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P2X4 and P2X7 Receptors in Evolution, Physiology, Inflammation, and Autoimmunity

Airi Rump

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Airi Rump

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P2X4 ja P2X7 retseptorid evolutsiooni, füsioloogia, põletiku ja autoimmuunsuse kontekstis

AIRI RUMP

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List of publications

The list of author's publications, on the basis of which the thesis has been prepared:

I **Rump, A.**, Smolander, O.-P., Rüütel Boudinot, S., Kanellopoulos, J. M., Boudinot, P. (2020) Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates. Front. Immunol. 11: 113.

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III **Rump, A.**, Ratas, K., Lepasepp, T. K., Suurväli, J., Smolander, O.-P., Gross-Paju, K., Toomsoo, T., Kanellopoulos, J., Rüütel Boudinot, S. (2023) Sex-dependent expression levels of VAV1 and P2X7 in PBMC of Multiple Sclerosis patients. Scand J Immunol. 98(2): e13283.

*- Equal contribution.

Author's contribution to the publications

Contribution to the papers in this thesis are:

I Airi Rump conceived the project, designed experiments and approaches, performed primary data analysis, wrote and edited the manuscript.

II Airi Rump conceived the project, designed experiments, performed wet-lab experiments, performed primary data analysis, provided resources, and edited the manuscript.

III Airi Rump conceived the project, designed experiments, collected samples, performed primary data analysis, wrote, edited and approved the manuscript.

Introduction

P2X receptors are trimeric ATP-gated ion channels that are broadly expressed in various cells and tissues, mostly in immune cells and neurons (North, 2002). Seven members (P2X1R – P2X7R) belong to the P2X receptors family. They participate in many physiological and pathological functions like energy metabolism modulation, intercellular communication, stimulation of secretion, differentiation, and proliferation, neuromodulation and -transmission, inflammation, musculoskeletal disorders, pain-sensing, diabetes, and cancer (Chaudhary et al., 2018, Burnstock, Kennedy, 2011, Illes et al., 2021).

P2X4 receptors are very sensitive receptors and activate at nanomolar concentrations of ATP when most of the P2X receptor family members need higher concentrations. P2X4 receptors act as non-selective cation channels with the highest permeability of Ca^{2+} in the P2X receptor family (Egan, Khakh, 2004). These receptors are abundantly expressed in peripheral and central neurons, microglia, several glandular tissues, endothelial cells, and vascular and cardiac smooth muscle cells (Burnstock, Kennedy, 2011, Ralevic 2015). P2X4 receptors can be found in the plasma membrane and intracellular compartments (Qureshi et al., 2007).

In the P2X receptor family, P2X7 receptors demonstrate the lowest sensitivity to ATP (Sadovnick et al., 2017). But at inflammation sites and in cancer, the concentrations of extracellular ATP can increase to the level needed to activate these receptors (Di Virgilio et al., 2018a). After long activation, the P2X7 receptors can open pores that are large enough to allow the passage of 900 Da molecules (Khakh et al., 1999). P2X7 receptor is the most structurally and functionally distinct P2XR subtype, containing two cytoplasmic elements: Cysteine-rich anchor and cytoplasmic ballast domain, while the other P2X receptors lack the two last features. Palmitoylation of the Cysteine-rich anchor prevents receptor desensitization and allows to initiate apoptosis in the presence of a high concentration of extracellular ATP (McCarthy et al., 2019). P2X7 receptors are expressed on immune cells like macrophages/ microglia, B and T cells, natural killer cells, and mast cells (Di Virgilio et al., 2017). Thus, they have an important role in the control of innate and adaptive immune responses (Di Virgilio et al., 2018b). P2X7 receptors are also expressed in the cells of the central nervous system like oligodendrocytes, microglia, and Schwann cells, probably in neurons and astrocytes (Sluyter, Stokes, 2011) and have been implicated in psychiatric disorders and neurodegenerative diseases (Cheffer et al., 2018, Savio et al., 2018).

P2X4 and P2X7 receptors are both promising therapeutic targets in neuroinflammatory disorders. Therefore, their precise roles and signalling processes in physiology and pathophysiology still need better understanding.

The purpose of this thesis was to improve the knowledge about the biology of P2X4 and P2X7 receptors in evolutionary, physiological, inflammatory, and autoimmunological contexts. The thesis is based on three publications. The first investigates the evolutionary origin of the P2X7 receptor. The second involves the production and validation of monoclonal antibodies against the P2X4 receptor and characterization of the P2X4 receptor in human peripheral blood leukocytes and some murine cancer cell lines. The third explores P2X4 and P2X7 gene expression in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis.

Abbreviations

AA	Amino acid
Ab	Antibody
AD	Alzheimer's disease
ATP	Adenosine-5'-triphosphate
BDNF	Brain-derived neurotrophic factor
bp	Base pair
C-cys	Cysteine-rich
CNS	Central nervous system
DAMP	Damage-associated molecular pattern
EAE	Experimental autoimmune encephalomyelitis
EGFP	Enhanced green fluorescent protein
eATP	Extracellular ATP
FITC	Fluorescein isothiocyanate
GA	Glatiramer acetate
HC	Healthy control
IFNβ	Interferon-β
IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon regulatory factor
KO	Knock out
mAb	Monoclonal antibody
MS	Multiple sclerosis
NLRP3	Leucine-rich repeat pyrin domain containing 3
NT	Not treated
P2RX	P2X gene
P2XR	P2X receptor
PAM	Positive allosteric modulator
PB	Peripheral blood
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PNI	Peripheral nerve injury
qPCR	Quantitative polymerase chain reaction
RGS16	Regulator of G protein signalling 16
SNI	Spared nerve injury
SNP	Single-nucleotide polymorphism
TM	Transmembrane
TNF	Tumour necrosis factor
WT	Wild-type
ZCD	Zn-coordinating cysteine-based domain

1 Review of the literature

1.1 P2X receptors

Adenosine-5'-triphosphate (ATP) is mainly known as a central energy source for use and storage at the cellular level. It has a diverse role in energy metabolism, biosynthesis processes, and intercellular signal transmission. In 1972, Geoffrey Burnstock proposed new roles for nucleotides as neurotransmitters. ATP is released from healthy and damaged cells and acts as an extracellular signalling molecule (Burnstock, 2012).

Extracellular ATP (eATP) is quickly degraded into ADP, then AMP and finally adenosine by ectonucleotidases present at the plasma membrane of many cells. Under normal physiological conditions, the levels of eATP are relatively low. ATP is released from damaged cells. In chronic inflammation, both eATP and adenosine may be present at high concentrations for extended periods (Cauwels et al., 2014). One role of ATP is to resolve inflammation by attracting macrophages that engulf dead cells (Boada-Romero et al., 2020).

Ion channel receptors for ATP have been found even in primitive invertebrates (*Dictyostelium*, *Schistosoma*) and also green algae (Agboh et al., 2004, Fountain et al., 2007, Fountain et al., 2008) suggesting that ATP could be one of the earliest extracellular messengers (Burnstock, Verkhratsky, 2009). ATP signalling has also been described in plants (Demidchik et al., 2003, Kim et al., 2006, Clark, Roux, 2009).

ATP binds to two classes of purinergic cell surface receptors: G-protein-coupled metabotropic P2Y receptors and ionotropic P2X receptors (P2XR) (Abbracchio, Burnstock, 1994) (Figure 1).

