



The P2X7 Receptor-Interleukin-1 Liaison

Anna Lisa Giuliani, Alba C. Sarti, Simonetta Falzoni and Francesco Di Virgilio*

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

Interleukin-1 β (IL-1 β) plays a central role in stimulation of innate immune system and inflammation and in several chronic inflammatory diseases. These include rare hereditary conditions, e.g., auto-inflammatory syndromes, as well as common pathologies, such as type II diabetes, gout and atherosclerosis. A better understanding of IL-1 β synthesis and release is particularly relevant for the design of novel anti-inflammatory drugs. One of the molecules mainly involved in IL-1 β maturation is the P2X7 receptor (P2X7R), an ATP-gated ion channel that chiefly acts through the recruitment of the NLRP3 inflammasome-caspase-1 complex. In this review, we will summarize evidence supporting the key role of the P2X7R in IL-1 β production, with special emphasis on the mechanism of release, a process that is still a matter of controversy. Four different models have been proposed: (i) exocytosis via secretory lysosomes, (ii) microvesicles shedding from plasma membrane, (iii) release of exosomes, and (iv) passive efflux across a leaky plasma membrane during pyroptotic cell death. All these models involve the P2X7R.

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*Correspondence:

Francesco Di Virgilio
fdv@unife.it

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THE INFLAMMATORY PROCESS

Inflammation has been the object of countless studies and experimental observations since its definition more than 2000 years ago (Celsus, *De Medicina*, 47 CE). Nevertheless, many aspects of this process are not fully understood, and therefore inflammation is still nowadays a field of extensive investigation, especially in view of its crucial role in the pathogenesis of many acute and chronic diseases. Accordingly, inflammation is a fertile ground of research for the development of novel drugs. Diverse chemical mediators with pro- or anti-inflammatory activity have been identified over the years. These range from histamine to bioactive lipids, e.g., prostaglandins and leukotriens, from free radicals, e.g., reactive oxygen species (ROS) and nitric oxide (NO), to cytokines, e.g., interleukins (ILs) and tumour necrosis factor (TNF). Among all these mediators, interleukin-1 β (IL-1 β) is recognized as one of the earliest and most potent pro-inflammatory agents synthesized and released in response to infectious agents and injuries, and therefore central to both septic and sterile inflammation (Gabay et al., 2010; Dinarello, 2011).

AN OVERVIEW ON INTERLEUKIN-1 (IL-1)

The term Interleukin-1 (IL-1), also known as leukocyte endogenous mediator, hematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor, was used in the past to indicate a factor mediating many different pro-inflammatory and catabolic effects.

The history of IL-1 begins with studies on the endogenous factor produced by activated leukocytes that causes fever. As such, IL-1 was originally described in Menkin (1943), who reported the isolation of a pyrogenic euglobulin from inflammatory exudate named “pyrexin” or “endogenous pyrogen.”

These initial studies were followed by the groundbreaking contributions of Beeson (1948) who confirmed Menkin’s observation and further reported that an endotoxin-free, protein-containing material, released from rabbit peritoneal leukocytes, caused the rapid onset of fever after injection into rabbits. This was the first time in which the mechanism behind fever, in the absence of infection, was described. After Beeson’s paper, there was a surge of studies on the links between infection/inflammation and fever, that culminated in the demonstration by Bodel and Atkins (1967) that human blood monocytes produced a pyrogen, similar to that released by rabbit neutrophils, by *de novo* synthesis.

Gery and Waksman (1972) described the effect on lymphocyte proliferation of soluble factors released in response to antigenic or mitogenic stimuli, and a few years later Dinarello and Bernheim (1981) purified the human leukocytic pyrogen from peripheral blood mononuclear cells (PBMCs) *in vitro* stimulated with heat-killed *Staphylococcus epidermidis*. Leukocytic pyrogen was also shown to enhance T cells responses to antigens and to promote synthesis of acute phase proteins (Kampschmidt et al., 1973).

Initially, the vast number of biological activities attached to a single molecule generated some confusion in the scientific community, however, with the cloning of IL-1 by Lomedico et al. (1984), the use of recombinant IL-1 established that IL-1 was indeed a pleiotropic cytokine mediating a great variety of inflammatory, as well as immunological, responses. Thanks to the seminal work of Dinarello, we now know that IL-1 is the founding member of a family of cytokines.

The IL-1 cytokine family consists of 11 members with different roles in inflammation. Seven of them, i.e., IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β and IL-36 γ , own well-demonstrated pro-inflammatory properties, whereas four members, IL-1Ra, IL-36Ra, IL-37 and IL-38, are anti-inflammatory (Garlanda et al., 2013; Borthwick, 2016). Cytokines of the IL-1 family ligate and activate specific plasma membrane receptors, the IL-1 receptor family, comprised of 10 members, named IL-1R1, IL-1R2, IL-1R3 (IL-1RAcP), IL-1R4 (ST2), IL-1R5 (IL-18R α), IL-1R6 (IL-1R36), IL-1R7 (IL-18R β), IL-1R8 (SIGIRR: single Ig IL-1R-related molecule or TIR8: three Ig domain-containing IL-1R related), IL-1R9 (IL-33R), IL-1R10 (TIGIRR-1) (Garlanda et al., 2013).

