Immunity
Review

The P2X7 Receptor in Infection and Inflammation

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Adenosine triphosphate (ATP) accumulates at sites of tissue injury and inflammation. Effects of extracellular ATP are mediated by plasma membrane receptors named P2 receptors (P2Rs). The P2R most involved in inflammation and immunity is the P2X7 receptor (P2X7R), expressed by virtually all cells of innate and adaptive immunity. P2X7R mediates NLRP3 inflammasome activation, cytokine and chemokine release, T lymphocyte survival and differentiation, transcription factor activation, and cell death. Ten human *P2RX7* gene splice variants and several SNPs that produce complex haplotypes are known. The P2X7R is a potent stimulant of inflammation and immunity and a promoter of cancer cell growth. This makes P2X7R an appealing target for anti-inflammatory and anti-cancer therapy. However, an in-depth knowledge of its structure and of the associated signal transduction mechanisms is needed for an effective therapeutic development.

Introduction

In complex multicellular organisms, cells communicate through molecules that diffuse across the intercellular space, are transferred from one cell to another via intercellular channels, or are dispersed into the extracellular milieu via extracellular vesicles. The immune system is no exception to this pattern: cytokines or growth factors are released into the extracellular space, gap junctions allow molecular transfer in lymphocytes and macrophages, and exosomes or microvesicles are released by virtually all immune cells. A host of signaling molecules have evolved over the ages, but it is generally acknowledged that purine nucleotides were among the primordial mediators of cell-to-cell communication (Burnstock and Verkhratsky, 2009; Verkhratsky and Burnstock, 2014). Phosphate compounds have a unique place in cellular energetics and in signal transduction due to the extraordinary energy content and the chemical reactivity of the pyrophosphate bond. Adenosine triphosphate (ATP) is a reactive compound that participates in a large number of biochemical reactions (Burnstock and Verkhratsky, 2009). Phosphate-based metabolism requires very low Ca^{2+} concentrations (because Ca^{2+}-phosphate precipitates will form easily), thus suggesting that cellular systems governing intracellular Ca^{2+} homeostasis (and Ca^{2+}-based signaling) and energy accumulation and metabolic regulation via phosphorylated compounds have evolved in parallel. However, despite early anecdotal evidence showing that phosphorylated compounds (e.g., ATP) trigger the generation of intracellular second messengers (i.e., large Ca^{2+} fluxes) in lymphocytes and macrophages (Sung et al., 1985; Lin et al., 1985), little attention was paid to their extracellular messenger role in immunity. A radical shift occurred over the latest few years due to the accumulation of a large body of evidence showing that nucleotide signaling governs some of the most essential responses in immunity, ranging from antigen-driven T lymphocyte proliferation to T helper 1 (Th1) and Th2 cell differentiation, from neutrophil and macrophage chemotaxis to intracellular pathogen killing, and from NADPH-oxidase activation to IL-1β maturation and release (Junger, 2011; Idzko et al., 2014; Cekic and Linden, 2016).

The role of extracellular ATP in inflammation and immunity is supported by the direct in vivo demonstration that inflammatory sites contain high (hundred micromolar) extracellular ATP concentrations, as opposed to the interstitium of healthy tissues where the ATP concentration is in the low nanomolar range (Pellegatti et al., 2008; Wilhelm et al., 2010; Barberà-Cremades et al., 2012). Almost all living organisms, from protozoa to higher mammals, have evolved a sophisticated receptor set for extracellular nucleotides. In higher mammals, the nucleotide receptor family (P2 receptors, P2Rs) is comprised of two subfamilies: G protein-coupled metabotropic P2Y (P2YR) and ligand (ATP)-gated ionotropic P2X (P2XR) receptors (see Table 1; Burnstock, 2007). The P2YR and the P2XR subfamilies consist of eight (P2Y1, 2, 4, 6, 11–14) and seven (P2X1–7) members, respectively. P2YRs have a rather mixed ligand nucleotide selectivity, P2Y11R being the only "true" ATP-selective receptor, while ADP, UDP, UTP, UDP-glucose, or UDP-galactose are preferred agonists at the other P2YRs (Jacobson and Müller, 2016). In contrast, the preferred agonist for P2XRs is ATP, whereas all other nucleotides are inactive (Jacobson and Müller, 2016). For some time it was thought that P2YRs were evolutionary more ancient than P2XRs, but cloning of P2XRs from *Dictyostelium*, *Schistosoma*, and algae suggests an earlier appearance of P2XRs during evolution (Verkhratsky and Burnstock, 2014). In addition, data from *Dictyostelium* hint to an original function of these receptors in ion exchange across the membrane of intracellular organelles, a role that might have been retained in eukaryotes by P2X4R (Robinson and Murrell-Lagnado, 2013).

P2XRs are expressed by several mouse and human immune cell types, but their function with a few exceptions is rather poorly understood. P2X1R has been associated to T lymphocyte metabolic regulation and proliferation (Woehrle et al., 2010; Lederose et al., 2016a) and to neutrophil extravasation (Maître et al., 2015). P2X4R has been implicated in NFAT activation

Table 1. P2 Receptors

Receptor	Preferred Agonist(s)	Coupling Mechanism	Subunit Assembly	Transduction Mechanism	Intracellular Signaling
P2YRs					
P2Y1	ATP, ADP	G_q/G_{11}	–	PLC activation and IP3 production	Ca^{2+} increase
P2Y2	ATP, UTP				
P2Y4	UTP				
P2Y6	UDP				
P2Y11	ATP	G_q/G_{11}-Gs	–	PLC activation and IP3 production; adenylate cyclase stimulation	Ca^{2+}/ cAMP increase
P2Y12	ADP	$G_i/_o$	–	adenylate cyclase inhibition	cAMP decrease
P2Y13	ADP				
P2Y14	UDP-glucose; UDP-galactose				
P2XRs					
P2X1	ATP	–	P2X1, P2X2, P2X4, P2X5	cation-selective ion channel	Ca^{2+} and Na^+ increase
P2X2		–	P2X2, P2X3, P2X6		
P2X3		–	P2X3, P2X2		
P2X4		–	P2X4, P2X6		
P2X5		–	P2X5, P2X1		
P2X6		–	P2X6, P2X2, P2X4		
P2X7		–	P2X7	cation-selective ion channel; non-selective pore	Ca^{2+} and Na^+ increase; K^+ decrease

and in the modulation of γδ T lymphocyte responses (Woehrle et al., 2010). In addition, P2X4R has an established role in central nervous system microglia, where it is pivotal in the pathogenesis of neuropathic pain (Tsuda et al., 2003; Coull et al., 2005). Even less is known about P2X5R and P2X6R in the immune system, except for the reported upregulation of P2X5R in $CD34^+$ leukemic myeloid cell subpopulations and in activated human T lymphocytes (Norde et al., 2009; Abramowski et al., 2014). In this landscape, the P2X7 receptor (P2X7R; encoded by *P2RX7*) stands out as the single member of the P2XR family with a firmly established role in multiple inflammatory and immune responses.

In this review we will discuss recent advances in P2X7R structure and in our understanding of P2X7R contribution to inflammation and host defense. The role of the P2X7R in typical (e.g., NLRP3) and atypical (e.g., caspase-11) inflammasome activation, modulation of energy metabolism, and macrophage and T lymphocyte differentiation will be highlighted. Finally, alternative P2X7R agonists will be described and the therapeutic perspective briefly discussed. The following notations will be used throughout: P2X7 to indicate the single subunit or monomer, whether human or mouse; *P2RX7* or *P2rx7* to indicate the human or mouse gene, respectively; and P2X7R, to indicate the oligomeric receptor.

The P2X7R Structure

Human *P2RX7* is located on the long arm of chromosome 12, at 12q24.31, centromeric and close to *P2RX4* (12.q24.32). Mouse *P2rx7* and *P2rx4* are both located on chromosome 5, 62.50 and 62.43 cM, respectively. Close proximity of *P2RX4* and *P2RX7*, together with protein sequence homology (41% identity), suggests an origin by gene duplication. Sequence homology of P2X7 and P2X4 monomers has turned out especially useful for the prediction of the tridimensional structure of the single P2X7 subunit as well as of the multimeric P2X7R. In fact, the crystal structure of the zebrafish P2X4R has been resolved in the closed and open (ligand-bound) state, providing a wealth of information on the ATP binding site, subunit interaction, and mechanism of pore opening (Kawate et al., 2009; Hattori and Gouaux, 2012). This information has been further implemented by recent tridimensional resolution of human P2X3R (Mansoor et al., 2016).