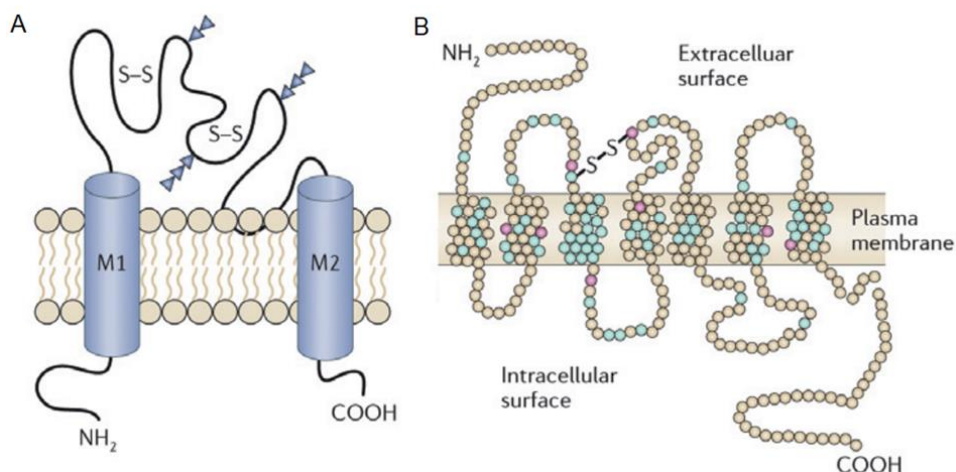

Figure 1. Molecular structures of purinergic receptors. A. The family of P2X receptors are ligand-gated ion channels. B. The family of P2Y receptors are G protein-coupled receptors. S–S: disulphide bond; M1 and M2: transmembrane domains; arrowheads: N-linked glycosylation sites; circles in B: AA residues (blue – conserved, red – functionally essential in other G-coupled receptors). From (Burnstock, 2012) with permission.

Mammalian P2X receptors are trimeric ion channels constructed as homomers or heteromers of seven subunit isoforms (P2X1R-P2X7R). All these subunits are 40-50% identical in amino acid (AA) sequence and display a common topology: intracellular C- and N-termini, two anti-parallel, hydrophobic transmembrane (TM) segments TM1 and TM2, and between them a large glycosylated and cysteine-rich extracellular domain (~280 AA) (North, 2002, Young, 2010). Structural data obtained from vertebrate and invertebrate P2X receptors demonstrate that they share also similar tertiary and quaternary architecture, confirming the hypothesis that all P2XRs are part of the same structural and evolutionary group (Illes et al., 2021).

Seven genes of the P2X receptor subunits contain 10–12 introns and are found on five chromosomes: P2RX1 and P2RX5 – Chr 17, P2RX2, P2RX4 and P2RX7 – Chr 12, P2RX3 – Chr 11, P2RX6 – Chr 22. The genes for P2X1 and PX5 receptors, and P2X4 and P2X7 receptors, are located adjacently suggesting relatively recent duplication (North, 2002, Rump et al., 2021).

The structure of exons and introns but also the major motifs of vertebrate P2X genes (P2RXs) are conserved well. The general shape of the individual subunits resembles a leaping dolphin, consisting of a body, head, tail, dorsal fin, and flippers (Figure 2C). The α-helical TM2 of each subunit participates directly in forming the channel pore while TM1 assists the pore formation. The TM domains correspond to the tail part of the dolphin. The large ectodomain (the dolphin body) contains β-sheet structures that form lateral access sites for ions. All ectodomains contain 10 conserved cysteine residues which interact with each other to form 5 disulfide bonds (Clyne, 2002).

The 3 ATP binding pockets are situated between two subunits and are built by the head and left flipper of one subunit and the dorsal fin of the neighbouring subunit (Mansoor et al., 2016) (Figure 2D). The phosphates of ATP bind to certain highly conserved residues (K70, K72, N296, R298, and K316 in zfP2X4R), while ATP surprisingly adopts a rare U-shaped conformation, allowing the adenine base to be deeply inserted into the binding pocket and recognized by polar (K70 and T189), non-polar (L217), and hydrophobic interactions (L191 and I232) (Figure 2D). Other agonists like 2-methylthio-ATP and CTP show similar binding forms and orientations with slight differences (Mansoor et al., 2016, Kasuya et al., 2017a). It is remarkable that the binding pocket entrance of the P2X7 receptor (<11Å) is much smaller than that of other P2X receptors, for example, the P2X3 receptor (17 Å) (McCarthy et al., 2019, Mansoor et al., 2016). This probably decreases the drug accessibility to the binding pocket and contributes to the much lower ATP potency of the P2X7R compared to other P2X receptors (Illes et al. 2021). The binding modes and orientations of P2X receptor antagonists like TNP-ATP or A-317491 are more varying but generally, the antagonists bind more deeply and adopt either Y-shape or an extended conformation (McCarthy et al., 2019, Mansoor et al., 2016). Their conformational flexibility when bound may explain why they do not lead to channel opening (Illes et al., 2021). P2X receptors can also be inhibited non-competitively by allosteric antagonists that prevent mechanical motions needed to channel gating (Coddou et al., 2011, Karasawa, Kawate, 2016, Wang et al., 2018).

The N-termini of all subunits are short (20–30 AA) and contain a consensus site (Thr-X-Lys/Arg) for protein kinase C (PKC) phosphorylation (Vial et al., 2006). The C-terminal has the most differences in AA sequence between subunits and it also varies considerably in length, from 28 AA in the P2X6R subunit to 242 AA in the P2X7R subunit. The C-termini of P2X receptors modulate the kinetics of carried currents and their permeation and desensitization properties (Khakh, 2001).

All P2XR subunits can form homomeric receptors (P2X6R only after glycosylation), whereas several functional heterotrimeric P2X receptors composed of two different subunits have been described – P2X1/P2X2R, P2X1/P2X4R, P2X1/P2X5R, P2X2/P2X3R, P2X2/P2X6R, P2X4/P2X6R, P2X4/P2X7R (North, 2002, Barrera et al., 2005, Haines et al., 1999, Lewis et al., 1995, King et al., 2000, Nicke et al., 2005, Brown et al., 2002, Guo et al., 2007).

P2X receptors mediate most of the functions of ATP (Khakh, North, 2006). After binding of 3 extracellular ATP molecules, these receptors change their conformational states, which leads to the opening of the TM pore (Browne et al., 2010, Jiang et al., 2013) (Figure 2A, 2B), allowing the flow of Na^{+}, K^{+} and Ca^{2+} across the membrane (Egan, Khakh, 2004). Prolonged exposure to ATP drives to the desensitized state – a temporary inactivation that aborts the ion flux, even though ATP is still bound to the receptor. The kinetics of desensitization differ between P2XR subtypes - P2X1 and P2X3 receptors desensitize quickly, whereas P2X2, P2X4, and P2X5 receptors desensitize slowly and P2X7 receptors present no apparent desensitization (North, 2002) or slow desensitization (Illes et al., 2021). After extended or repeated ATP activation, a major conformational rearrangement occurs in some P2XR subtypes and the pore becomes wider and permeable to large organic molecules of up to 900 Da like N-methyl-D-glucamine (NMDG). ATP dissociation returns the channel to the closed state and it is capable to be activated again. This process is known as resensitization (Khakh et al., 1999, Virginio et al., 1999). Besides the activation by ATP, P2X receptors activity is also regulated by allosteric modulators like small cations and lipophilic molecules (Jarvis et al., 2009).