Interleukin-1 β (IL-1 β), a crucial factor of host defense in response to infections and injuries, is the best characterized and most extensively studied member of the IL-1 family (Dinarello, 1996). In the last decade, IL-1 β has also emerged as a causative agent and a therapeutic target for an expanding number of systemic and local inflammatory conditions named “auto-inflammatory diseases.” The auto-inflammatory diseases include rare hereditary conditions as well as common pathologies. Recently, increasing evidence shows that the same pathogenetic mechanisms responsible for the activation of innate immunity in inherited auto-inflammatory diseases may also play a key

role in sustaining inflammation in several frequent multifactorial pathologies, such as type II diabetes, gout, pseudogout, and atherosclerosis (Ginaldi et al., 2005).

IL-1 β , usually not expressed by healthy resting cells, is mainly produced by activated inflammatory cells of the myeloid lineage. Production of IL-1 β is a multistep process involving synthesis of immature pro-IL-1 β , proteolytic cleavage to mature IL-1 β and, finally, release into the extracellular environment. Synthesis of the immature full-length pro-IL-1 β is started with the recognition via Toll-like receptors (TLRs) of molecules derived from invading micro-organisms [pathogen-associated molecular patterns (PAMPs)] (Janeway, 2001). Once synthesized, the 31 kD pro-IL-1 β undergoes a proteolytic cleavage catalyzed by caspase-1 (casp-1) which removes 116 N-terminal aminoacids to generate the 17 kD bioactive form, now ready to be secreted. If conversion to the 17 kD form does not occur, pro-IL-1 β is polyubiquitinated and targeted for proteasomal degradation (Ainscough et al., 2014). Activation of casp-1, in turn, depends on assembly and activation of inflammasomes, multisubunit organelles that convert pro-casp-1 to active casp-1 (Thornberry et al., 1992; Martinon et al., 2002; Ogura et al., 2006).

The NLRP3 inflammasome has been investigated in depth and recognized as a very, likely the most, efficient machinery for pro-IL-1 β maturation, and the biology of this cytokine has been intimately intertwined with that of the inflammasomes and of inflammasome-activating agents (Martinon et al., 2002; Di Virgilio, 2013). Inflammasomes are high molecular weight protein complexes assembled in the cytosolic compartment in response to a variety of stimuli, either of exogenous (PAMPs) or endogenous [danger/damage associated molecular patterns (DAMPs)] origin. PAMPs include bacteria- as well as virus derived components, whereas DAMPs encompass different classes of molecules normally segregated inside the cells (Venereau et al., 2015). DAMPs are released in response to invasion by micro-organisms (septic inflammation) as well as to physical, chemical, metabolic non-infectious agents (sterile inflammation) (Gallucci et al., 1999). DAMPs released in the extracellular milieu fulfill the task of alerting surrounding cells, especially of immune lineages, of an incumbent danger or a damage (Venereau et al., 2015; Nie et al., 2016). Among DAMPs, extracellular ATP and other nucleotides play an undisputed role.

Nucleotide signaling is central in IL-1 β maturation and release, as well as in other immune responses, such as neutrophil and macrophage chemotaxis, intracellular microbe killing, NADPH-oxidase activation, T lymphocyte proliferation and differentiation (Di Virgilio, 1995; Bours et al., 2006; Ferrari et al., 2006; Junger, 2011; Eltzschig et al., 2012; Idzko et al., 2014; Cekic and Linden, 2016). Extracellular ATP acts at plasma membrane purinergic P2 receptors, chiefly the P2X7 receptor (P2X7R) subtype, to drive NLRP3 inflammasome activation and IL-1 β processing and release (Ferrari et al., 1997). ATP is released into extracellular environment during inflammation, ischemia, hypoxia, or other harmful events, via lytic (e.g., cell necrosis) or non-lytic (e.g., exocytosis, plasma membrane channels or pores) pathways. Pathways for non-lytic ATP release include pannexins (Dahl, 2015), connexins (Evans et al., 2006), ABC transporters (Cantiello, 2001), secretory vesicles (Sneddon and

Westfall, 1984; Wang et al., 2013), and the P2X7R (Pellegatti et al., 2005; Suadicani et al., 2006).