P2RX7 encodes a 595-aa protein (the P2X7 subunit or monomer) that assembles into a trimeric complex to form the functional P2X7R (Surprenant et al., 1996; North, 2002). The P2X7 monomer has a short intracellular N-terminal residue (26 aa), a bulky extracellular domain (282 aa), two transmenbrane helices (about 24 aa each), and a long cytoplasmic carboxy-terminal tail (239 aa) (Figure 1; Surprenant et al., 1996). Ten cysteines present in the extracellular loop are likely to form intrachain disulfide bridges. Five N-glycosylation sites have also been identified in human P2X7 (Lenertz et al., 2010). The short N terminus contains a protein kinase C (PKC) phosphorylation consensus site [TX(K/R)] conserved in all P2X subtypes (Boué-Grabot et al., 2000). The most interesting domain in the P2X7 subunit is no doubt the carboxy-terminal tail (aa 356–595), unique to this receptor subtype. Multiple lipid and protein binding motifs have been identified in this domain: region 441–460 is homolog to SRC homology 3 (SH3)-binding domains; region 436–531 is homolog to a stretch of the tumor necrosis factor receptor 1 (TNFR1) that includes the death domain; regions 389–405 and 494–508 are homolog to regions found in proteins that bind the cytoskeleton; and residues 573–590 show homology with the endotoxin (LPS)-binding region of serum LPS-binding protein

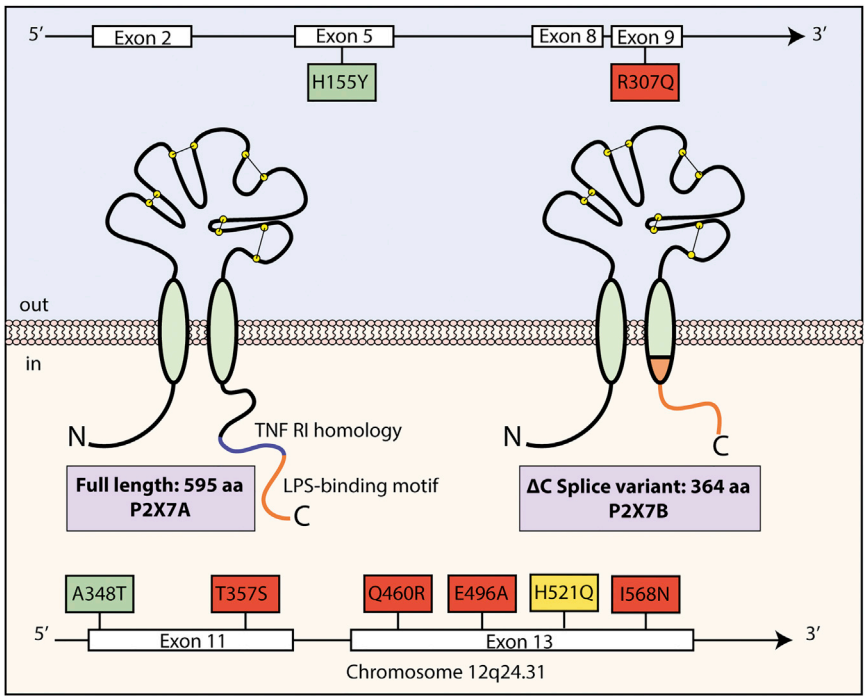

Figure 1. Schematic Rendition of the P2X7 Monomer

The full-length (P2X7A) and a common truncated splice variant (P2X7B) are shown. TNF RI homology and putative LPS-binding domains in the carboxy-terminal tail of P2X7A are shown in blue and orange, respectively. The carboxy-terminal tail of P2X7B lacks the last 249 aa of the full-length receptor and shows an insertion of 18 extra aa after residue 346. Most common gain- (green) and loss- (red) of-function SNPs are shown. No effect on function, yellow. dbSNP ID: H155Y, rs208294; R307Q, rs28360457; A348T, rs1718119; T357S, rs2230911; Q460R, rs2230912; E496A, rs3751143; H521Q, rs2230913; I568N, rs1653624.

(LBP) (Denlinger et al., 2001). The carboxy-terminal tail is an absolute requirement for the formation of the large conductance pore typical of the P2X7R as truncation at residue 418 in the rat receptor abolishes large pore function (Surprenant et al., 1996). Likewise, the short P2X7B human splice variant generates a receptor that lacks ability to promote uptake of low-molecular-weight aqueous solutes but retains ion channel activity (Adinolfi et al., 2010).

Functional P2X7R is a trimer, as shown by biochemical data on blue-native polyacrylamide gel electrophoresis, by functional experiments based on co-expression of concatamers (see review by North, 2002), and by analogy to crystallized P2X4R and P2X3R (Kawate et al., 2009; Mansoor et al., 2016). This structural feature places P2X7R in a distinct trimeric ionotropic receptor family, together with acid-sensing ion channels (ASICs) (Baconguis et al., 2013). Since protons (H^+), together with ATP, are thought to be the earliest danger signals released by unicellular organisms, this suggests a common evolution of receptors for damage-associated molecular patterns (DAMPs). P2XRs and ASICs have no sequence homology but show common functional features, such as slow desensitization and the ability to undergo an increase in pore size during prolonged exposure to the agonist (this dynamic selectivity is the most intriguing functional feature of the P2X7R). Scattered evidence suggests that the P2X7R oligomer can incorporate additional subunits to form higher-order complexes (hexamers) (Kim et al., 2001), especially in the presence of positive allosteric modulators such as the antibiotic polymixin B (Ferrari et al., 2004). The dynamics of subunit stoichiometry and pore opening of the P2X receptors as derived from crystallization studies and biochemical and molecular analysis will be very informative for the clarification of the mechanism responsible for formation of the large conductance P2X7R pore.

Structural information on the general architecture of the P2XR family in both the closed (apo) and open (ATP-bound) states derives from the obtained and solved crystal structures of zebrafish P2X4R (Kawate et al., 2009; Hattori and Gouaux, 2012), of an invertebrate P2XR (Kasuya et al., 2016), of human P2X3R (Mansoor et al., 2016), and of panda (*Ailuropoda melanoleuca*) P2X7R (Karasawa and Kawate, 2016). The tridimensional monomer structure has been compared to the shape of a dolphin (Hattori and Gouaux, 2012), where the two transmembrane (TM) domains make the dolphin flukes and the different segments of the large extracellular loop make various sections of the dolphin body (i.e., head, flippers, dorsal fin) (Figure 2A). The "dolphin anatomy" is now generally adopted to identify specific regions in the tertiary structure of P2X subunits (Kaczmarek-Hájek et al., 2012) and also to describe P2X7R architecture. Crystal structure from P2X4R has been previously used for theoretical modeling of the P2X7R by means of homology modeling (see Browne et al., 2013; Dal Ben et al., 2015; De Marchi et al., 2016; Jiang et al., 2013). Availability of X-ray structures of the apo (PDB: 5SVJ) and ATP-bound (PDB: 5SVK) human P2X3R now allows a more faithful rendition of the 3D organization of the P2X7R (Figure 2B; Mansoor et al., 2016). Unfortunately, due to lack of direct structural data, homology modeling provides no information on the C-terminal cytoplasmic tail unique to P2X7R, nor does it predict interaction of the tail with other domains of the same or adjacent monomers.

