of RT on immunocompetent syngeneic murine tumor model. To this end, we injected subcutaneously melanoma B16-F10

melanoma cancer-derived syngeneic cell lines in two different hosts: *Pml* WT and *Pml*$^{-/-}$ C57BL/6 mice, that display low and high levels of inflammation and we compared the tumor growth. Our data demonstrated that treatment with low or high doses of RT that exacerbate the inflammatory response, did not affect the reduction of tumor growth, while high doses of RT clearly provoke a reduction on tumor progression.

Moreover, to ascertain if the effect of IL-1β released from macrophages after RT directly affect tumor cells, we established a co-culture model. As we expected, cancer cells co-cultured with peritoneal macrophages from PML KO mice and treated with RT 2Gy grow faster compared to cells left untreated confirming that IL-1β release by low doses of RT is the main actor of inflammation RT-induced tumor progression.

Drugs targeting IL-1 downstream of the inflammasome pathway are available and are currently in use or undergoing trial analysis for the management of many auto-inflammatory disorders. However, despite their promising efficacy, anti-IL-1 therapies do not resolve the disease. Therefore, we decided to test in our model the anti-inflammatory effects of nimodipine, previously known as an inhibitor of IL-1β release in cells from microglia (Sanz, Chiozzi et al. 2012), and of the specific NLRP3 inhibitor MCC950 (Coll, Robertson et al. 2015) in combination with RT. As above confirmed low doses of RT alone activate NLRP3 inflammasome, however pre-treatment with nimodipine or MCC950 in combination with RT 2Gy significantly affects IL-1β release as demonstrated both *in vitro* in peritoneal macrophages and in PBMCs, both *ex vivo* in peritoneal supernatant of mice.

Considering the great ability of Nimodipine to reduce IL-1β production, we tested the antitumor activity in association with RT in terms of delaying tumor growth. We subcutaneously injected syngeneic melanoma cancer cells (B16-F10) in WT and PML KO mice and we treated with Nimodipine and different doses of radiation. Our data reveal that nimodipine treatment didn't affect RT efficacy on tumor growth in a PML WT host; conversely in PML KO mice with high level of inflammation, nimodipine treatment in combination with RT drastically reduced tumor growth increasing anti-cancer treatment.

Taken together, these data reveal that low doses of RT induces a hyper-activation of NLRP3 inflammasome through a high IL-1β release in the microenvironment in absence of PML. Although IL-1β can has a potent stimulatory effect on immune response, it can also stimulate the tumor

growth. Inhibiting NLRP3 inflammasome activation could improve the efficacy of RT protocols by limiting the possible undesirable side effects consisting in the promotion of tumor growth.

Future perspectives

About 14 million new cases of cancer occurred globally in one year with about 8 million deaths. Thus, it is of the utmost importance to understand signaling pathways deregulated to decrease the rate of mortality increasing the cure rate of malignant tumors. Radiotherapy is a critical point in cancer therapy to cure more than 50% of patients and reduce its impact on patient health. Numerous lines of evidence have integrated the consolidated traditional notion of radiation having inhibitory effect on immunity system with the outstanding observation that RT can strongly stimulate the immunity response. The cooperation between RT and immunitary response is testified by the so-called abscopal RT effect (Kalbasi, June et al. 2013). Immunostimulatory activity of RT can achieve tumor rejection or also stimulate the tumor growth. Our aim is to determine, at the preclinical level, how to properly modulate the impact of anticancer therapies on tumor growth and the immune system in order to better profile the use of radiotherapy and improve patient outcomes.

Single nucleotide polymorphisms, frequently called SNPs, are single nucleotide variations between different individuals of the same species and are the most common type of genetic variation among human individuals. SNPs in the protein coding regions were classified into synonymous SNPs (without amino acid change), non-synonymous SNPs (with amino acid change) and nonsense mutations (where the SNP codes for a stop codon). We will select the SNPs that occur within the coding regions of the PML gene in the human database and will address the measure of their frequency. This procedure will be used to prioritize the investigation of those SNPs that could play a more direct role by affecting the gene's function.