THE P2X7R

Several reports underscore the pivotal role of ATP-mediated P2X7R activation in IL-1 β release from activated immune cells (monocytes, macrophages, and microglia) (Di Virgilio et al., 1998; Pelegrin et al., 2008; Sanz et al., 2009). Macrophages from genetically modified mice lacking the P2X7R, ASC or NLRP3, do not release IL-1 β in response to ATP (Solle et al., 2001; Mariathasan et al., 2004, 2006). Moreover, oxidized ATP, an irreversible blocker of the P2X7R (Murgia et al., 1993) abrogates ATP-induced IL-1 β release from immune cells (Ferrari et al., 1997). P2X7R stimulation also induces fast release into the cytosol of oxidized mitochondrial DNA (mitoDNA) that promotes NLRP3 inflammasome assembly by direct interaction (Nakahira et al., 2011; Shimada et al., 2012).

The P2X7R is a bi-functional ATP-gated plasma membrane ion channel that upon sustained stimulation undergoes a transition that generates a non-selective pore permeable to aqueous solutes of MW up to 900 Da (Di Virgilio, 2000). The P2X7R is widely distributed in human tissues, the highest expression being in cells of the immune and inflammatory systems, especially of the myeloid lineage (Di Virgilio, 1995, 2015; Karmakar et al., 2016). The P2X7R is the seventh, and latest to be cloned, member of the P2X receptor (P2XR) subfamily activated by an agonist concentration about 100 fold higher than the other members of the family. P2XRs are ATP-gated channels permeable to monovalent (Na⁺, K⁺) and divalent (Ca²⁺) cations formed by the assembly of the same (homo) or different (hetero) P2X subunits. Six homomeric (P2X1R-P2X5R and P2X7R) and six heteromeric (P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R, P2X2/6R, and P2X4/6R) functional P2XRs have been described so far (Dubyak, 2007; North, 2016). Among P2X subunits, the P2X7 is generally thought not to assemble with the others, and thus forming P2X7 only homomeric channels. High sequence homology of P2X7R with the P2X4R (41% identity, 71% similarity), suggests a common origin by gene duplication. Therefore, the solved crystal structure for zebrafish P2X4R (Kawate et al., 2009; Hattori and Gouaux, 2012) has been used to model the 3D conformation of the P2X7R (Jiang et al., 2013). Useful insights as to ATP binding pocket, ion permeation pathway, site of antagonist binding and interaction with allosteric modulators are also derived from the crystal structure of the panda P2X7R (Karasawa and Kawate, 2016). Further information are ensued by recent 3D resolution of the human P2X3R (Mansoor et al., 2016).

The P2X subunits are characterized by a large extracellular loop, which includes agonist- and antagonist-binding sites, two short transmembrane domains, and intracellular N- and C-termini. The P2X7R with an extended C-terminal tail of 239 aa and an overall length of 595 aa, is the largest in the P2XR family. Transmembrane domains are responsible for the interactions among subunits and the formation of the ion-permeation pathway (Hattori and Gouaux, 2012; Grimes and Young, 2015).

The intracellular C-tail interacts with different intracellular molecules such as heat shock proteins (HSP), cytoskeletal components, kinases and possibly also with membrane proteins. Among these latter, pannexin-1 and connexin-43 hemichannels have been variably implicated in the formation of the P2X7R-associated large-conductance pore and therefore in P2X7R-dependent IL-1 β secretion, and in the release of extracellular ATP (Pelegrin and Surprenant, 2007; Baroja-Mazo et al., 2013). P2X7R has also been found to interact directly with components of inflammasomes, such as NLRP2, ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (Minkiewicz et al., 2013; Franceschini et al., 2015; Salaro et al., 2016). P2X7R activation by ATP is one of the most potent stimuli for NLRP3 inflammasome activation (Mariathasan et al., 2006; Munoz-Planillo et al., 2013).

THE NLRP3 INFLAMMASOME

Inflammasomes are cellular organelles with a fundamental role in inflammation and cell death (Martinon et al., 2002; Guo et al., 2015; Rathinam and Fitzgerald, 2016). The basic scaffold subunit is a nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) that contains a C-terminal leucine-rich repeat (LRR) domain, a central NACHT nucleotide-binding domain (NOD) and an N-terminal pyrin domain (a CARD domain in the NLRC4 inflammasome). The pyrin domain of the NLR scaffold subunit interacts with the pyrin domain of an adaptor molecule named ASC. NLR-driven ASC recruitment drives pro-casp-1 activation via CARD domains present on both ASC and pro-casp-1, thus resulting in pro-casp-1 cleavage and casp-1 activation. Casp-1 then cleaves pro-IL-1 β and pro-IL-18 to produce the mature forms of both cytokines (Benko et al., 2008; Schroder and Tschopp, 2010; Broz and Dixit, 2016; Prochnicki et al., 2016). Inflammasomes play a cardinal role in innate immunity thanks to their ability to sense PAMPs and DAMPs (He et al., 2016a; Kim et al., 2016). Within the subfamily of NLRP inflammasomes (i.e., inflammasomes based on NLR scaffold molecules with an N-terminal pyrin domain) NLRP3 is currently enjoying the widest popularity as crucial sensor for a large number of danger signals and as the main platform for IL-1 β processing. Activating stimuli for the NLRP3 inflammasome include bacterial toxins, flagellin, muramyl dipeptide, viral nucleic acids and fungal products, as well as endogenous components such as ATP, cholesterol crystals, monosodium urate, glucose and amyloid β , environmental pollutants, such as silica, asbestos or physical agents such as UV radiations (Kim et al., 2016).