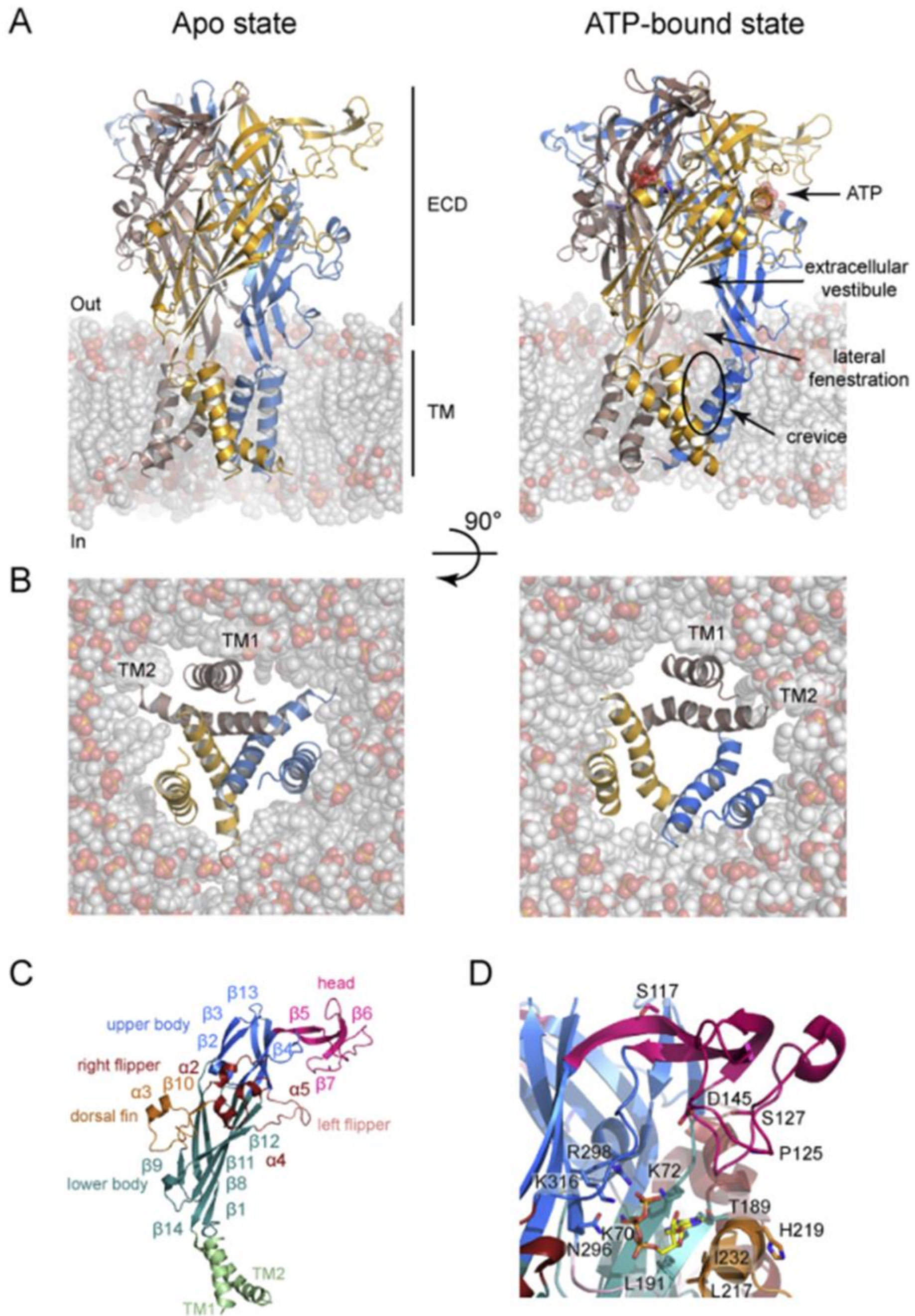

Figure 2. Molecular organization of P2XRs. A. Crystal structures of DzfP2X4 receptor in a closed state (left) and in an ATP-bound state (right) embedded in a lipid bilayer. Each subunit is shown in cartoon representation and different colour. ATP is in stick representation, the membrane is represented by spheres. ECD - extracellular domain, TM - transmembrane domain. B. The same structures from the extracellular side, forward the axis of the central channel. C. Ribbon representation of one subunit folding. Domains are named according to a homology comparison to a dolphin. D. Proximate view of the ATP-binding pocket (domains are coloured as in panel C). From (Habermacher et al., 2016) with permission.

In humans, P2X receptors are expressed all over the body and they participate in several physiological functions and pathological reactions like platelet aggregation, macrophage differentiation, proliferation, and activation, muscle contraction, synaptic transmission, chemotaxis, inflammation, chronic and acute pain conduction (Suurväli et al., 2017, Illes et al., 2021). The comparison of properties of different P2XR subtypes is presented in table 1.

Table 1. General properties of P2X receptors. Based on (Illes et al., 2021) with permission.

PROPERTIES	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7
Human chromosome location	17p13.3	12q24.33	11q12.1	12q24.32	17p13.3	22q11.21	12q24.31
Protein length (AA)	399	471	397	388	444	441	595
C tail length (AA)	41	113	56	29	82	87	240
Membrane expression	Good	Good	Good	Good	Poor	Usually no expression	Good
Desensitization (complete)	Fast (<1s)	Slow (>20s)	Fast (<1s)	Slow (>20s)	Slow (>20s)	-	Slow (>20s)
Activation-dependent	Yes	No	-	Yes	-	-	-
Major cellular expression	Smooth muscle	Neurons	Pain sensing neurons	Neurons, microglia	Skeletal muscle	Broad expression	Immune cells
Major role	Neuroeffector transmission	Taste, hearing	Pain, bladder reflexes, taste	Vascular remodeling, neuropathic pain	Inflammatory bone loss	-	Inflammation, neurodegenerative diseases
Model native cell type	Vas defrens, myocytes	SCG and myenteric plexus neurons	Small DRG neurons	Macrophages	Skeletal myocytes	-	Monocytes, macrophages, microglia
KO available	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ATP agonism (μM)	0,56 - 0,7	2 - 8	0,5 - 1	1 - 10	0,44 - 10	12	100(rat)

1.2 P2X1 receptor

The human P2X1 gene (ID: 5023, ENSG00000108405) is 2662 base pairs (bp) long, has 12 exons and encodes a protein of 399 AA (NCBI, Cunningham et al., 2022). It is located on chromosome 17p13.3 reverse strand (Cunningham et al., 2022). Orthologues of P2RX1 have been detected in 492 species. The orthologues are present in mammals, birds, reptiles, and fish, but not in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (NCBI).

The homomeric P2X1 receptor is a non-selective cation channel that desensitizes quickly (Egan, Khakh, 2004). Such fast desensitisation reduces its nanomolar potency for ATP (Rettinger, Schmalzing, 2004). P2X1Rs have higher fractional Ca^{2+} currents compared to other P2X receptors and very high Ca^{2+} permeability (Egan, Khakh, 2004). The only functional heteromeric receptor of P2X1 found in vivo is formed together with P2X5R. But also, heteromultimers with P2X2R and P2X4R have been detected (Kennedy, 2015).