For those SNPs that are expressed at a higher frequency in the population, we will determine if they could be responsible for a mutant inflammatory phenotype.

In this way, we could design a population-based research, rationalizing combinations of RT and target therapy, nowadays used only empirically.

The purpose of this study is to continue in order to obtain a correlation among IL-1β production, oncological outcomes and toxicity profile after RT. We want to improve the efficacy of RT protocol by defining its role in promoting tumor growth and selecting IL-1β inhibitors specifically designed to get the highest immunity response and the lowest tumor growth.

Materials and Methods

Cell cultures and transfections

B16-F10 melanoma cells were grown in RPMI medium supplemented with nonessential amino acids (Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The LL/2 mouse Lewis lung carcinoma cell line was obtained from Sigma; LL/2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 1% glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO2.

Only cells that were tested negative for mycoplasma contamination were used in experiments. For BMDMs and peritoneal macrophages, mice were killed by excess anesthesia, and 5 mL sterile PBS was injected into the peritoneal cavity. The peritoneum was gently massaged, and fluid recovered. Peritoneal macrophages were collected by centrifugation at 200 x g for 5 minutes at 4°C then a total of $3x10^5$ cells/well were added to 24-well tissue culture plates; a total of $1x10^6$ cells were added to 6-well tissue culture plates. Macrophages were transfected with siRNA NLRP3 and siRNANLRC4 using lipofectamine RNAiMAX (Invitrogen) or with the following plasmid erPML (21030605) using jetPEI-Macrophage (Polyplus transfection).

Cocolture assay

For coculture cell assays, the macrophage from WT and Pml-/- mice were seeded in 6-well plates until 80% confluence and cocultured in an insert chamber placed on top of the B16-F10 cells. The bottom of the insert chamber has 0.4-μm pores, allowing cytokines and growth factors produced by macrophages to reach the lower chamber where B16-F10 cells were cultured. The cells were kept in culture up to 1 week and macrophages were treated every day with LPS (1ug\ml) and BenzoilATP (100nM) alone or with anti-IL1β (50 pg/ml); control received the equal amounts of PBS; for RT treatment,cells were exposed to ^{60}Co radiation source to attain the desired doses. After one week inserts were removed and B16-F10 cells were stained with crystal violet. After lysis with 10% acetic acid, the absorbance was read at 595 nm.

PBMc isolation

PBMCs were isolated from human blood from healthy donors by Ficool-Paque density gradient centrifugation. Blood was diluted with an equal volume of PBS. Diluted blood was layered over 25 ml of the Ficoll-Paque PLUS. Gradients were centrifuged at 400 × g for 30 min at room temperature in a swinging-bucket rotor without the brake applied. The PBMC interface was carefully removed by pipetting and washed with PBS by centrifugation at 250 × g for 10 min then a total of $5x10^5$ cells/well are added to 24-well tissue culture plates.

Inflammasome activation assays

We seeded peritoneal macrophages from WT, Pml-/-, Pml-/-/P2X7-/ -and Pml-/-/NLRP3-/- mice at 3x105, 1x106 in 24 or 6 well plates, respectively. The following day, the overnight medium was replaced and cells were stimulated with 1µg/ml LPS for 2h alone or in combination with 100µM BenzoilATP (30min), 10µM nigericin (1h). Cells were also pretreated with nimodipine 100nM (30min) then stimulated with 1µg/ml LPS for 2h and 100µM BenzoilATP (30min). Supernatants were removed and analyzed using ELISA kits according to the manufacturer's instructions.

Radiotherapy treatments

Mice were anesthetized and exposed to whole-body radiation by timed exposure to ^{60}Co radiation source. After 1h, mice were sacrificed and supernatants from peritoneal cavity were isolated. For mice with tumor injection, they were anesthetized and only tumor mass was exposed to radiation by timed exposure to ^{60}Co radiation source. MU and exposition time will be calculated by the Treatment Planning System.