The identity of the activating stimulus of the NLRP3 inflammasome has been a hot issue ever since its discovery. Nowadays there is basically general consensus on the key role played by K⁺ efflux, which seems to be the final common pathway for many different agents (Munoz-Planillo et al., 2013). Most efficient NLRP3 activators include extracellular ATP, K⁺ ionophores, and several extracellular crystals, all known to decrease the cytosolic K⁺ level. The mechanism whereby these different agents lower K⁺ is not entirely clear, but many converge

on P2X7R activation (Alves et al., 2014; Prochnicki et al., 2016). In fact, while P2X7R opening or nigericin, a carboxylic K⁺ ionophore, directly allow K⁺ efflux along its concentration gradient, the mechanism by which crystals, such as monosodium urate, deplete intracellular K⁺ is obscure. To support the contribution of K⁺ depletion, drugs inhibiting the Na⁺/K⁺-ATPase also trigger NLRP3 inflammasome activation (Walev et al., 1995; Munoz-Planillo et al., 2013). Albeit inhibition of Na⁺/K⁺-ATPase also causes plasma membrane depolarization, there is no evidence that depolarization itself may trigger P2X7R pore opening and/or IL-1 β release (Di Virgilio, 2013). The central role of intracellular K⁺ is further supported by the finding that a K⁺ drop is also necessary to allow recruitment of the Nima-related kinase (NEK)7 protein to the NLRP3 inflammasome (He et al., 2016b). On the other hand, the mechanism by which the drop in the K⁺ concentration drives NEK7 recruitment, NLRP3 inflammasome assembly and activation is utterly unknown.

The NLRP3 inflammasome can be also activated by a non-canonical pathway involving casp-11. Casp-11, and its human orthologs casp-4 and -5, function as cytosolic LPS sensors (Shi et al., 2014). Once activated by LPS, casp-11 induces cleavage of the plasma membrane channel pannexin-1 (Yang et al., 2015) producing two events consisting of K⁺ efflux, that activates NLRP3, and release of ATP that acts as a P2X7R agonist to promote further NLRP3 activation and cell death (Yang et al., 2015). The casp-11/pannexin-1/NLRP3 inflammasome axis is proposed to promote IL-1 β /IL-18 production (Yang et al., 2015). In addition, active casp-11 triggers pyroptosis via cleavage of Gasdermin D (GSDMD) leading to accumulation of free active N-terminal domains of this protein which disrupt cellular functions by forming plasma membrane pores (He et al., 2015; Vince and Silke, 2016). Casp-11-mediated cell death, like casp-1-induced pyroptosis, requires cleavage of the GSDMD pyroptotic factor (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Casp-11 mediated cell death is indeed abrogated in GSDMD deficient cells and, although it is not clear if GSDMD is the terminal pyroptotic factor, its N-terminal domain released following casp-11-dependent cleavage is sufficient to cause pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). It has been proposed that, since casp-1 is required for both pyroptosis and IL-1 β cleavage, IL-1 β is passively released alongside DAMPs following plasma membrane rupture (Vince and Silke, 2016). The finding that in macrophages lack of GSDMD has no effect on NLRP3-stimulated IL-1 β processing by casp-1 but prevents IL-1 β secretion (He et al., 2015; Shi et al., 2015), suggests that casp-1 is necessary for IL-1 β cleavage whereas GSDMD is indispensable for its release. Recent findings have revealed that in human monocytes stimulated with LPS casp-4 and -5 act as key determinants in one-step non-canonical NLRP3 inflammasome activation culminating with IL-1 β release (Vigano et al., 2015). This one-step pathway has been suggested to require Syk activity and Ca²⁺ influx due to CD14/TLR4-mediated LPS internalization (Vigano et al., 2015). NLRP3 activation and IL-1 β release can also be driven by K⁺ independent mechanisms involving ROS generation or RIPK1/FADD/casp-8 recruitment (Zhou et al., 2011; Heid et al., 2013; He et al., 2016a; Sanman et al., 2016). Converging experimental findings seem to rule out a role for cytosolic Ca²⁺

increases (Brough et al., 2003; Rada et al., 2014; Katsnelson et al., 2015). In some non-immune cells, e.g., astrocytes, IL-1 β maturation has been reported to be due to P2X7-dependent NLRP2 stimulation via a process involving direct NLRP2, P2X7R, pannexin-1 interaction (Minkiewicz et al., 2013). Finally, IL-1 β can also be processed independently of inflammasome/casp-1 activation, as shown in casp-1 deficient mice, where pro-IL-1 β to IL-1 β extracellular conversion is catalyzed by various neutrophil proteases such as elastase, proteinase-3, granzyme A and cathepsin G (Fantuzzi et al., 1997; Joosten et al., 2009).