The binding site of ATP is located at the interface of each pair of monomers, between the dorsal fin of one subunit and the head and the left flipper domain of the adjacent subunit (Figure 2C). The ATP binding domain is rather atypical due to a set of residues conserved in all human and rodent P2X subtypes (Chataigneau et al., 2013; Dal Ben et al., 2015). Several charged amino acids (Lys64, Lys66, Lys193, Arg294, and Lys311) involved in coordination of the three phosphate groups of ATP and a threonine residue (Thr189) involved in interaction with the adenine moiety are present at the bottom of the ATP-binding pocket of the human P2X7R (Figure 2D). Presence of four lysines in the ATP-binding domain might explain the exquisite sensitivity of P2X7R to blockade by oxidized ATP (Murgia et al., 1993), a widely used di-aldehyde P2X7R inhibitor that forms Schiff bases

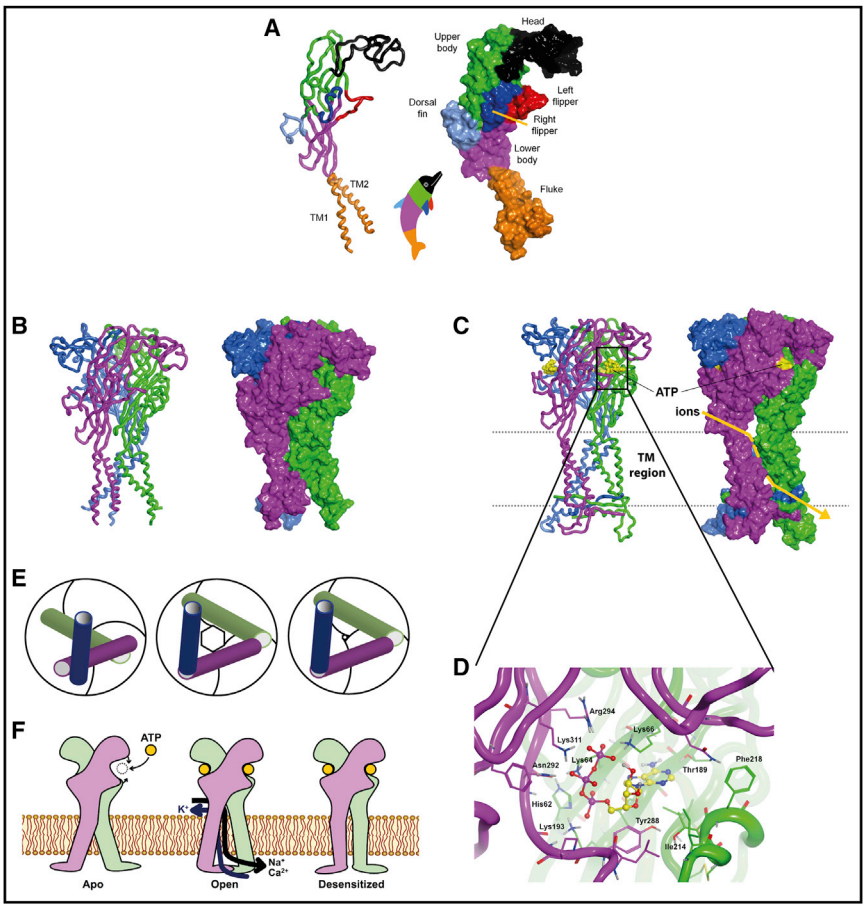

Figure 2. Architecture of Human P2X7R Built by Homology Modeling on the Basis of the Crystal Structure of Human P2X3R

(A) The P2X7 monomer represented as secondary structure (left) and molecular surface (right) styles built by using the crystal structure of the apo-form of the human P2X3 as a template. The shape of the monomer is compared to a dolphin with the subdivision of the monomer regions according to Hattori and Gouaux (2012).

(B and C) Schematic view of the P2X7R trimer in the closed (B) and open (C) states. For each state, the receptor trimer is shown as secondary structure (B and C, left) and molecular surface (B and C, right) style. The three monomers are colored in blue, purple, and green. The ATP molecule is colored in yellow, and its position in the trimer is indicated.

(D) Detailed view of the ATP binding site. Two adjacent monomers (represented in purple and green, respectively) contribute to the binding site. Key residues for ligand interaction are shown. The ATP molecule is represented with ball & stick style.

(E) Schematic representation of the transition of the ion channel pore from closed (left), to open (center), to desensitized (right).

(F) Cartoon model summarizing the conformational changes occurring during receptor opening and desensitization, and the ion pathways (after Mansoor et al., 2016).

with un-protonated lysines. Sequence analysis and tertiary structure of the entrance of the ATP-binding pocket show lack of positively charged amino acids and a restricted access, suggesting a rather small volume of the binding cavity and difficult entry for hydrophilic compounds, which might explain why P2X7R has typically low affinity for ATP and for several negatively charged drug-like compounds active at other P2XRs. On the contrary, several P2X7R ligands are small, often hydrophobic, compounds. The comparison of the apo and the ATP-bound crystal structures suggests a mechanism for channel opening (“gating”): ATP binding triggers a large conformational rearrangement whereby the monomers change the reciprocal orientation without significantly altering conformation, thus behaving as rigid bodies (Hattori and Gouaux, 2012; Mansoor et al., 2016). During this process, the head domain of the monomers get closer to the dorsal fin of the adjacent units, while the TM domains change the mutual orientation, thus generating a pore formed by lateral fenestrations lined by TM2 of each monomer (Figures 2E and 2F). The gating process allows the rapid inward flux of Na^+ and Ca^{2+} and the outward flux of K^+ (Figure 2F). Both inward and outward ion fluxes are fundamental in P2X7R-mediated responses. Current onset is followed by an inactivation phase (desensitization) and by recovery. In the P2XR subfamily, desensitization may be fast (P2X1R and P2X3R) or slow (P2X2R, P2X4R, and P2X7R) (Coddou et al., 2011; North, 2002). Prolonged exposure to the agonist causes receptor inactivation, but in P2X7R (and possibly also in P2X2R and P2X4R) may also promote further increase in permeability (maybe by receptor rearrangement) with the formation of a large pore (the paradigmatic “P2X7R pore”) that allows transmembrane fluxes of large hydrophilic molecules (Falzoni et al., 1995; North, 2002). Homology modeling based on the P2X3R structure predicts that ATP binding to P2X7R causes a reorientation of the three monomers and formation of a so-called “cytoplasmic cap,” consisting in N- and C-terminal segments partially covering the cytoplasmic end of the trans-membrane pore (Mansoor et al., 2016). Analysis of trans-membrane helices movement in the activated, ATP-bound, state may help understand how and if the cation-selective P2X7R channel might enlarge to form the large conductance pore.

Recruitment of auxiliary pore-forming partners (e.g., pannexin-1) might also participate in the permeability increase to higher-molecular-weight solutes, but converging evidence from different laboratories suggest that permeability to high-molecular-weight molecules is an intrinsic property of P2X7R as macrophages from pannexin-1-deficient mice, or silenced for pannexin-1, undergo ATP-stimulated plasma membrane permeability increases to about the same extent as macrophages from wild-type mice (Qu et al., 2007; Alberto et al., 2013; F.D.V. and D. Cavagna, unpublished data). A detailed electrophysiological analysis of human and rat P2X7R now shows that permeability to large organic cations is an intrinsic property of the P2X7R channel that does not require progressive pore dilatation (Pippel et al., 2017; Harkat et al., 2017). However, the extended C-terminal P2X7R tail is an absolute requirement for large permeability increases (Surprenant et al., 1996; Adinolfi et al., 2010). This

might mean that the tail is needed to support TM2 helices movement to generate the large pore, to stabilize the "cytoplasmic cap" formed by the interaction between the N and C termini (Mansoor et al., 2016), or to allow maximal ion permeability of the P2X7R channel. An interesting property of the P2X7R-dependent plasma membrane permeability increase is its reversibility. Removal of ATP within a few minutes from its addition allows full resealing of the plasma membrane. This property has been exploited to trap in the cytoplasm of different cells various low-molecular-weight normally impermeant aqueous solutes such as nucleotide analogs, fluorescent probes, and antitumor drugs (Di Virgilio et al., 1988; Munerati et al., 1994).