P2X1 receptors are broadly expressed all over the body. It is the predominant P2XR family member in arteries, smooth muscles (including vas deferens), and the urinary bladder. P2X1Rs are very mobile in the plasma membrane and are probably present in the lipid rafts that are involved in receptor trafficking and signalling (Kennedy, 2015).

When ATP is excreted as an excitatory cotransmitter (together with noradrenaline or acetylcholine) from sympathetic or parasympathetic nerves, P2X1R mediates its actions (Kennedy, 2015). In arteries such purinergic cotransmission leads to vasoconstriction (Harhun et al., 2014) and in the urinary bladder to urinary voiding (Vial, Evans, 2000). Thus, P2X1 receptors play an important role in male reproductive function and they are also potential targets for oral non-hormonal contraceptives (White et al., 2013).

In platelets, P2X1Rs mediate Ca^{2+} influx and participate in the formation of thrombi (Hechler et al., 2003) as P2X1R activation evokes changes in platelet shape, amplifies platelet signalling, and causes aggregation of platelets (Hechler, Gachet, 2011, Mahaut-Smith et al., 2011). Low doses of collagen induce platelet activation through P2X1 receptor activation (Oury et al., 2001). ATP is released from these collagen-stimulated platelets and this further promotes platelet aggregation and degranulation in an autocrine manner. P2X1Rs also potentiate platelet aggregation induced by thrombin and thromboxane 2 (Erhardt et al., 2006).

As platelets promote the recruitment and activation of immune cells, P2X1Rs might also play a role in the inflammation, but these mechanisms are not described sufficiently. It is proposed that neutrophil and platelet P2X1 receptors might have a protective role in intestine inflammation (Wéra et al., 2020). Studies of their role in sepsis have given controversial results (Lecut et al., 2012, Maître et al., 2015). In inflammation, contrary to normal physiological conditions, lack of P2X1Rs might have a pro-thrombotic effect due to poor regulation of neutrophils (Oury, Wera, 2021).

The activation of P2X1Rs regulates neutrophils activation and stimulates their chemotaxis (Lecut et al., 2012, Lecut et al., 2009). A functional expression of P2X1Rs has been established in macrophages and mast cells, too (Vargas-Martínez et al., 2020, Wareham et al., 2009).

Several agonists and antagonists with differing potency and selectivity levels have been identified and created for P2X1 receptors. Agonist α,β-meATP has high selectivity for P2X1Rs and P2X3Rs among homomeric P2X receptors (Chen, Lin, 1997). Another agonist BzATP has the highest potency for P2X1Rs but it is also an agonist for P2X7Rs (Bianchi et al., 1999). The most potent antagonist of P2X1Rs is NF449 that is also selective over other P2 receptors (Rettinger et al., 2005). Low molecular weight compounds are more suitable candidates for drug development. Such antagonists of P2X1Rs with nanomolar potency are aurintricarboxylic acid (Obrecht et al., 2019), MRS2159 (Kim et al., 2001), PSB-2001 (Tian et al., 2020), and compound 1 (Jung et al., 2013). A small molecule positive allosteric modulator (PAM) MRS2219 has been identified to potentiate and antagonise P2X1Rs (Jacobson et al., 1998).

Tens of single-nucleotide polymorphisms (SNPs) of P2RX1 have been found in multiple types of cancer and some of them are considered „probably damaging“. It is unknown how or if they contribute to the cancer progression. A notable decrease of P2X1 mRNA expression has been identified in multiple cases, but it is still unidentified whether it is the effect or cause of the cancer (Illes et al., 2021).

Following and in the next chapters are discussed the most common mouse models.

P2RX1 knock-out (KO) $P2rx1^{tm1Chn}$ (mutation in exon 1) mice display the smooth muscle (slightly increased systolic blood pressure, 90% reduced male fertility, azoospermia) and the antithrombotic phenotype (Mulryan et al., 2000, Darbousset et al., 2014).

To study P2X1 receptor expression, a knock-in mouse model $P2RX1^{em1(DPPVATTeYFP)}$ was created. In this model, the enhanced yellow fluorescent protein (eYFP) was added to the P2X1R C-terminus to produce a fused P2X1-eYFP (Mahaut-Smith et al., 2019).

1.3 P2X2 receptor

The human P2X2 gene (ID: 22953, ENSG00000187848) is 1873 bp long, has 11 exons and encodes a protein of 471 AA. It is located on chromosome 12p24.33 forward strand (NCBI, Cunningham et al., 2022). Orthologues of P2RX2 have been detected in 261 species and the gene is highly conserved between humans and rats (NCBI).

During their synthesis, P2X2R subunits assemble into homotrimers (Aschrafi et al., 2004). But in several cell types, stable and functional heterotrimers of P2X2/1R, P2X2/3R, P2X2/5R, and P2X2/6R have been found (Hausmann et al., 2015).

Homotrimeric P2X2 receptors are fast activated by eATP. Among all P2XR subtypes, P2X2R channels show the most constant steady-state current with no or slow desensitization in the course of prolonged ATP exposure (North, 2002).

P2X2Rs are widely expressed in the central nervous system (CNS) but can also be found in the rest of the body (Cockayne et al., 2005). Their expression is especially high in the hippocampal formation (Uhlén et al., 2015). In the process of neuronal differentiation, P2X2Rs participate in neurogenesis (Yuahasi et al., 2012).

P2X2 receptors are involved in the stimulation of the sinus nerve afferent fibres, which is crucial to the carotid body hypoxia sensitivity (Rong et al., 2003). They also participate in the epithelial-neuronal signalling mode in taste perception, when ATP released by a taste stimuli activates co-expressed P2X2R and P2X3R (both homo- and heterotrimeric) in the tongue taste buds (Cockaine et al., 2005).

In the auditory system, ATP that is released after sustained elevated noise levels activates P2X2 receptors on epithelial cells in the endolymph compartment, leading to a reduction of hearing sensitivity (Mittal et al., 2016). P2X2R positive modulation in the inner ear might be protective against injury generated by loud noise and may relieve balance disorders (Thorne et al., 2004, Takimoto et al., 2018).

P2X2Rs play a role in the release of vasopressin from the hypothalamus (Custer et al., 2012) and luteinising hormone from the pituitary gland (Zemkova et al., 2006). In the hypothalamus, P2X2 receptors are involved in food intake regulation (Collden et al., 2010).

Mice experiments demonstrate that P2X2 receptors mediate susceptibility to chronic stress by regulating neuroplasticity. Chronic stress state promotes the upregulation of P2X2R expression that may transmit the anti-depressant impact of ATP (Kuang et al., 2022, Cao et al., 2013). In mice, autism-like behaviour may be regulated by ATP released from astrocytes and acting through P2X2 receptors (Wang et al., 2021).

Steroid hormones can act as PAMs for P2X2Rs. Dehydroepiandrosterone is a positive modulator for the homomeric P2X2R channel but also the heteromeric P2X2/3R channel (De Roo et al., 2003, De Roo et al., 2010). Progesterone selectively potentiates only the homomeric P2X2 receptors (De Roo et al., 2010). Also, several synthetic testosterone derivates (testosterone valerate, testosterone butyrate), but not testosterone itself, act as PAMs of P2X2Rs (Sivcev et al., 2019).