Subcellular fractionation

WT and Pml-/- C57BL/6 mice were injected intraperitoneally with 250ng/gr LPS for 1h before i.p. injection of nigericin 2,5mg/Kg (1h). After mice were euthanized and livers were isolated. Fractionations were performed as described previously (19816421, 2332429). IP3R, Sigma 1-R and Tubulin were used as markers for the ER, MAMs, and cytosol, respectively.

Western blot

Total cell lysates were prepared in RIPA buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol (DTT)) supplemented with proteases and phosphatase inhibitors (2 mM Na3VO4, 2 mM NaF, 1 mM PMSF and protease inhibitor cocktail). Surnatant from peritoneal macrophages was concentrated using Pierce Protein Concentrators PES 10K MWCO (ThermoFisher). The surnatant was centrifugated at 3000 x g for 15 minutes.

A total of 20 μg of protein or a total of 10 μl of concentrated medium was separated by SDS/PAGE and transferred to nitrocellulose membranes for standard western blotting. Antibodies were purchased from the following sources and used at the indicated dilutions: PML anti-mouse (1:1000) from Chemicon (Merck Millipore, Billerica, MA, USA); β-actin (1:3000), β-tubulin (1:3000) and Sigma-1R (1:1000) from Sigma-Aldrich; IP3R3 (1:1000) from BD Biosciences (San Jose, CA, USA); NLRP3 (1:1000) from Adipogen; P2X7 (1:500) from Alomone; Caspasi1 m-20 (1:500) and IL-1β (M-20) (1:500) from Santa Cruz (Santa Cruz, CA, USA). Isotype-matched horseradish peroxidase-conjugated secondary antibodies were used, followed by detection using chemiluminescence (PerkinElmer).

In Vivo LPS challenge

WT and Pml-/- C57BL/6 mice were injected intraperitoneally with nimodipine (100nM) or vehicle control (PBS) 30 minutes before i.p. injection of 1mg/Kg LPS. After 1h or 4h mice were euthanized and supernatants from peritoneal cavity and organs were isolated. For caspase 1 assay WT and Pml-/- C57BL/6 mice were injected intraperitoneally with 250ng/gr LPS for 1h before i.p. injection of nigericin 2,5mg/Kg. After 1h mice were euthanized and livers were isolated and homogenized in lysis buffer (300 mM sucrose, 1 mM K2HPO4, 5.5 mM D-glucose, 20 mM Hepes, 1 mM phenylmethylsulfonylfluoride, and 0.5% IGEPAL) with a Potter pestle. Tissue extracts were then centrifuged at 12,000 g at 4°C for 15 min. Protein extracts at 200 μg were subjected to caspase 1 assay.

ELISA

Mouse plasma was collected after blood centrifugation (1,000 x g, 10 minutes at 4°C). Tumor specimens were homogenized in lysis buffer (300 mmol/L sucrose, 1 mmol/L K2HPO4, 5.5 mmol/L D-glucose, 20 mmol/L Hepes, 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L benzamidine, 0.5% IGEPAL) with a Potter pestle. Tumor extracts were then centrifuged at 12,000 g at 4°C for 15 min.

IL-1β ELISA was performed according to the manufacturer's instructions.

Tumor generation and in vivo drug administration

Procedures involving animals and their care were in conformity with institutional guidelines, and Animal Ethics Committee approved all experimental protocols (Authorization N°481/2017-PR approved by Italian Ministry of Health).

Pml-/- C57BL/6 mice were crossed with P2X7-\- C57BL/6 mice to generate Pml-/-/P2X7-/- C57BL/6 mice. Pml-/- C57BL/6 mice were crossed with NLRP3-\- C57BL/6 mice to generate Pml-/-/NLRP3-/- C57BL/6 mice.