P2X7R stimulation by itself has no or little effect on pro-IL-1 β cytoplasmic accumulation, therefore cells need priming by agents that promote IL-1 β gene transcription, which mainly occur via NF κ B activation. Typical priming agents are bacterial lipopolysaccharide, zymosan and poly(I:C) (Ferrari et al., 1996; Facci et al., 2014).

IL-1 β RELEASE

The canonical pathway for the export of cellular proteins into the extracellular space involves the ER and the Golgi apparatus that together form the endo-membrane system through which the vast majority of proteins are either targeted to the extracellular space or to specialized sub-cellular compartments. At variance with other cytokines, IL-1 β lacks the conventional leader/signal peptide and therefore is not targeted to the conventional ER-Golgi secretory pathway (Rubartelli et al., 1990). This leads to IL-1 β accumulation into the cytosol after translation on free ribosomes. Moreover, conversion of pro-IL-1 β to the mature form by inflammasomes also takes place in the cytosol. Therefore, release of mature IL-1 β requires non-classical mechanisms of export from the cytosolic compartment (Rubartelli et al., 1990; Wewers, 2004; Eder, 2009). A number of different possible mechanisms have been proposed (Dubyak, 2012) and summarized in **Figure 1**. They include exocytosis via secretory lysosomes (Andrej et al., 1999; Andrej et al., 2004), microvesicle shedding from plasma membrane (MacKenzie et al., 2001; Bianco et al., 2005; Pizzirani et al., 2007), release of exosomes (Qu et al., 2007), and, lastly, passive efflux across a leaky plasma membrane during pyroptotic cell death (Bergsbaken et al., 2009; Martin-Sanchez et al., 2016). The P2X7R has been implicated in all these processes.

EXOCYTOSIS OF IL-1 β -CONTAINING SECRETORY LYOSOMES

Rubartelli et al. (1990) presented the first evidence for a non-classical secretory pathway for IL-1 β release. Blockade of protein transport and secretion through the ER-Golgi complex did not affect IL-1 β release, thus pointing to the involvement of secretory lysosomes. Secretory lysosomes are unusual organelles found principally in hematopoietic cells with a dual-function, degradative and secretory (Blott and Griffiths, 2002). The exocytic process can be triggered by different stimuli among which ATP, possibly via the increase in the intracellular Ca²⁺