Two single disease-causing P2RX2 mutations (DFNA41 (V60L) and G353R-hP2X2R) have been identified that cause autosomal dominant deafness. Both of these mutations affect the ATP sensitivity of the P2X2R channel (Yan et al., 2013, George et al., 2019).

P2rx2^{tm1Ckn} (Exons 2…11 deleted and replaced by a neomycin resistance cassette in embryonic stem cells) mice have sufficient P2X2 receptor expression in neuronal and non-neuronal tissues and present a mild phenotype with defective neurotransmission, for example in pelvic afferent nerves, sensory ganglia, and carotid sinus. They show a considerable exacerbation of permanent threshold shift after being exposed to loud

noise. P2rx2^{tm1Ckn} mice have bladder hyporeflexia, impaired peristalsis and changed hippocampal synaptic plasticity (Cockayne et al., 2005).

KO mice P2rx$^{em1(MPC)H}$ have lower circulating glucose levels and reduced locomotor activity and P2rx$^{tm1b(EUCOMM)Hmgu}$ smaller testes and decreased exploration in the new environment compared to wild-type (WT) mice (International Mouse Phenotyping Consortium).

A knock-in mouse model P2X2^{Cre} enables to study the P2X2R expression. To reveal the expression, these mice need to be crossed with cre-sensitive reporter mice (Kim et al., 2020).

Another tracing model P2rx2$^{em1(2A\text{-}tdTomato\text{-}WPRE\text{-}polyA)Smoc}$ has a 2A-tdTomato-WPRE-polyA expression cassette knocked into the stop codon site of the P2X2 gene (Shanghai Model Organisms Center, Inc.).

1.4 P2X3 receptor

The human P2X3 gene (ID: 5024, ENSG00000109991) is 3792 bp long, has 16 exons and encodes a protein of 397 AA. It is located on chromosome 11p12.1 forward strand (NCBI, Cunningham et al., 2022). Orthologues of P2RX3 have been detected in 427 species (NCBI).

The crystal structure of the human P2X3 receptor has been determined in a resting, an agonist-bound/open-pore, a desensitized/agonist-bound/closed-pore, and two antagonist-bound states (Mansoor et al., 2016).

Functional heterotrimeric P2X2/P2X3R channels may consist of two P2X2R and one P2X3R subunits (in the sensory neurons of dorsal root ganglia) or vice versa (in nodose ganglia) (Jiang et al., 2003, Kowalski et al., 2015). Homotrimeric P2X3R channels desensitize rapidly but heterotrimeric P2X2/P2X3R channels slowly after ATP exposure (Chen et al., 1995, Lewis et al., 1995).

In sensory neurons, P2X3Rs and P2X2/3Rs may be presynaptic (enhancing glutamatergic neurotransmission from the central terminals) or postsynaptic (receiving the signals in the cell bodies) (Khakh, North, 2012).

Homotrimeric P2X3 and heterotrimeric P2X2/3 receptors of gustatory sensory neurons mediate the taste signals to the gustatory cortex (Finger et al., 2005, Vandenbeuch et al., 2015).

In pathological conditions, these receptors may be both – up- or down-regulated, depending on particular context and influences, including interactions with other P2X receptors, biochemistry, and neuroanatomical structure (Bernier et al., 2018).

In carotid body neurons, P2X3 receptors participate in regulating the chemoreflex sympatho-excitatory response that controls blood pressure. The rise of P2X3 receptor expression probably contributes to high blood pressure and hyperreflexia in the pathophysiology of hypertension (Pijacka et al., 2016).

When ATP is released after tissue damage, P2X3Rs and P2X2/3Rs at nerve endings are activated and function as acute pain generators (Cook et al., 1997, Cook, McCleskey, 2002). In inflamed tissues, where ATP concentrations are high, P2X3Rs and P2X2/3Rs have an essential role in mediating chronic pain (Jarvis et al., 2002, Wu et al., 2004) (Figure 3).

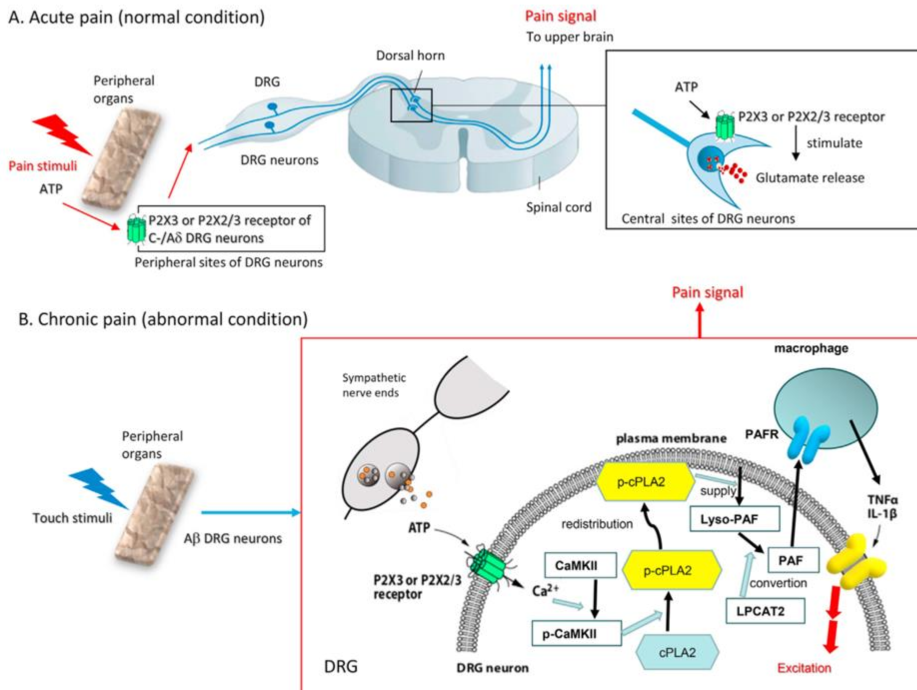

Figure 3. P2X3 and P2X2/3 receptors in dorsal root ganglia neurons in pain. A. When ATP is released from damaged tissue, P2X3 and P2X2/3 receptors in peripheral points of DRG neurons are activated and operate as pain generators. These receptors in the central terminals transmit the excitatory synaptic signals by raising the glutamate release from nerve endings. As a result, pain signalling is induced. B. After peripheral nerve injury (PNI), sympathetic axons branch abnormally around DRG neurons. ATP released together with noradrenaline activates P2X3 and P2X2/3 receptors in the DRG neurons bodies increasing internal Ca^{2+} concentrations that lead to the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation, which phosphorylates Ca^{2+}-dependent cytosolic phospholipase A2 (cPLA2). Phospho-cPLA2 cuts off lyso - platelet activating factor (PAF) from the cell membrane, that is then converted into PAF by lyso-PAF-acetyltransferase (LPCAT2). PAF triggers its receptors on macrophages, evoking the production and release of IL-1β and tumour necrosis factor (TNF) α that enhances DRG neurons' excitability resulting in tactile allodynia. From (Inoue, 2022) with permission.

In vivo studies using selective antagonists show that blocking these receptors leads to reduced nociceptive sensitivity with symptoms like enhanced bladder volume threshold, decreased bladder reflexes, and diminished airway sensitivity in cough models (Ford, Undem, 2013, North, Jarvis, 2013).