WT, Pml-/-, Pml-/-/NLRP3-/- and Pml-/-/P2X7-/- mice were housed in a temperature-controlled environment with 12 h light/dark cycles and received food and water ad libitum.

A total of $1x10^6$ B16-F10 melanoma cells and 1x106 LL/2 cells were subcutaneously inoculated into female, 6- to 8-week-old C57BL/6 mice, respectively. Tumor growth was monitored daily and tumor volumes were measured every other day with calipers using the following equation: Volume = π/6 x (a x b2), where a is the major diameter and b is the minor diameter. The mice were randomly divided into the treatment groups and the control group, with 6 mice per group. For NLRP3 inflammasome inhibition mice were injected intraperitoneally with nimodipine (100nM thrice weekly); for cytokine neutralization experiments, mice were injected i.p. with 50μg twice weekly anti IL-1β. Control mice received equal amounts of isotope control antibodies or equal volumes of PBS. After 2 weeks all mice were sacrificed, and tumors were excised.

Histology and Immunohistochemistry

Tissue samples (tumor masses including peri-tumoral stroma, and lungs) were collected at 14 days post-inoculation, fixed in Bouin's liquid for 7 h at 4 °C, dehydrated in cold graded ethanol series and embedded in paraffin. For each specimen, at least ten sets (50 µm away) of five serial sections (6 µm-thick) were stained with Harris' haematoxylin and eosin (H/E) for general histology, or processed for immunohistochemistry (IHC). A qualitative inflammatory score (0= no inflammation; 1= weak; 2= moderate; 3= strong) of peri-tumoral stroma and distal lungs was assigned to sections stained with H/E by an observer unaware of treatments.

IHC was performed by ABC-peroxidase. Adjacent sections were incubated for 1 h at room temperature with monoclonal antibodies raised in rat against mouse CD8 (Thermoscientific, MA1-70041, clone YTS 169AG101HL) or mouse F4/80 (CI:A3-1) (Santa Cruz Biotech; sc-59171), or a rabbit polyclonal antiserum anti-human CD3ε (synthetic peptide aa. 156-168) (Abcam; ab 5690). The antibodies were diluted 1:10 to 1:50 in 0.01 M, pH 7.3 PBS containing 0.1% sodium azide. A hybridoma irrelevant supernatant or normal rabbit serum substituted the primary antibody in negative controls. The antibodies were tested on sections after or without antigen retrieval steps (40 min at 95 °C in 0.01 M, pH 6.0 sodium citrate buffer containing 0.05% Tween 20, followed by cooling at RT and rinses in PBS Tween 20). Thereafter, sections were incubated for 60 min with biotinylated goat anti-rat IgG (mouse adsorbed) or biotinylated goat anti-rabbit IgG sera (Vector Labs., Burlingame, USA), diluted 1:200 or 1:1000, respectively, with PBS containing 0.1% sodium azide and 1% BSA. Some sections (not treated with any primary antibodies) were incubated with biotinylated horse anti-mouse IgG serum (Vector) diluted 1:1000 to label B-cells. Thereafter sections were incubated for 60 min with avidin-biotinylated peroxidase complex (ABC, Vectastain Elite, Vector). Following rinses and staining (diaminobenzidine), sections were slightly counterstained with Harris' haematoxylin, rinsed, dehydrated, mounted and examined under bright-field illumination.

Counts of immunoreactive cells (nucleated only) in 1 mm2 areas of peri-tumoral stroma were done by an observer unaware of treatments using a computer-assisted image analysis system, which includes a Nikon Eclipse E600 microscope equipped with DS-5M digital colour video camera. Images were captured through a PC interface using the software package Lucia G 4.81 (Laboratory Imaging Ltd., Praha, Hostivar). The number of immunoreactive cells per group were then calculated by averaging the cell numbers from all specimens. Mean and SD were calculated for each parameter. Two-way ANOVA was used to determine the effect of treatment, and one-way analysis

of variance and post hoc Tukey's multiple comparison test were used to determine differences among groups. The level for accepted statistical significance was $P < 0.05$.