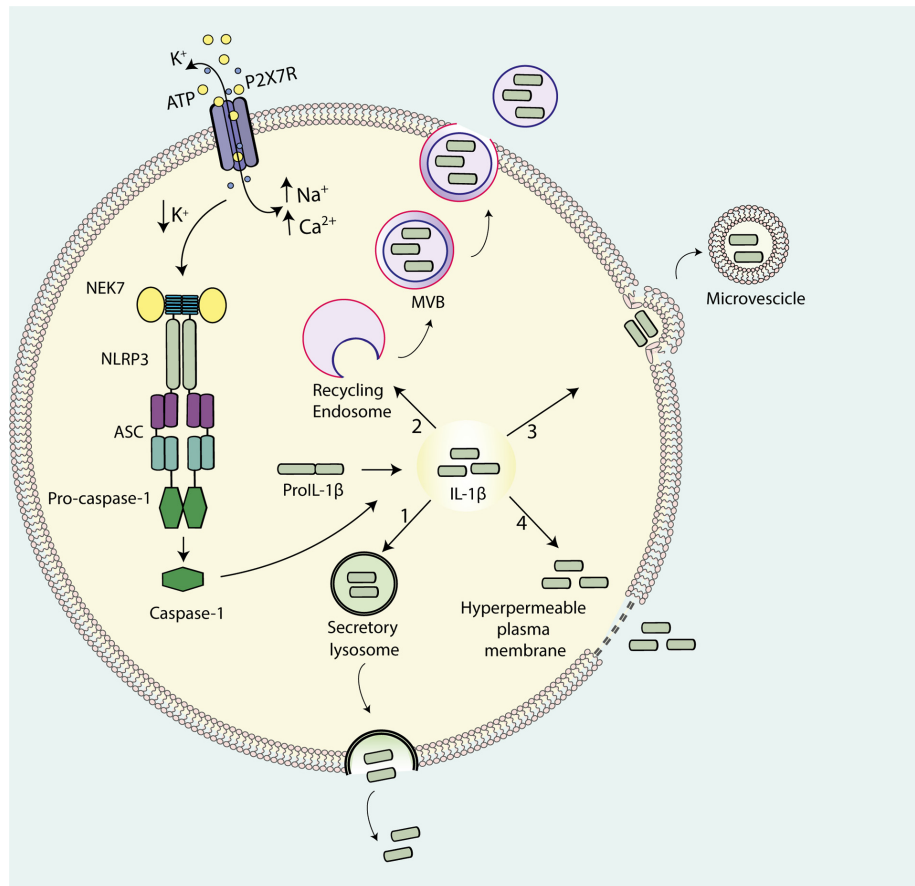


FIGURE 1 | Pathways for IL-1 β release from activated immune cells. IL-1 β maturation is catalyzed by ATP-mediated stimulation of the P2X7R that drives NLRP3 inflammasome assembly and casp-1 recruitment. Four models have been proposed for IL-1 β release: (1) exocytosis of secretory lysosomes; (2) shedding of plasma membrane-derived microvesicles; (3) exocytosis of multivesicular body (MVB)-derived exosomes; (4) passive efflux across hyperpermeable plasma membrane during pyroptotic cell death.

concentration. Migration of exocytic lysosomes to the plasma membrane is a microtubule-dependent process that brings the lysosomes close to the plasma membrane allowing fusion and release of their content into the extracellular space. This model for IL-1 β secretion is mainly based on morphological evidence from ATP-stimulated monocytes where IL-1 β was found to be trapped within organelles akin to late endosomes and early lysosomes (Andrei et al., 1999). In human monocytes and mouse macrophages, ATP-stimulated, P2X7R-dependent release of mature IL-1 β and casp-1 strongly correlated with secretion of the lysosomal markers cathepsin B, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1) (Andrei et al., 1999; Carta et al., 2006). Both IL-1 β and casp-1 are found in the extracellular medium 20 min after ATP stimulation, suggesting a similar time course. According to Rubartelli and coworkers a fraction of intracellular pro-IL-1 β is co-stored together with pro-casp-1 within the secretory lysosomes, ready to be secreted in response to P2X7R stimulation (Rubartelli et al., 1990). The triggering stimulus is thought to be the P2X7R-induced loss of intracellular K⁺, which activates a phosphatidylcholine-specific phospholipase C, which in turn causes an increase in

cytosolic Ca²⁺, Ca²⁺-dependent phospholipase A₂ activation and finally exocytosis of the IL-1 β -containing lysosomes. These events are blocked by inhibitors of phospholipase A₂ or phosphatidylcholine-specific phospholipase C. This model suggests that, whereas the massive K⁺ efflux due to P2X7R activation has a key role in the maturation of pro-IL-1 β , the intracellular Ca²⁺ increase is more directly responsible for IL-1 β secretion.

SHEDDING OF IL-1 β -CONTAINING PLASMA MEMBRANE MICROVESICLES

Surprenant and coworkers proposed a different vesicular mechanism for IL-1 β release from THP-1 monocytes (MacKenzie et al., 2001). According to this mechanism, P2X7R stimulation induces mature IL-1 β accumulation at discrete sub-plasmalemmal sites, where from it is then trapped into small plasma membrane blebs that are finally rapidly shed as microvesicles into the extracellular space (Figure 2). Microvesicle shedding is preceded by flip of phosphatidylserine