Gefapixant (AF-219, MK-7264) is a reversible and potent noncompetitive P2X3 receptor antagonist that is about 3 times less potent for P2X2/3 receptors (Richards et al., 2019). Gefapixant and its analogues inhibit P2X3R-dependent signals in peripheral tissues innervating afferent neurons in several disease models (Ford, Undem 2013, Richards et al., 2019). The agent can be administered orally and it is peripherally restricted, making it a potential therapeutic for humans (Ford, Undem, 2013). The side effect called dysgeusia appears to be dose-dependent, suggesting potential for the optimal dosage that has a therapeutic effect with minimal side effects (Smith et al., 2020).

There are also recently discovered next-generation P2X3R antagonists that are more selective and have a much lower affinity for heteromeric P2X2/3Rs – BLU-5937 and imidazopyridine. As dysgeusia is probably mediated by heterotrimeric receptors, these antagonists are expected to have fewer side effects (Garceau, Chauret, 2019). BLU-5937 is currently in a phase 3 clinical trial for refractory chronic cough (National Library of Medicine).

P2rx3^{tm1Ckn} (mutation in exon 1) and P2rx3^{tm1Jwo} have decreased sensitivity to inflammatory pain but not to mechanical nor acute noxious thermal (hot plate) stimuli. Thermal hyperresponsiveness has been detected in these mice (Cockaine et al., 2005, Souslova et al., 2000).

P2rx3-/- mice (as P2rx2^{tm1Ckn} mice) show bladder hyporeflexia, impaired peristalsis and changed hippocampal synaptic plasticity (Cockaine et al., 2000).

P2rx2/P2rx3Dbl-/- mice show gustatory deficiencies and have developmental abnormalities and very high lethality (>90%) through weaning due to pneumonia, probably due to reduced ventilatory responses to hypoxia. Surviving mice do not differ from WT controls (Eddy et al., 2009).

P2X2/P2X3$^{Dbl-/-}$ mice neurons have minimal or no response to ATP (Cockaine et al., 2005).

1.5 P2X4 receptor

The human P2X4 gene (ID: 5025, ENSG00000135124) is 1760 bp long, has 14 exons and encodes a protein of 388 AA (NCBI, Cunningham et al., 2022). It is located on chromosome 12p24.32 forward strand close to the P2X7 gene (Cunningham et al., 2022). Orthologues of P2RX4 have been detected in 393 species (NCBI). P2RX4 is one of the most conserved P2RX among vertebrates (North, 2002).

Functional P2X4 receptor is found in homotrimeric and heterotrimeric (with P2X1R, P2X2R, P2X5R, and P2X6R) form. Human and mouse P2RX4 have alternatively spliced variants but the shorter forms are not able to develop into functional channels (Kaczmarek-Hajek et al., 2012, Syed, Kennedy, 2012, Antonio et al., 2014).

The P2X4 receptor crystal structure was determined in zebrafish (Kawate et al., 2009, Hattori, Gouaux, 2012) and verified in rat (Igawa et al., 2015).

Homotrimeric P2X4 receptors activate quickly and desensitize slowly depending on ATP concentrations. The deactivation of these receptors is fast and independent of ATP concentrations. They are very sensitive receptors and activate at nanomolar concentrations of ATP, when most of the P2XR family members need higher concentrations. P2X4Rs act as non-selective cation channels with the highest permeability of Ca^{2+} in the P2XR family (Egan, Khakh, 2004).

The homotrimeric as well as heterotrimeric P2X4Rs, but not other P2XR family members, respond to ivermectin which is a PAM for these receptors (Priel, Silberberg, 2004). Ivermectin also potentiates nicotinic α7 and GABA(A) receptors (Krause et al., 1998, Krušek, Zemková, 1994). Besides ivermectin, other avermectin analogues (avermectin, doramectin, emamectin, abamectin, moxidectin, and selamectin) also have PAM activity for P2X4R (Silberberg et al., 2007, Asatryan et al., 2014, Huynh et al., 2017).

The P2X4R antagonist NC 2600 targeting glial cells entered clinical trials for chronic pain (Nippon Chemiphar). There are several other compounds discovered to antagonize P2X4Rs: selective allosteric antagonists benzodiazepine derivative 5-BDBD (Coddou et al., 2019) and NP-1815-PX (Matsumura et al., 2016), N-(benzyloxycarbonyl)phenoxazines PSB-12054 and PSB-12062 (Hernandez-Olmos et al., 2012), urea derivative BX430

(Ase et al., 2015), highly selective sulfonamide BAY-1797 (Werner et al., 2019), and selective and brain-penetrant PSB-15417 (Teixeira et al., 2019). P2X4Rs are inhibited also by multiple clinically used antidepressants like fluoxetine, fluvoxamine, paroxetine, nortriptyline, clomipramine, and desipramine (Nagata et al., 2009) and their analgesic effects in relieving neuropathic pain might be at least partly mediated by P2X4Rs (Nagata et al., 2009, Zarei et al., 2014, Yamashita et al., 2016).

P2X4Rs can be found in the plasma membrane and intracellular compartments as vacuoles, vesicles, lysosomes, and lamellar bodies (Qureshi et al., 2007). When in the membrane, they can rapidly internalize into lysosomes and early endosomes. The process might be constitutive or induced by an agonist and is dynamin- and clathrin-dependent, involving receptor c-terminus interactions with adaptor protein 2 (AP2). Mutations in the AP2 Tyr binding pocket or receptor endocytic motif cause the accumulation of functional P2X4R in the plasma membrane. Native P2X4 receptors reside mainly in lysosomes. The internalized receptors move out of the intracellular compartments and to the plasma membrane when the pH is increased to 7,4 and they are activated by the intracellular ATP (Bobanovic et al., 2002, Kaczmarek-Hajek et al., 2012, Xu et al., 2014) (Figure 4).

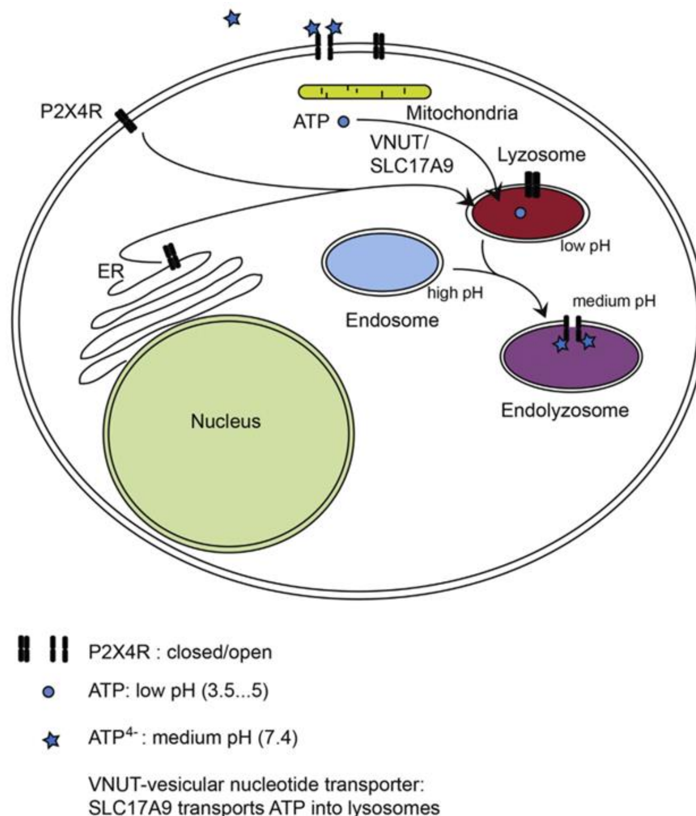

Figure 4. The intracellular distribution of P2X4R. P2X4 receptors are mostly residing in lysosomes and are engaged in the lysosome - late endosome fusion. Arrows – P2X4R transfer between compartments. From (Suurväli et al., 2017) with permission.