Immunofluorescence assay

Peritoneal macrophages from WT and Pml-/- mice were seeded on 24-mm glass coverslips; the following day, the overnight medium was replaced and cells were stimulated with 1µg/ml LPS for 2h alone or in combination with 10µM nigericin (1h). Then cells were fixed in 4% paraformaldehyde in PBS for 10 min and washed three times with PBS. Cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS and blocked in PBS containing 5% BSA and 0.1% Triton X-100 for 1 h. Cells were incubated overnight at 4 °C with the indicated antibodies: PDI (1:100) from Abcam (ab3672), NLRP3 (1:50) from Adipogen (cat.#: AG-20B-0014-C100). The appropriate isotype-matched, AlexaFluor-conjugated secondary antibodies [Life Technologies, A-11010 (546 goat anti-rabbit, 1:1000) and A11001 (488 goat anti-mouse, 1:1000)] were used.

The coverslips were mounted with ProLong Gold Antifade reagent (Life Technologies), and the analysis of immunofluorescence was performed with a confocal laser scanner microscopy Zeiss LSM 510 (Carl Zeiss, Gottingen, Germany) equipped with a 63x oil objective (n.a.1.42) and a Zen lite 2.0 software. Quantificazione NLRP3 speckle/NLRP3 total.

Co-immunoprecipitation.

Co-immunoprecipitations were carried out using protein G-coated sepharose beads (GE Healthcare, Chalfont St. Giles, UK) following the manufacturer's instructions. Protein extractions in the ER fraction were carried out by adding 50 mM NaCl and 1% NP-40 to the homogenization buffer. All the buffers were supplemented with proteases and phosphatases inhibitors (2 mM Na3VO4, 2 mM NaF, 1 mM PMSF and protease inhibitor cocktail). Extracted proteins (1000 mg) were first precleared by incubating lysates with sepharose beads for 1 h at 4°C and the supernatant (referred as Input) was incubated overnight with NLRP3 or P2X7 antibody at 4°C. Precipitation of the immune complexes was carried for 3 h at 4°C. Afterwards, beads were washed with 50 mM Tris-HCl pH 7.4, 0.1% NP-40 4°C supplemented with phosphatases inhibitors and PMSF. Samples were proceed by SDS-PAGE and analyzed by standard western blotting technique.

RT-PCR

Total RNA was extracted with Trizol reagent (Life Technologies), and then purified with the Pure link RNA Mini Kit (Invitrogen) as per manufacturer's instructions. RNA content was quantitated 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 NLRP3, NLRC4 and G3PDH as a housekeeping gene.

PCR

DNA was prepared from tail biopsy and it was used in 50 □l reactions that included three primers. P2X7 gene was amplified by using the primers 5'-TGCCCATCTTCTGAACACC, 5'-CTTCCTCTTACTGTTTCCTCCC, 5'-GCAAGGCGATTAAGTTGGG. The 568-bp-specific product corresponds to the WT P2X7 gene and the 393-bp product corresponds to the KO P2X7 gene. NLRP3 gene was amplified by using the primers 5'-GTCCAGGACATACGTCTGGA, 5'-TGAGGTCCACATCTTCAAGG, 5'-TTGTAGTTGCCGTCGTCCTT. The 327-bp-specific product corresponds to the WT NLRP3 gene and the 589-bp product corresponds to the KO NLRP3 gene. PML gene was amplified by using the primers 5'-TTGGACTTGCGCGTACTGTC, 5'-CGACCACCAAGCGAAACA, 5'-TTTCAGTTTCTGCGCTGCC. The 400-bp-specific product corresponds to the WT PML gene and the 700-bp product corresponds to the KO PML gene. The reactions were analyzed on 2% agarose gel containing midori green.