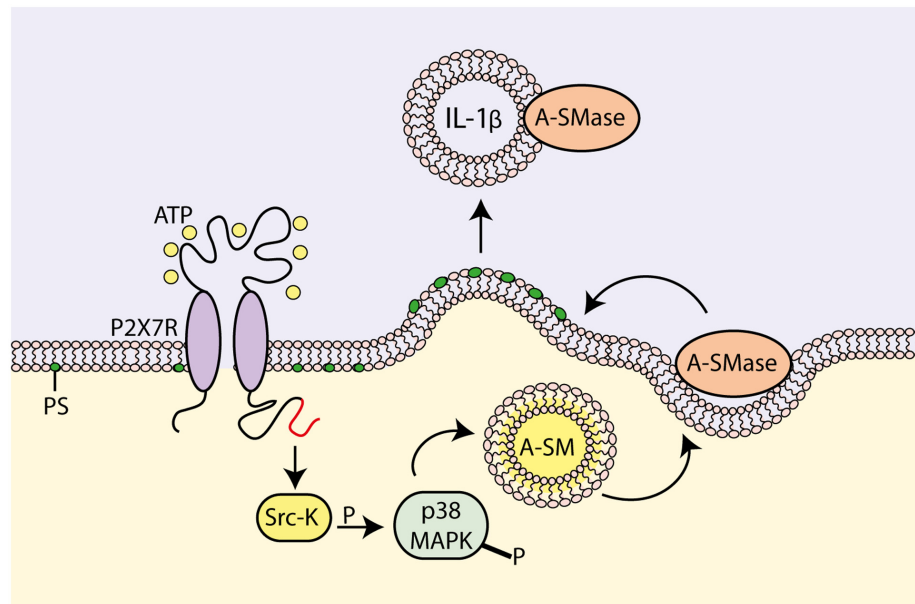


FIGURE 2 | Molecular mechanism for P2X7R-dependent microvesicle shedding. P2X7R activation promotes interaction of the C-terminal domain with a src-protein tyrosine kinase (Src-K), which in turn phosphorylates P38 MAP kinase (P38 MAPK). P38 MAPK induces flip of acidic sphingomyelinase (A-SMase) from the inner to the outer plasma membrane leaflet. On the outer plasma membrane leaflet, A-SMase hydrolyzes sphingomyelin to generate ceramide that in turn alters membrane fluidity, drives formation of plasma membrane blebs and promotes shedding of IL-1 β -containing microvesicles (modified from Bianco et al., 2009).

(PS) to the outer leaflet of the plasma membrane. Microvesicles size ranges from 200 nm to 1 μ m, which makes them distinct from the much larger apoptotic bodies derived from apoptotic cells (1–4 μ m size), and the smaller exosomes derived from intraluminal vesicles of endosomal multivesicular bodies (MVBs). A similar mechanism for IL-1 β release has also been observed in human monocyte-derived dendritic cells (DCs) and mouse microglia (Bianco et al., 2005; Pizzirani et al., 2007). Shed microvesicles contain (a) plasma membrane phospholipids, e.g., PS (MacKenzie et al., 2001), (b) membrane intrinsic proteins, such as P2X7R, CD63, CD39, MHC-II, LAMP1 (Andrei et al., 1999; Pizzirani et al., 2007) and (c) cytoplasmic proteins, such as pro-IL-1 β , pro-casp-1, IL-1 β , casp-1, casp-3 and cathepsin D (Gudipaty et al., 2003; Andrei et al., 2004; Bianco et al., 2005; Carta et al., 2006; Pizzirani et al., 2007; Qu et al., 2007). It is not clear how and if IL-1 β finally effluxes out of the microvesicles, thus fulfilling its role as an extracellular signaling molecule, or alternatively is delivered intracellularly following microvesicle fusion with the plasma membrane of target cells. Verderio and coworkers provided ample evidence showing that microvesicles released from P2X7R-stimulated microglia fuse with the plasma membrane of target cells (e.g., neurons), deliver their content and affect target cell responses (e.g., synaptic activity) (Antonucci et al., 2012; Turola et al., 2012; Verderio et al., 2012). We reported some time ago that microvesicles shed from P2X7R-stimulated DCs express the P2X7R and are lysed by exposure to extracellular ATP, thus releasing their cargo of IL-1 β (Pizzirani et al., 2007). This observation led us to propose that IL-1 β is released in the vicinity of the target cell plasma membrane by ATP-stimulated and P2X7R-dependent microvesicle rupture (Pizzirani et al.,

2007). In fact, it is known that due to continuous ATP release into the extracellular space, cells are surrounded by an “ATP halo” that generates an ATP concentration higher in the vicinity of the plasma membrane than in the bulk solution. Thanks to this ATP gradient, microvesicle journey across the interstitial space should be relatively safe until they reach the target cell surface where they are supposed to find an ATP concentration sufficient to activate the P2X7R and trigger lysis.

EXOCYTOSIS OF IL-1 β -CONTAINING EXOSOMES

In mouse bone marrow-derived macrophages (BMDMs) the main mechanism for non-classical IL-1 β release has been reported to be neither secretory lysosomes nor microvesicle shedding, but rather P2X7R-stimulated MVBs formation and exosome release (Qu et al., 2007). Exosomes are small vesicles (30–100 nm) released upon fusion of MVBs with the cell plasma membrane. Exosomes originate as intraluminal vesicles during the process of MVBs formation. MVBs or late endosomes are components of the endocytic pathway that range from 250 to 1000 nm in diameter. MVBs can either be degraded or fuse with the plasma membrane, releasing the intraluminal vesicles into the extracellular space. Intraluminal vesicles are then referred to as exosomes following their extracellular release. During the process of formation, transmembrane and peripheral membrane proteins are incorporated into the exosome membrane, while cytosolic components are enclosed within the vesicles. Exosomes released from macrophages, DCs or B-lymphocytes contain

soluble proteins present in the cytosol, such as pro-IL-1 β , pro-casp-1 and the respective mature form IL-1 β and casp-1, and plasma membrane proteins such as MHCI and MHCII, a feature of exosomes derived from antigen presenting cells. From P2X7R-stimulated BMDMs two distinct types of membrane-bound vesicles are shed: (a) plasma membrane-derived microvesicles carrying P2X7R and LAMP1, and (b) MVB-derived exosomes lacking both P2X7R and LAMP1. However, both types of vesicles are able to present peptide-MHCII complexes to T cells (Ramachandra et al., 2010). Secretion of IL-1 β and MHCII are strongly inhibited in mice deleted of ASC and NLRP3, suggesting the possibility that inflammasome complex regulate the formation of MVBs and the accumulation of IL-1 β and casp-1, although the mechanism remains unclear (Qu et al., 2009).