P2X7R Variants

Ten (P2X7A–J) and four (P2X7a, P2X7k, P2X713b, and P2X713c) splice variants of human *P2RX7* and mouse *P2rx7*, respectively, are known (Cheewatrakoolpong et al., 2005; Feng et al., 2006; Kaczmarek-Hájek et al., 2012; Masin et al., 2012). Four human splice variants (P2X7B, P2X7C, P2X7E, and P2X7G) are C-terminally truncated. P2X7G and P2X7H have an inserted additional exon (exon N3) that causes deletion of the first transmembrane region (TM1). In addition, P2X7C lacks exon 4, P2X7D exon 5, P2X7E exons 7 and 8, and P2X7F exons 4 and 8. P2X7I results from a point mutation in the first intron of *P2RX7* that generates a null allele (Sluyter and Stokes, 2011). The P2X7J variant is truncated after exon 7 and is non-functional. P2X7B has attracted the most interest because it is the predominant transcript in many tissues. The receptor made by its assembly (P2X7RB) lacks the pore function but retains channel activity, stimulates growth, and heteromerizes with P2X7A subunits to produce an enhanced response compared to the homomeric P2X7RA (Cheewatrakoolpong et al., 2005; Adinolfi et al., 2010; Liang et al., 2015). The mouse P2X7k variant is fully functional and bears alternative N terminus and TM1 (Nicke et al., 2009). Receptor formed by P2X7k monomers show increased pore formation activity. P2X13b and P2X713c are C-terminally truncated.

Human *P2RX7* is highly polymorphic. More than 150 non-synonimous SNPs have been identified in the extracellular loop and in the cytoplasmic tail, and functional effects of a few have been analyzed (Wiley et al., 2011; Bartlett et al., 2014). At least eight loss-of-function SNPs have been identified (Fuller et al., 2009). The best characterized is the replacement of Glu 496 with Ala (E496A), which causes reduced receptor activity (Gu et al., 2001). Another SNP located in the C-terminal domain, I568N, impairs P2X7 subunit translocation to the plasma membrane and decreases receptor activity. A SNP located in the ATP-binding pocket, R307Q, severely decreases affinity for the agonist (Gu et al., 2004). Three gain-of-function SNPs have been so far identified: H155Y, H270R, and A348T (Cabrini et al., 2005; Sun et al., 2010; Stokes et al., 2010). Association of various SNPs to bipolar disorders or increased susceptibility to tuberculosis infection, osteoporosis, age-related macular degeneration, and cardiovascular diseases has been suggested, but available evidence is as yet rather preliminary (Miller et al., 2011; Jørgensen et al., 2012). Caution should be exercised in the interpretation of SNP-disease association studies since strong linkage disequilibrium exists between SNPs, which generates distinct haplotypes (Fuller et al., 2009; Stokes et al., 2010).

Investigation of pathophysiological functions of the P2X7R has benefited from the generation of three P2X7R-deficient mouse lines according to different gene disruption strategies (Solle et al., 2001; Sim et al., 2004; Bartlett et al., 2014). The first P2X7R-deficient mouse line, established at the former Glaxo-Wellcome Geneva Biomedical Research Institute in the mid 1990s but not published until 2004, was generated by inserting a lacZ/Neo reporter cassette into exon 1 of *P2rx7*. The P2X7k splice variant may escape inactivation and therefore cells overexpressing this variant may exhibit full P2X7R-dependent responses (Taylor et al., 2009; Kaczmarek-Hájek et al., 2012). The second P2X7-deficient mouse was generated at Pfizer by deleting a portion of exon 13 by insertion of a Neo resistance cassette (Solle et al., 2001). Concerns have been raised that C-terminally deleted P2X7 variants might still be expressed in this model (Kaczmarek-Hájek et al., 2012). A third P2X7R-deficient mouse has been reported where exons 2 and 3 have been replaced with lacZ/Neo reporter cassette (Basso et al., 2009). Limited information is available on this P2X7R-deficient mouse model. Analysis of *P2rx7*-deleted mice showed that lack of P2X7R hinders inflammation, cytokine release, immune response against pathogens, allogeneic cells and transplanted tumors, and development of neuropathic pain. Developmental and skeletal deficits have also been reported. The reader is referred to the excellent reviews by others (Miller et al., 2011; Kaczmarek-Hájek et al., 2012; Syberg et al., 2012; Bartlett et al., 2014) for a more detailed account.

P2X7R in Inflammation

The association of the P2X7R with inflammation and immunity is long standing. Well before it was cloned in 1996, investigators were intrigued by its dramatic effects on lymphocyte and macrophage responses (Steinberg and Silverstein, 1987; Di Virgilio et al., 1989). The discovery of the NLRP3 inflammasome has finally placed P2X7R in the appropriate context. However, evidence suggests that its role 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 et al., 2015). Furthermore, P2X7R blockade inhibits allograft rejection (Vergani et al., 2013), graft-versus-host disease (Wilhelm et al., 2010), choroidal neovascularization, geographic atrophy, sterile liver inflammation (Fowler et al., 2014), and retinal ganglion cell loss due to experimentally elevated intraocular pressure (a model of glaucoma) (Resta et al., 2007). P2X7R targeting also inhibits the generation of epileptogenic foci (Jimenez-Mateos et al., 2015). Macrophage P2X7R might also be implied in spreading of HIV infection because its stimulation by autocrine ATP promotes release of HIV-laden microvesicles (Graziano 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 heightened pro-thrombotic response (Moore and MacKenzie, 2007; Baroni 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 et al., 1999; Kurashima et al., 2012; Shieh et al., 2014), TNF from human dendritic cells (DCs) and

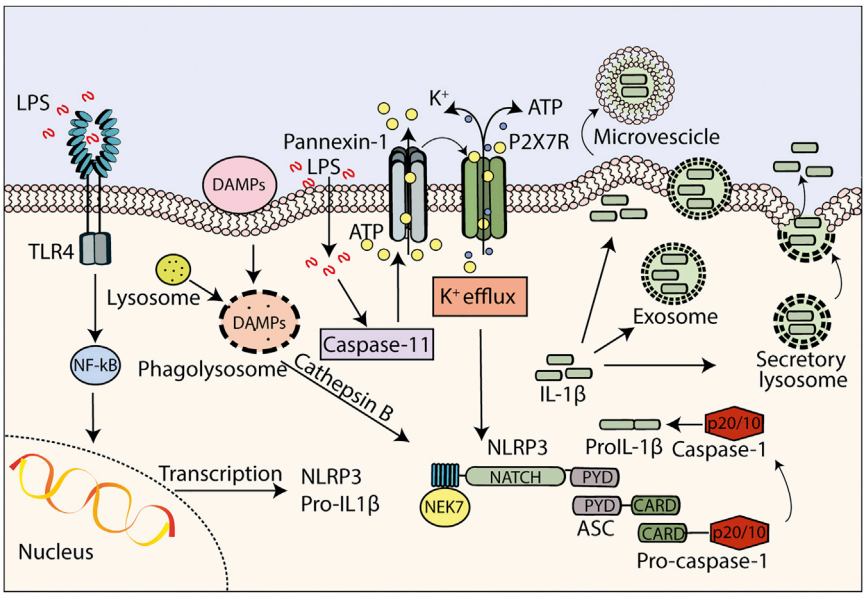

Figure 3. Central Role of P2X7R in IL-1β Processing and Release

PAMPs (LPS) promote transcription of the genes encoding IL-1β and inflammasome components such as NLRP3. DAMPs, either acting at the plasma membrane or in the cytosol, trigger NLRP3 inflammasome activation (for example by causing release of additional factors such as cathepsin B). Alternatively, cytoplasmic DAMPs and PAMPs may cause pannexin-1 opening, thus allowing ATP release. Extracellular ATP feeds back on P2X7R to trigger K^+ efflux, and thus NEK7, NLRP3, ASC, and caspase-1 assembly. The activated NLRP3 inflammasome catalyzes pro-IL-1β cleavage. Mature IL-1β will then be released via secretory lysosomes, exosomes, or plasma membrane-derived microvesicles. Opening of the large conductance P2X7R pore will also allow ATP efflux, thus amplifying the activation signal. Finally, P2X7R can also be directly activated by extracellular ATP, in the absence of stimulation by intracellular or extracellular PAMPs or DAMPs. Yellow circles, ATP; small blue circles, K^+.

mouse microglia (Ferrari et al., 2000; Shieh et al., 2014), and activation and shedding of matrix metalloproteases from human peripheral-blood mononuclear cells (Gu et al., 1998; Gu and Wiley, 2006). In vivo and in vitro release of anti-inflammatory agents, such as mouse IL-10 and TGF-β (Chessell et al., 2005; Monção-Ribeiro et al., 2014; Bianchi et al., 2014), is also stimulated by P2X7R, but in human monocytes inhibition of IL-10 and HLA-G release has been reported (Rizzo et al., 2009).