These receptors are abundantly expressed in peripheral and central neurons, microglia, several glandular tissues, endothelial cells, and vascular and cardiac smooth muscle cells (Burnstock, Kennedy, 2011, Ralevic 2015).

P2X4 receptors are primarily associated with neuropathic and inflammatory pain (Ransohoff, Perry, 2009, Ulmann et al., 2010). They evoke the secretion of two pain inducers – prostaglandin E2 and brain-derived neurotrophic factor (BDNF). Both of the pain pathways are associated with raised P2X4R expression on the cell surface (Baroja-Mazo et al., 2013, Khakh, North, 2012). P2RX4-deficient mice do not have any inflammatory prostaglandin E2 in their tissue exudates (Ulmann et al., 2010).

Several studies have demonstrated that P2X4R participates in the mediation of chronic neuropathic pain in allodynia resulting from processing pathway changes in the spinal cord (Inoue, 2019) (Figure 5). After nerve injury, P2RX4 KO mice do not display pain behaviour (Ulmann et al., 2008). In WT mice, P2X4R antagonist NP-1815-PX inhibits chronic pain caused by mechanical damage or herpesvirus infection (Matsumura et al., 2016).

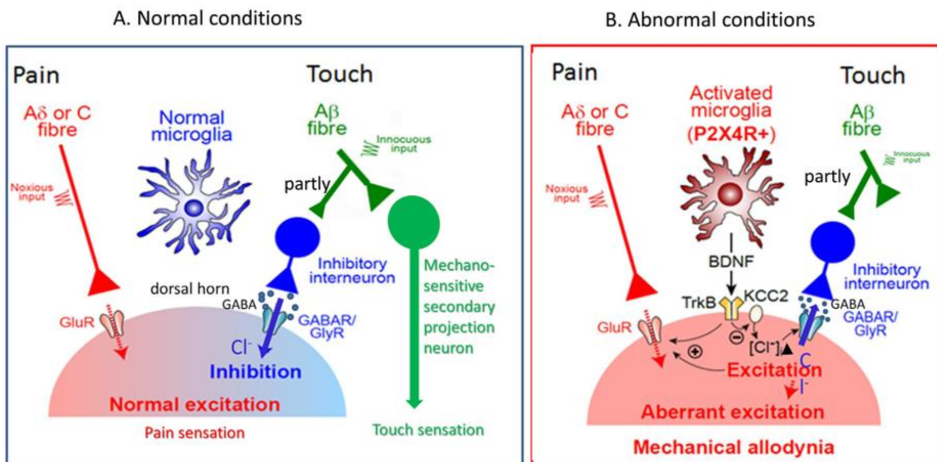

Figure 5. Pain signalling. A. In normal conditions, the signal is induced in C-fibres or Aδ-fibres of dorsal root ganglion neurons by painful stimuli and transmitted to dorsal horn secondary sensory neurons and the cortex. Touch stimuli signal is evoked in Aβ-fibres and a part of it inhibits secondary neurons by interneurons release of g-aminobutyric acid (GABA) and the pain signal is not induced. B. After nerve injury, P2X4 receptors are overexpressed in dorsal horn microglia. Dorsal horn neurons release ATP, that stimulates microglial P2X4Rs, which in turn, release the BDNF. This causes tyrosine kinase B (TrkB) of secondary sensory neurons to down-regulate the potassium-chloride transporter (KCC2). As a result, the intracellular Cl^- level is increased. In this case, when GABA is released from interneurons, it opens Cl^- channels on secondary neurons and Cl^- starts to flow out, resulting in signals that arrive at the cortex. Thus, harmless touch stimuli are changed into pain sensations called allodynia. From (Inoue, 2019) with permission.

Still, the pain studies have mostly used male rodents. Recent findings display that microglia's role in pain mediation is sexually divergent. In females, microglial P2X4 receptors are not upregulated and pain hypersensitivity is mediated by adaptive immune cells (Figure 6, Figure 7). In the deficiency of adaptive immune cells, the pain-mediating system of females may be switched to the male system (Mapplebeck et al., 2016).

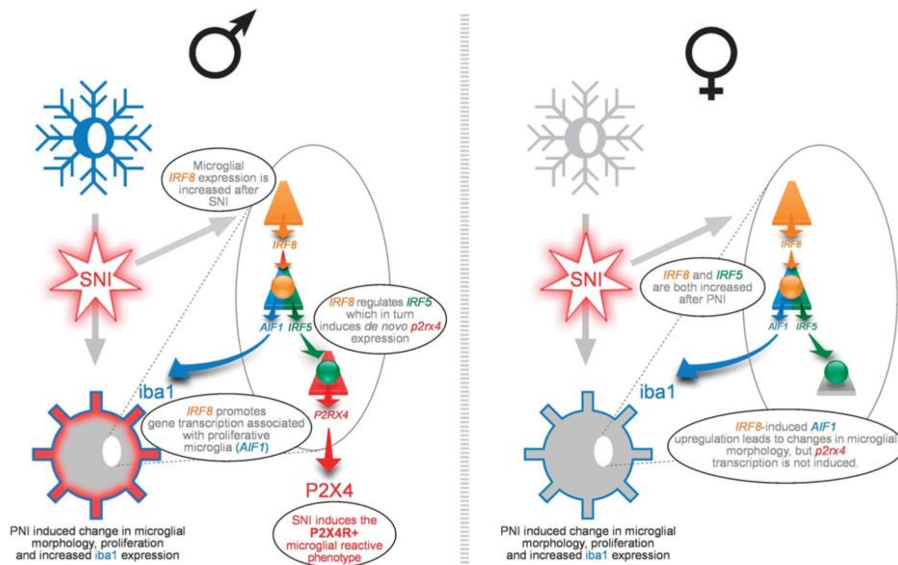

Figure 6. Sex-dependent differences in transcriptional P2RX4 induction in microglia. In male mice, P2X4 gene expression is controlled by interferon regulatory factors (IRFs). After spared nerve injury (SNI), expression of IRF8 is increased and evokes gene expression changes. Allograft inflammatory factor 1 (AIF1) and IRF5 expressions are raised. AIF1 codes a protein called ionized calcium-binding adapter molecule 1 (iba1) and regulates microglia morphology and proliferation. IRF5 binds to the promoter of P2RX4 and increases its transcription leading to overexpression of P2X4 receptors in microglia. In female mice, upregulation of IRF8 after SNI leads to microglia activation through iba1. Also, IRF5 expression is increased, but it does not induce the transcription of P2RX4. Therefore, the P2RX4 induction is the point of sexual divergence in the pathways activated after nerve injury. Other morphological and proliferative changes related to the spinal glial response after PNI are similar in males and females. From (Mapplebeck et al., 2016) with permission.