IL-1 β RELEASE AS A CONSEQUENCE OF PLASMA MEMBRANE DAMAGE AND CELL DEATH

A model for IL-1 β release involving plasma membrane damage and cell death (whether by necrosis or apoptosis) has been proposed several years ago (Hauser et al., 1986; Hogquist et al., 1991). A major obstacle for the acceptance of this model is the need for proteolytical activation of pro-IL-1 β , which is assumed to occur coordinately with its secretion, and the consistent observation that cytoplasmic mature IL-1 β levels are very low (Perregaux et al., 1992). Of course, it is possible that extracellular proteases, e.g., trypsin or cathepsins might do the job, but *in vivo* relevance of extracellular pro-IL-1 β maturation is dubious. However, in a recent paper, Pelegrin and co-workers have re-visited the cell permeabilization/cell death model for IL-1 β release from BMDMs taking advantage of novel, highly sensitive, fluorescence-based technique to measure IL-1 β secretion and of a novel inhibitor, punicalagin (Martin-Sanchez et al., 2016). Rigorous analysis of release of the cytoplasmic marker lactic dehydrogenase and of IL-1 β revealed that the kinetics of two processes were closely over-imposed. Furthermore, punicalagin, a polyphenolic compound that efficiently prevents plasma membrane permeabilization in response to a number of membrane-perturbing agents, fully abolished ATP-dependent IL-1 β secretion but not its processing, thus showing that pro-IL-1 β cleavage and mature IL-1 β secretion can be dissociated, and that a “leaky membrane” is needed for IL-1 β release. Since casp-1 activation is also a major driver of pyroptotic cell death, Pelegrin and co-workers suggested that in macrophages IL-1 β secretion occurs via a non-specific increase in plasma membrane permeability associated to cell death (Martin-Sanchez et al., 2016).

IS THE P2X7R-TARGETING A THERAPEUTICALLY LIVE OPTION?

Several studies show that P2X7R blockade efficiently antagonize IL-1 β release in different disease experimental models (Bartlett

et al., 2014). However, similar evidence from human studies is lacking. Measurement of serum IL-1 in autoimmune and autoinflammatory diseases is seldom significantly elevated, and is not thought to be a reliable indicator of inflammation (Dinarello, 2005). Therefore, it is not possible to verify in humans whether P2X7R blockade has any effect on IL-1 β release. Assessing the *in vivo* effect of P2X7R blockade on IL-1, and in general, all cytokines, release, is made even more complex by the disappointing results of most clinical trials so far carried out (De Marchi et al., 2016; Jacobson and Muller, 2016).

CONCLUSION

Extracellular ATP is now acknowledged to be one of the earliest most ubiquitous DAMPs (Di Virgilio, 2013; Kepp et al., 2014; Hammad and Lambrecht, 2015; Venereau et al., 2015). Its remarkable efficiency and plasticity as an alarm signal strongly depends on the diverse of ATP-selective plasma membrane receptors expressed by immune cells. Very interestingly, even before all ATP receptors (P2 receptors) expressed by immune cells were cloned and fully characterized, it was clear that stimulation with extracellular ATP was able to cause a dramatic acceleration of pro-IL-1 β processing and release from monocytes/macrophages, as well as from microglial cells, and this was very likely a receptor-mediated event (Perregaux and Gabel, 1994; Di Virgilio et al., 1996; Ferrari et al., 1996). About at the same time the P2X7R was cloned (Surprenant et al., 1996), and soon after identified as the molecule responsible for ATP-dependent mature IL-1 β release (Ferrari et al., 1997). Thus, the association between IL-1 β and the P2X7R is rock solid and long standing. However, this has not led to the introduction of any P2X7R-targeted anti-inflammatory therapy, despite large effort by virtually all major Pharma Industries. Are we missing some crucial information of P2X7R and IL-1 β biology, or is there a recurrent fault in P2X7R-targeting drug design and development, or both?

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FDV coordinated writing and reviewed the MS. AG wrote sections of the MS. AS wrote sections of the MS. SF wrote sections of the MS.

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Conflict of Interest Statement: FDV serves as a member of the Scientific Advisory Board of Biosceptre International Limited.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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