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 et al., 2001; Kurashima et al., 2012), IL-8 in rat C6 glioma cells (Wei et al., 2008), CC-chemokine ligand 3 (CCL3) in mouse microglial MG-5 cells (Kataoka et al., 2009), and CXCL2 in mouse microglia and mast cells (Shiratori et al., 2010; Kurashima et al., 2012). Prostaglandin E2 (PGE2) release is also dependent on P2X7R expression and is accordingly stimulated by P2X7R agonists in mouse osteoblasts and in mouse and human macrophages (Panupinthu et al., 2008; Barberà-Cremades 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 et al., 1998; Garcia-Marcos et al., 2006; Bianco et al., 2009). P2X7R is also a potent stimulus for activation of the NFATc1, NF-κB, HIF-1α, and PI3K-AKT-GSK-3β pathways (Di Virgilio and Adinolfi, 2017). But more importantly, P2X7R has a special place in IL-1β release for its role in NLRP3 inflammasome activation.

P2X7R Is a Major Driver of Inflammasome Activation

P2X7R was initially described as ATP^{4-} receptor or P2Z in mast cells, lymphocytes, and macrophages and thought to be mainly if not exclusively expressed in immune cells (Cockcroft and Gomperts, 1979; Di Virgilio et al., 1989; el-Moatassim and Dubyak, 1992). 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-β from mouse macrophages (Perregaux and Gabel, 1994). This was thought to be due to the potent cytotoxic effect caused by ATP on these cells. A few years later, we identified P2X7R as the receptor mediating the ATP effect (Ferrari et al., 1996, 1997) and highlighted the key role of K^+ efflux in ATP-mediated caspase-1 activation and therefore in pro-IL-1β processing. A role of K^+ efflux in LPS-stimulated IL-1β processing had been identified a few years earlier (Walev et al., 1995). The discovery of the NALP1 (NLRP1) inflammasome (Martinon et al., 2002) 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 et al., 2006). As of now, P2X7R is one of the most potent activators of the NLRP3 inflammasome, and therefore of caspase-1 cleavage and of mature IL-1β release (Figure 3).

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-IL-1β and the second ATP-dependent stimulation of P2X7R promoting inflammasome-mediated caspase-1 activation (Perregaux and Gabel, 1998; Perregaux et al., 2000; Ferrari et al., 2006). This model fits many but not all experimental observations and is certainly dependent on the cell type, culture conditions (type of serum, presence of specific cytokines or growth factors), and TLR agonist used. Gabel and co-workers carried out a thorough investigation of the various factors affecting ATP-stimulated IL-1β release from human monocyte and macrophage cells (Laliberte et al., 1997). 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 (Ferrari et al., 1996; Solle et al., 2001; Qu et al., 2007). This is also true of human monocyte and macrophage cells, whether tested in RPMI-diluted whole blood or as Ficoll-purified culture (Ferrari et al., 1997; Perregaux et al., 2000), and of mouse and human DCs (Ferrari et al., 2000), although more recent findings suggest that in mouse DCs the second stimulus (P2X7R activation) is dispensable during prolonged incubations in the presence of LPS (He et al., 2013). Requirement of P2X7R expression for in vivo IL-1β release has been tested in a few mouse models. In P2X7-deficient mice intra-peritoneally (i.p.) injected with LPS, no IL-1β was detected in the peritoneal fluid (Solle et al., 2001). In contrast, Nunez and co-workers reported no effect of *P2RX7* genetic ablation on LPS-stimulated IL-1β serum levels (He et al., 2013). Other investigators observed reduced levels of brain IL-1β in P2X7R-deficient mice systemically stimulated with LPS, and we have reported that tumor-bearing P2X7R-deficient mice have lower serum and intra-tumor IL-1β levels compared to wild-type (Adinolfi et al., 2015). Accordingly, DC cultures established from P2X7R-deficient mice and in vitro challenged with tumor cells release less IL-1β compared to wild-type (Adinolfi et al., 2015). Interleukin-18 secretion is also promoted by P2X7R activation, but the coupling mechanism is less clear (Perregaux et al., 2000).

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 (Muñoz-Planillo et al., 2013). Recent evidence suggests that P2X7R and NLRP3 might physically interact at discrete subplasmalemmal cytoplasmic sites. P2X7R and NLRP3 can be co-localized by confocal microscopy and co-immunoprecipitated in both mouse microglia and mouse peritoneal macrophages (Franceschini et al., 2015). Co-localization of NLRP2 with P2X7R has also been reported (Minkiewicz et al., 2013). These findings suggest that P2X7R channel or pore opening causes a localized modification of the intracellular ion microenvironment that drives recruitment of inflammasome components and facilitates their assembly in the vicinity of P2X7R itself. 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 Nima-related kinase (NEK)7 protein (He et al., 2016), 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 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. Recently, an alternative route not involving the classical "second signal" and the cytosolic K^+ drop has been described in LPS-stimulated human monocytes by Hornung and coworkers (Gaidt et al., 2016). This alternative inflammasome activation pathway involves activation of caspase-8 and cleavage of an as yet unknown caspase-8 substrate.

The molecular mechanism by which K^+ depletion causes inflammasome assembly is obscure. There is a precedent in the field of cell death, where early observations showed that apoptosis can be triggered by K^+ efflux via a mechanism possibly involving facilitation of Apaf-1 apoptosome formation (Bortner et al., 1997; Karki et al., 2007). Thus, it is possible that assembly of the fully active NLRP3 inflammasome complex (NLRP3-ASC-NEK7-caspase-1), in analogy to Apaf-1 apoptosome formation, is precipitated by a local decrease in the K^+ concentration. Since several microbial toxins are known to activate the inflammasome by causing depletion of intracellular K^+ (Muñoz-Planillo et al., 2013), 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 IL-1β 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 (Dinarello, 2002). 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 et al., 2001; Lopez-Castejon and Brough, 2011; Piccioli and Rubartelli, 2013). P2X7R is a main driver for all these routes (Bianco et al., 2005; Pizzirani et al., 2007; Qu et al., 2007), further emphasizing its fundamental contribution to the release of bioactive IL-1β.

The ability of P2X7R to trigger the release of multiple proinflammatory factors, some of them bona fide endogenous pyrogens, raises the issue of its involvement in a complex systemic response such as fever. An early paper by Gourine et al. (2005) showed that P2X7R blockade attenuated the febrile response induced in rats by LPS inoculation. These findings were later confirmed and extended by Pelegrin and coworkers who showed the inability of P2X7R-deficient mice to start a febrile response after i.p. LPS injection (Barberà-Cremades et al., 2012).

Alternative Inflammatory Activation of the P2X7R

Most available evidence supports a model whereby ligation of plasma membrane or cytoplasmic sensors for pathogen-associated molecular patterns (PAMPs) activates a mechanism for controlled ATP release (e.g., pannexin-1 activation) and autocrine P2X7R stimulation. At inflammatory sites, where extensive immune cell activation and tissue damage cause large ATP release, extracellular ATP by itself can be an effective pro-inflammatory stimulus. The associated P2X7R stimulation on one hand turns on intracellular effector systems (e.g., the NLRP3 inflammasome) and on the other further amplifies the effect of other pro-inflammatory agents by supporting additional ATP release,

since P2X7R is itself a pathway for ATP release (Pellegatti et al., 2005). One of the most serious criticisms raised against this model for immune cell activation is the low affinity for ATP, and therefore the high activation threshold of P2X7R. However, a reasonable answer is found in the peculiar biochemical composition of the inflammatory microenvironment since direct measurements performed at inflammatory or tumor sites have revealed that ATP concentrations as high as a few hundred micromolar are not unusual, and therefore in the range of the Km for P2X7R activation (Pellegatti et al., 2008; Wilhelm et al., 2010). In addition, data by Nunez and coworkers show that presence of LPS (and therefore reasonably of endotoxin-producing bacteria) in the macrophage cytoplasm "sensitizes" P2X7R by lowering the ATP threshold for activation (Yang et al., 2015).