Flow cytometry

Tumors were cut into 1-2 mm3 pieces and digested with DNAase (100□g/ml, Sigma) and collagenase A (20 units/ml, Roche) in RPMI at 37°C for 30 minutes. Tumor-infiltrating macrophages were blocked with anti-mouse FcR antibody (CD16/CD32, BD) for 5 min at 4°C, subsequently cells were stained for 15 min at 4°C with surface antibodies. The following mAbs were purchased from Biolegend (http://www.biolegend.com/): PE/Cy7-conjugated anti-CD11b

(clone M1/70, cat.#: 102515 working dilution 1:200), APC-conjugated anti-CD11c (clone N418, cat.#: 117309 working dilution 1:200), Fitc-conjugated anti-CD206 (clone C068C2, cat.#: 141703 working dilution 1:200), APC/Cy7-conjugated anti-F4/80 (clone, cat.#:BM8 123117 working dilution 1:200). Samples were acquired on a BD FACSCantoII (BD Biosciences) flow cytometer. Data were analysed using Kaluza Flow Analysis Software (Beckman Coulter).

Bone Marrow Replacement Experiment

Bl6 Cd45.1, Pep Boy recipients received 950 cGy total body irradiation, followed by intravenous injection of 106 bone marrow mononuclear cells derived from C57BL/6J WT or Pml-/- donor mice.

Reconstitution of donor cells and repopulation of donor myeloid and lymphoid cells were monitored by staining peripheral blood cells with antibodies against CD45.1, CD45.2, CD3 (T cell), B220 (B cell), CD11b and Gr-1 (myeloid) every 4 weeks starting at 4 weeks after transplantation. At 16 week post-transplantation the engraftment was considered stable and mice were subjected to tumor engraftment. Experiments were performed with authorization from the Institutional Review Board for Human Research at Albert Einstein College of Medicine.

Proximity Ligation Assay

Peritoneal macrophages from WT and Pml-/- mice were seeded on microarray slide 16-well (Thermo Fisher) at density of 100000. Twenty-four hours after seeding cells were stimulated with 1µg/ml LPS for 2h alone or in combination with 10µM nigericin (1h). Then cells were fixed in 4% paraformaldehyde in PBS for 10 min and washed three times with PBS. Cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS and blocked in PBS containing 5% BSA and 0.1% Triton X-100 for 1 h. Then cells were hybridized with conjugated primary antibodies (PLA probes): one MINUS (P2X7) and one PLUS (NLRP3) for P2X7-NLRP3 interaction, one MINUS (PML) and one PLUS (NLRP3) for PML-NLRP3 interaction, in a humidity chamber overnight at 4°C. The day after Proximity ligation assay was concluded according to manufacturer instruction. After PLA assay, cells were fixed in Methanol 100% for 20 min at -20°C and then permeabilized for 10 min with 0.1% Triton X-100 in PBS. Cells were blocked in PBS containing 5% Milk and 0.1% Triton X-100 for 1 h and then incubated overnight at 4 °C with antibody PDI (1:100) from Abcam

(ab3672). The appropriate isotype-matched, AlexaFluor-conjugated secondary antibody [Life Technologies, A11008 (488 goat anti-rabbit, 1:1000)] was used.

PLA signal was detected by an Olympus Xcellence widefield system, and deconvolved using Fiji. After 3D digital deconvolution PLA signal was quantified as dots within each PDI positive cell or DAPI positive cell.

Statistical analysis

The data were analysed by Prism 6 (GraphPad) or using Microsoft Excel (Microsoft Co.). Unless otherwise noted in figure legends, data are representative of at least three biologically independent experiments. Two-group datasets were analysed by Student's unpaired t-test. For three or more group analysis, one-way ANOVA Tukey's multiple comparison test was used. One asterisk was used for $P< 0.05$, two asterisks for $P< 0.01$, three asterisks for $P< 0.001$, and four asterisks for $P< 0.0001$.

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