At inflammatory sites, P2X7R can also be activated by alternative ligands. The nucleotide NAD^+ accumulates at sites of inflammation and tissue damage, where it can be used by the plasma membrane enzyme ARTC2.2 to ADP-ribosylate the P2X7Rk splice variant mainly expressed by mouse T lymphocytes (Seman et al., 2003; Rissiek et al., 2014). This modification allows long-lasting opening of the P2RX7 channel and negatively affects Treg and NKT cell survival, but is operative only in the mouse since human cells lack ARTC2.2. Unique among all other P2 receptors, non-nucleotide agonist are also active at P2X7R. Of special relevance in the context of inflammation are bactericidal peptides released by inflammatory cells such as the cathelicidin LL-37 (Elssner et al., 2004; Tomasinsig et al., 2008), ALU-derived RNAs (Kerur et al., 2013; Fowler et al., 2014), the amyloidogenic 1-42 β-amyloid peptide (Sanz et al., 2009), and serum amyloid (Niemi et al., 2011). In addition, the peptide antibiotic polymixin B, which by itself is not an agonist, lowers the activation threshold for ATP of the human P2X7R heterologously expressed in HEK293 or K562 cells, as well as of the native P2X7R in human macrophages, thus acting as a positive allosteric modulator (Ferrari et al., 2004). These findings suggest that P2X7R might be a sensor of many more signals of danger or distress than not just ATP, and therefore a receptor for multiple DAMPs. Such a "ligand infidelity" might better help understand how P2X7R opening is tuned at inflammatory sites.

The P2X7R in Cancer-Associated Inflammation

A large body of literature has investigated the role of P2X7R in cancer (see Roger et al., 2015; Di Virgilio and Adinolfi, 2017, for recent reviews). A detailed appraisal is outside the scope of this review, but the crucial role of P2X7R in immunogenic cell death needs to be briefly addressed. Immunogenic cell death 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 (Kroemer et al., 2013; Galluzzi 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 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 et al., 2007; Ghiringhelli et al., 2009). Immunogenic cell death turned out to be a major pathway for anti-cancer immune response that may deeply affect the outcome of anti-cancer therapy. This has raised hot interest on the endogenous molecules involved. 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 IL-1β (Ghiringhelli et al., 2009). These preclinical data were confirmed by analysis of subjects affected by breast cancer and stratified for *P2RX7* SNPs. Patients with the loss-of-function E496A substitution had a significantly shorter metastatic disease-free survival compared to subjects bearing the normal E496 *P2RX7* allele (Ghiringhelli et al., 2009). The key role of P2X7R in host response against tumors is further highlighted by recent experiments from our group showing that transplanted B16 melanoma or CT26 colon carcinoma tumors grow much faster and metastasize more readily in P2X7R-deficient versus P2X7R wild-type mice (Adinolfi et al., 2015). Histology shows near total lack of tumor-infiltrating inflammatory cells and low IL-1β levels in the absence of P2X7R.

P2X7R Is a Cytotoxic Receptor

P2X7R is a potent activator of cell death that may occur via different mechanisms, depending on the cell type, agonist concentration, and duration of the challenge. P2X7R-dependent cell death was initially described as necrotic (Di Virgilio et al., 1989), but it was soon clear that the mechanism was cell type dependent (Zanovello et al., 1990). Combination of different cell death mechanisms has also been described, for example in mouse lymphocytes where P2X7R stimulation has been reported to trigger a fast initial shrinkage followed by swelling that eventually culminates in lysis (Taylor et al., 2008). P2X7R has also been shown to promote pyroptosis in response to direct activation by ATP, stimulation with allosteric modulators, or challenge with pathogens (Dubyak, 2012; Yang et al., 2015). While there are no data on a direct effect of P2X7R on apoptosome assembly, there is scattered evidence that ATP stimulation (likely binding to P2X7R) causes cytochrome *c* release and caspase-3, -8, and -9 cleavage (Ferrari et al., 1999). Lack of an in-depth investigation of the effect of P2X7R activation on the overall apoptotic machinery is surprising given that P2X7R is widely considered a pro-apoptotic receptor. The pyroptotic pathway has been more thoroughly investigated. In mononuclear cells, pyroptosis is a form of caspase-1-dependent cell death generally precipitated by intracellular parasites, especially gram-negative bacteria. In mouse cells pyroptosis is efficiently triggered by the non-canonical caspase-11 inflammasome, and in human cells by caspase-1, caspase-4, and caspase-5 (Wallach et al., 2016). P2X7R has a crucial role in the sequence of events downhill to non-canonical inflammasome activation since caspase-11 causes ATP release via pannexin-1 activation and therefore P2X7R stimulation and pyroptosis, a process shown to be gasdermin-D dependent (Yang et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). In an as yet to be defined fashion, P2X7R is an obligatory step in this process since caspase-11 is unable to trigger pyroptosis in mononuclear cells from P2X7R-deficient mice (Yang et al., 2015). In the absence of bacterial products, as for example during sterile inflammation, the most common type of death triggered by P2X7R is necrosis. Long-standing evidence shows that P2X7R stimulation causes cell and intracellular organelles swelling, karyolysis, and irreversible plasma membrane damage (Di Virgilio et al., 1998). Although

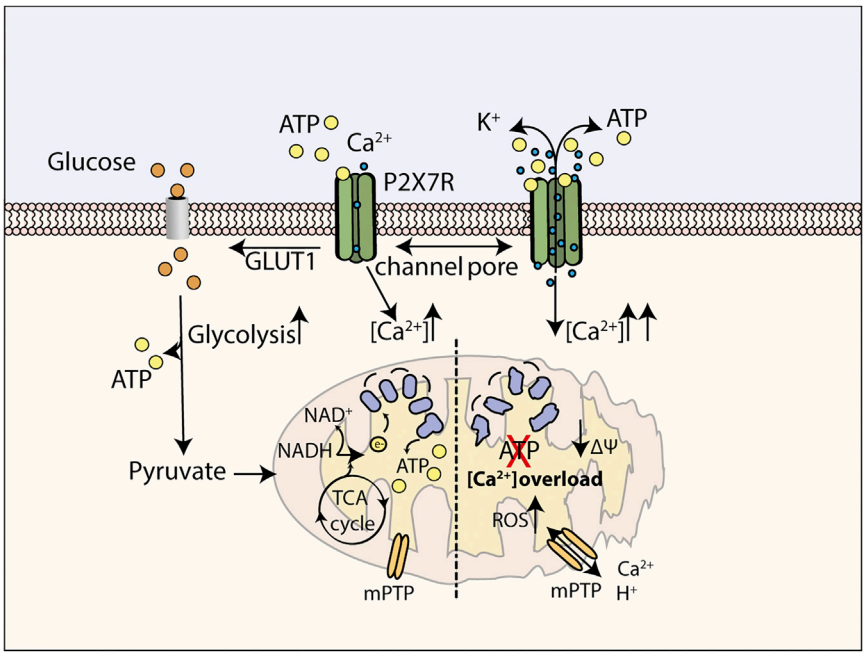

Figure 4. The P2X7R Is a Powerful Modulator of Cellular Energy Metabolism

Moderate P2X7R activation stimulates both glycolysis and oxidative phosphorylation. Stimulation of oxidative phosphorylation is likely the consequence of a mild increase in cytoplasmic Ca^{2+} that translates into a moderate elevation of the Ca^{2+} concentration in the mitochondrial matrix. The mitochondrial Ca^{2+} increase enhances the activity of the NADH dehydrogenase, and therefore of oxidative phosphorylation. Maximal (pharmacological?) P2X7R activation drives a large and uncontrolled Ca^{2+} influx that causes Ca^{2+} overload of the mitochondria, collapse of the mitochondrial potential ($\Delta\Psi$), opening of the mitochondrial permeability transition pore (mptp), and eventual mitochondrial fragmentation. Yellow circles, ATP; small blue circles, K^+.

P2X7R-dependent necrosis is widely documented, little effort has been made to investigate whether this type of necrosis might indeed be a form of incomplete or aborted pyroptosis.

P2X7R in Host-Pathogen Interaction

Evidence implicating P2X7R in the effector mechanisms downstream of caspase-11 underlines the central role of P2X7R in host-microbe interaction. P2X7R is activated in response to lethal factor (LF) from *Bacillus anthracis* (Ali et al., 2011), to *Porphyromonas gengivalis* (Park et al., 2014), to cytolethal distending toxin from *Actinobacillus actinomycetemcomitans* (Shenker et al., 2015), and to *Chlamidia* (Omosun et al., 2015). Furthermore, P2X7R may enhance resistance to sepsis (Csóka et al., 2015) and have a role as a pathogen sensor for *Entamoeba histolytica* (Mortimer et al., 2015). A thorough investigation of P2X7R expression and function in neutrophils highlights its pivotal role in host defense to *Streptococcus pneumoniae* (Karmakar et al., 2016). Several studies suggest that P2X7R might also participate in the elimination of intracellular parasites such as *Chlamidia*, *Leishmania*, *Toxoplasma* (Coutinho-Silva et al., 2003; Coutinho-Silva and Ojcius, 2012), and *Mycobacterium tuberculosis* (Lammas et al., 1997). However, relevance of this latter activity for host defense against tuberculosis infection is controversial. While the role of P2X7R in defense against *M. tuberculosis* is unclear, it has been recently shown that P2X7R expression, due to its strong pro-inflammatory and cytotoxic activity, aggravates tuberculosis disease progression and the associated tissue damage (Amaral et al., 2014).

Altogether, these findings place P2X7R at a central crossroad in inflammation. To make the picture more appealing and complex, P2X7R expression supports both oxidative phosphorylation and glycolysis (Adinolfi et al., 2005; Amoroso et al., 2012; Ledderose et al., 2016b), with intriguing implications for immune cell differentiation.

P2X7R Modulates Energy Metabolism and T Cell Growth and Differentiation

P2X7R (formerly known as P2Z) has attracted attention from the very beginning as a cytotoxic receptor (Di Virgilio et al., 1998). However, studies in human lymphoid cells made it clear that while its expression sensitizes cells to enhanced ambient ATP concentrations, under less harsh conditions P2X7R expression affords a growth advantage (Baricordi et al., 1999). This is epitomized by the opposite effect that P2X7R overstimulation versus basal stimulation has on mitochondrial structure and function. Pharmacological stimulation of P2X7R-transfected HEK293 cells triggers a "mitochondrial catastrophe" characterized by fragmentation of the mitochondrial network, uncoupling of oxidative phosphorylation, and cytochrome C release (Mackenzie et al., 2005), while low-level, tonic P2X7R stimulation has a striking trophic effect as shown by thicker mitochondrial network, higher mitochondrial potential, and ATP synthetic efficiency (Figure 4; Adinolfi et al., 2005). The Ca^{2+} concentration of the mitochondrial matrix is also moderately increased in P2X7R-expressing versus P2X7R-deleted cells, a condition that likely enhances activity of the NADH-dehydrogenase and therefore overall efficiency of the respiratory chain. Such a trophic effect is also reflected on glycolysis. In HEK293 cells transfected with the P2X7R and in tumor cells expressing the native receptor, level of key glycolytic transporters and enzymes such as Glut-1, glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase M2, and pyruvate dehydrogenase kinase 1 is enhanced, and GSK-3β is inactivated, thus expanding intracellular glycogen stores (Amoroso et al., 2012, 2015). End result of this profound effect on energy metabolism is the expansion of the intracellular ATP pool and the promotion of anaplerotic pathways. Stimulation of cell growth and mitochondrial metabolism by P2X7R (in association with the P2X1R) has also been described in human leukemic cell lines (Ledderose et al., 2016b).

Energy metabolism modulation suggests that P2X7R might have a role in skewing immune cell differentiation. An enhanced oxidative metabolism is associated, and likely determines, a preferential M2 macrophage and Treg cell differentiation, while glycolysis is more closely associated to M1 macrophage and

Th17 cell differentiation. P2X7R expression promotes both glycolysis and mitochondrial respiration, so in principle it is difficult to envisage how it might skew macrophage or T lymphocytes in one direction or the other. However, since P2X7R activity can be modulated by several factors, such as intracellular or extracellular bacterial or host products, it can be hypothesized that depending on its level of activation, glycolytic substrate availability, or oxygen tension, P2X7R might preferentially support one metabolic pathway (e.g., respiration) rather than another (e.g., glycolysis).

Available evidence supports a role for P2X7R in T lymphocyte proliferation and differentiation (Baricordi et al., 1996; Schenk et al., 2011), but whether Treg or Th17 cell differentiation is promoted is controversial. Treg cells overexpress P2X7R during differentiation, but P2X7R activation seems to play an inhibitory role in this cell type, as it inhibits suppressive functions and, in the presence of IL-6, promotes conversion into Th17 cells (Schenk et al., 2011). To confirm an inhibitory role of P2X7R in Treg cell maturation, *P2rx7*-deleted mice have a higher number of Treg cells compared to the wild-type counterpart (Hubert et al., 2010). Administration of NAD^+ can also be used to deplete Treg and NKT cells in vivo (Kawamura et al., 2006). P2X7R is also a signature of mouse Th follicular cells, where it regulates survival and at the same time is downmodulated by TCR stimulation (Proietti et al., 2014). Peyers's patches from *P2rx7*-deleted mice show enhanced Th follicular cell number and germinal centers.

More recent data that extend P2X7R regulatory function to Th1 and Th17 effector cells further highlight the role of this receptor in immune tolerance in the intestine (Hashimoto-Hill et al., 2017). P2X7R is reported to be upregulated in intestinal $\alpha\beta$ and $\gamma\delta$ T cells, Th1 and Th17 cells included, in a retinoic acid-dependent manner due to presence of a retinoic acid-binding region in *P2rx7*. As previously shown for Treg cells, P2X7R activation by ATP or NAD^+ causes T effector cell depletion, and vice versa genetic deletion of P2X7R expands the intestinal T effector cell population. The end result on intestinal inflammation of P2X7R-mediated depletion of both Treg and T effector cells is not easily anticipated. It might be that constitutive expression of P2X7R by Treg cells, as opposed to naive T effector cells that almost entirely lack this receptor, renders these immune cell populations differentially sensitive to ATP and NAD^+ since the very early phases of differentiation.

How lack of P2X7R promotes Treg and T effector cell population expansion and differentiation is not known. The easiest explanation could simply be that P2X7R expression sensitizes these cell types to the high ATP and NAD^+ concentrations present in the intestinal mucosa (which likely reflects gut lumen concentrations) and thereby promotes T lymphocyte-selective elimination by apoptosis. However, mitochondrial metabolism might also have a role. For example, differentiation of Treg cells has been shown to be modulated by metabolism (Beier et al., 2015), so it is possible that these cells are eminently susceptible to P2X7R-mediated mitochondrial damage, and therefore in the absence of P2X7R a healthier mitochondrial metabolism drives differentiation. In the mouse, extracellular ATP and P2X7R have been shown to be necessary for Th17 cell differentiation (Atarashi et al., 2008; Fan et al., 2016), a process enhanced by glycolytic metabolism and thus possibly promoted by a lower sensitivity to mitochondrial damage, or by a preferential coupling of P2X7R to glycolysis in this cell type. In bone-marrow-derived macrophages, ATP stimulation promotes a preferential M2 differentiation (Barberà-Cremades et al., 2016), a process driven by oxidative metabolism, and possibly also supported by P2X7R. In the overall mechanism of P2X7R-modulated T lymphocyte and macrophage differentiation, HIF-1α is likely to play a central role. In fact, this transcription factor, which is activated by P2X7R in different cell types (Amoroso et al., 2012, 2015; Hirayama et al., 2015), inhibits Treg cell differentiation by promoting proteasomal degradation of Foxp3 (Dang et al., 2011), a subset-specific transcription factor that controls the expression of genes determining the Treg cell suppressive phenotype. In addition, HIF-1α promotes lactic acid-dependent M2 polarization in the tumor microenvironment (Colegio et al., 2014). Interestingly, the P2X7R is a strong stimulant of aerobic glycolysis and of lactic acid production (Amoroso et al., 2012). A role for P2X7R and HIF-1α is also likely in the differentiation of IL-10-producing type 1 regulatory T (Tr1) cells. Quintana and co-workers have shown that the P2X7R triggers HIF-1α-mediated destabilization of the aryl hydrocarbon receptor, a transcription factor that drives the late phase of Tr1 cell differentiation, thus causing inhibition of differentiation of this T cell subpopulation (Mascanfroni et al., 2015). Earlier work from the same laboratory suggests that the P2X7R might also be implicated in the modulation of DC-mediated Th1 and Th17 cell differentiation via the NLRP3 inflammasome (Mascanfroni et al., 2013). All these data provide very strong evidence for the participation of the P2X7R in T lymphocyte differentiation. While available evidence points to multiple direct and indirect roles, it is possible that the P2X7R also participates in immune cell differentiation by biasing the inflammatory microenvironment due to its ability to promote release of Treg- or Th17-, M1-, or M2-skewing cytokines, as shown by convergent observations in human inflammatory settings (Killeen et al., 2013; Pandolfi et al., 2016).

P2X7R: A Therapeutic Target?

P2X7R has attracted interest for its potential therapeutic developments soon after cloning and the demonstration of its role as a trigger of inflammatory cytokine release. Many pharmaceutical companies have synthesized small-molecule (drug-like) P2X7R inhibitors (see Gunosewoyo and Kassiou, 2010; Sluyter and Stokes, 2011; Park and Kim, 2017, for a review of patents on P2X7R antagonist or on P2X7R SNPs and variants) that have been taken to phase I and II clinical studies for a few chronic inflammatory diseases. More than 30 clinical studies have been performed so far to test efficacy of P2X7R blockade in osteoarthritis, rheumatoid arthritis, chronic obstructive pulmonary disease, and Crohn's disease (Arulkumaran et al., 2011; Stock et al., 2012; Keystone et al., 2012; Eser et al., 2015). Zhejiang Conba Pharmaceutical in partnership with Evotec is currently testing a P2X7R antagonist in rheumatoid arthritis (phase I) and other unspecified inflammatory diseases (phase II) (https://www.biocentury.com/companies/zhejiang_conba_pharmaceutical_co_ltd). Currently, eight studies investigating the effect of P2X7R blockade in rheumatoid arthritis and chronic allograft vasculopathy or exploring the suitability of P2X7R as a biomarker in cancer or the association of P2X7R SNPs with osteoporosis are listed on ClinicalTrials.gov. One

Table 2. Ongoing Clinical Studies Investigating P2X7R

Status	ID	Title	Disease
Completed, has results	NCT02587819	Investigation of the Safety and Tolerability of BSCT (Anti-nf-P2X7) 10% Ointment	basal cell carcinoma
Terminated	NCT00471120	Feasibility Study: Accuracy of Biomarker in Detection of Endometrial Cancer	uterine cancer, endometrial cancer
Unknown	NCT00293189	Gene-Polymorphisms in the P2X7 Gene in Patients With Osteoporotic Fractures	hip fracture
Unknown	NCT02082821	A P2X7R Single Nucleotide Mutation Promotes Chronic Allograft Vasculopathy	cardiac allograft vasculopathy
Completed	NCT00628095	Study of CE-224,535 A Twice Daily Pill To Control Rheumatoid Arthritis In Patients Who Have Not Totally Improved With Methotrexate	rheumatoid arthritis
Unknown	NCT02293811	Decoding of the Expression of Tumor Suppressor P2RX7 in Inflammatory and Malignant Colonic Mucosa	Crohn disease-associated colorectal adenocarcinoma
Completed	NCT00697983	Cohort Study on Associations Between Purinergic Receptor SNPs and Osteoporosis Risk	osteoporosis
Completed	NCT00849134	First Time in Human Study Evaluating the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics and the Effect of Food of Single Assending Doses of GSK1482160.	inflammatory pain
Completed, has results	EudraCT n. 2008-000327-25, NCT00628095	A Phase 2a, Randomized, Double-Blind, Placebo-Controlled, Parallel-Group Study Of Ce-224,535, An Antagonist Of The P2X7 Receptor, In The Treatment Of The Signs And Symptoms Of Rheumatoid Arthritis In Subjects Who Are Inadequately Controlled On Methotrexate	rheumatoid arthritis

study reported there is also listed in the European Clinical Trial Register and has results (see Table 2). An authoritative update of most important chemical families of P2X7R antagonists is provided in the recent reviews by Jacobson and Müller (2016) and by Adinolfi and co-workers (De Marchi et al., 2016). In general, no serious concerns were raised on safety profile of P2X7R antagonist so far tested, the main complaints reported being dizziness, headache, diarrhea, and nausea.

Despite the favorable safety profile, clinical efficacy has been disappointing in rheumatoid arthritis, osteoarthritic pain of the knee, and chronic obstructive pulmonary disease. More encouraging results were obtained in Crohn's disease (Arulkumaran et al., 2011; Eser et al., 2015). Availability of high-affinity antagonists has allowed development of P2X7R radioligands suitable for positron emission tomography (PET) studies (Ory et al., 2016; Territo et al., 2017; Fantoni et al., 2017). Preclinical studies show that these tracers specifically label P2X7R in mouse, rat, and monkey brains, thus setting the basis for the study of the role of P2X7R in neuroinflammation and neurodegenerative diseases.

Thus, with a few exceptions, P2X7R has proven to be a rather disappointing therapeutic target, in contrast to the striking abrogation of release of inflammatory mediators (chiefly IL-1β) seen in vitro and the encouraging results from animal studies. Should we conclude that P2X7R is not a suitable in vivo target for anti-inflammatory intervention, or are we missing something? The disappointing outcome of several clinical studies might be due to several reasons, among which a marginal contribution of P2X7R to the pathogenesis of the human diseases so far investigated should be seriously taken into consideration. In addition, the more we learn about P2X7R genetics, the more we realize that the *P2RX7* haplotype, not just single SNPs, will drastically affect responses to P2X7R-targeted drugs (Fuller et al., 2009). However, it might be that the very intrinsic properties of the antagonists used make it difficult to unveil P2X7R therapeutic potential. Many compounds tested in the clinic are non-competitive, negative allosteric modulators (e.g., CE-224535 or GSK1482160), which allow a much better tuning of receptor blockade and minimize unwanted side effects. However, at inflammatory sites, where the extracellular ATP concentration can be 4–5 orders of magnitude higher than in healthy tissues, negative allosteric modulators might not be in the best condition to inhibit P2X7R-mediated responses. This hypothesis finds support in the recently published structural data from the paper by Karasawa and Kawate (2016) investigating conformational changes occurring during ATP-mediated activation in an artificially truncated version of panda P2X7R. In this study the interaction of five structurally unrelated antagonists with P2X7R is reported. Quite surprisingly, all the tested antagonists bind at an allosteric site distinct from the ATP-binding pocket. Furthermore, this site narrows when ATP is bound and restricts accessibility to the allosteric drugs.

Disappointing clinical efficacy of small drug-like chemical compounds may pave the way to the development of biological drugs. Biosceptre International Limited has developed antibodies targeting a non-functional (nf) variant of P2X7R to treat cancer. A highly purified sheep IgG raised against human nfP2X7R was recently shown to reduce lesion size when applied as an ointment to basal cell carcinoma (Gilbert et al., 2017; ClinicalTrials.gov identifier NCT02587819). Ablynx nv has developed nanobodies (single-domain antigen-binding fragments from camelid Abs) that bind with high-affinity mouse or human P2X7R. Inoculation of the 13A7 nanobody in mice improved experimental glomerulonephritis and allergic contact dermatitis, and more importantly the human P2X7R-selective Dano1 nanobody was highly effective in preventing IL-1β release from endotoxin-treated human blood (Danquah et al., 2016). P2X7R-targeted biologics might thus be an appealing alternative route to antagonize P2X7R activity.

Conclusions

The realization that ATP is a basic constituent of the inflammatory and tumor microenvironment has greatly widened our understanding of the molecular mechanisms underlying the activation of the first steps of inflammation and has opened an entirely novel perspective for the design and development of anti-inflammatory and anti-cancer drugs. In the nucleotide receptor family, P2X7R has emerged as the most closely involved in inflammation and cancer and the most appealing potential drug target. These anticipations have not yet been entirely fulfilled, but we are confident that in the end these efforts will be successful, for the advancement of science and for the sake of patients.

AUTHOR CONTRIBUTIONS

F.D.V. supervised the overall organization and wrote the final version of the review. D.D.B. wrote the section dealing with P2X7R structure. A.L.G., A.C.S., and S.F. specifically wrote sections on P2X7R and inflammasome activation, P2X7R and cancer-associated inflammation, and P2X7R and cytotoxicity.

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