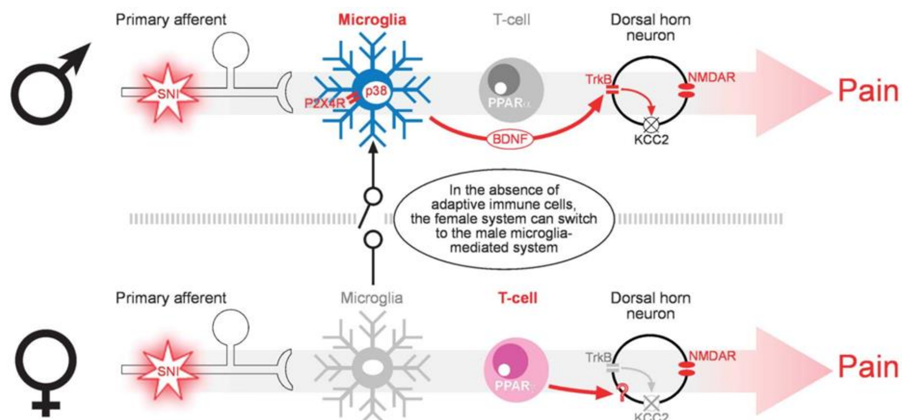

Figure 7. Male and female neuroimmune pathways mediating pain hypersensitivity induced by SNI. In male mice, nerve injury causes microglial P2X4R overexpression that stimulates BDNF release. BDNF binds and activates tyrosine kinase B (TrkB), which induces potassium-chloride transporter (KCC2) downregulation and following spinal nociceptive circuitry disinhibition. In female mice, neuroimmune interactions involving T cells mediate pain hypersensitivity instead of microglial BDNF after nerve injury. The specifics of these interactions are not determined yet. From (Mapplebeck et al., 2016) with permission.

In the course of inflammation, ATP released from damaged or stressed cells operates as a damage-associated molecular pattern (DAMP) signal via P2X4Rs, P2X7Rs, P2Y1Rs, P2Y2Rs, and P2Y6Rs to evoke proinflammatory responses in neutrophils and macrophages (Di Virgilio et al., 2020). In chronic inflammation, both eATP and adenosine might have raised levels for extended periods of time (Cekic, Linden, 2016). In macrophages, P2X4R regulates ATP-induced CXCL5 expression and secretion, which promotes the recruitment of neutrophils to the inflammation site (Layhadi et al., 2018). In T lymphocytes, P2X4 receptors mobilize rapidly to the immune synapse after T cell receptor stimulation and mediate their activation and migration (Woehrle et al., 2010, Ledderose et al., 2018).

Post nerve damage, overexpression of P2X4 receptors in Schwann cells benefits the sensory and motor functions recovery and remyelination through the secretion of the BDNF. Also, brain ischemia, spinal cord injury, and trauma up-regulate P2X4R expression in microglia, which probably plays a role in resolving the inflammation (Montilla et al., 2020).

P2X4Rs are involved in neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease, and multiple sclerosis (MS), where they participate in microglia activation in neuroinflammation processes. Activated P2X4Rs also encourage Ca^{2+} signalling, electrical activity, and secretion of neuro-hormones in neuroendocrine cells (Bjelobaba et al., 2015).

Expression of P2X4 receptors is upregulated in the optic nerves of MS patients and activated microglia of MS animal model experimental autoimmune encephalomyelitis (EAE) rats, where they participate in the repair responses and remyelination (Domercq, Matute, 2019, Montilla et al., 2020). P2X4R expression is increased at the peak of EAE and stays high during the recovery period. In the course of EAE, P2X4R inhibition leads to raised expression of pro-inflammatory genes, pro-inflammatory microglia polarization, and low oligodendrocytes remyelination. On the other hand, positive modulation of P2X4R by ivermectin (administered daily post EAE onset) considerably improves axon conduction latencies in the corticospinal tract and motor deficits of model animals. In vitro studies of microglia show that ivermectin decreases the expression of pro-inflammatory genes, increases the expression of anti-inflammatory genes, potentiates myelin degradation, and stimulates remyelination (Zabala et al., 2018).

In rheumatoid arthritis, P2X4R elevates inflammation in joints. Antisense RNA targeting P2X4 mRNA suppresses pro-inflammatory cytokines production (Li et al., 2014) and P2X4R inhibition reduces joint inflammation (Sakaki et al., 2013). Therefore, blocking P2X4R is a potential strategy in rheumatoid arthritis treatment (Li et al., 2014).

The P2X4R also plays a role in cardiac function by contributing to the control of large vessel tone through smooth muscle relaxation of arteries and endothelial-dependent nitrogen oxide release (Braganca, Correia-de-Sa, 2020).

After partial liver removal in the rat, the high level of eATP contributes to liver regeneration through P2X4 receptors. In P2RX4 KO mice, the regeneration is delayed, hepatocytes are prone to necrosis, and bile flow is disturbed (Gonzales et al., 2010, Besnard et al., 2016).

P2X4Rs are involved in lung surfactant release (Miklavc et al., 2013). Their expression is raised in asthmatic patients and also in the asthmatic disease mouse model (Zech et al., 2016). In the mouse model, antagonizing P2X4Rs relieves many of the asthmatic symptoms in the airways like inflammation, mucus production, eosinophilia, and cellular infiltration (Zech et al., 2016, Chen et al., 2016). Thus, P2X4R in the lungs is a promising therapeutic target for asthma and other airway disease treatment.

The role of the P2X4 receptor in protecting the host cells in infections has not been well studied. Still, bacteria *C. Trachomatis* growth in HeLa cells is inhibited through stimulation of P2X4Rs (Pettengill et al., 2012), P2X4Rs promote leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome activation and production of reactive oxygen species in gingival epithelial cells (Hung et al., 2013). P2X4R and P2X7R activation has a protective effect against inflammation and sepsis during E. coli infection (Greve et al., 2017, Csoka et al., 2018).

P2X4 receptors regulate alcohol-induced reactions in microglia. Ethanol induces the expression of P2X4 in both – mRNA and protein levels (Gofman et al., 2014) but also allosterically restricts the functions of already expressed P2X4Rs (Asatryan et al., 2010). Ivermectin antagonizes the effect ethanol causes on P2X4 receptors in vivo and in vitro and considerably decreases ethanol preference and intake in mouse experiments of alcohol self-administration (Wyatt et al., 2014, Asatryan et al., 2010, Kosten, 2011). Therefore, P2X4R is a potential therapeutic target for the treatment of alcohol use disorders (Asatryan et al., 2011).

The non-synonymous SNP Tyr315Cys in the human P2X4 gene has been identified to change the receptor function causing raised blood pressure (Stokes et al., 2011, Braganca, Correia-de-Sa, 2020) and increased predisposition for osteoporosis (Wesselius et al., 2013). P2RX4 variants may also contribute to raised risks for developing MS, HIV-associated sensory neuropathy, and defective immune responses (Kanellopoulos et al., 2021).

P2rx4^{tm1Ando} mice show abnormal common carotid artery morphology and blood vessel physiology, resulting in high blood pressure (Yamamoto et al., 2006).

P2X4R^{tm1Rass} mice display high bone mass phenotype with stronger bones and improved bone microstructure (Ellegaard et al., 2021). They also show perceptual and socio-communicative deficits and changed hippocampal synaptic plasticity (Sim et al., 2006). These mice carry a passenger mutation P2X7L451P and their CD4 + and CD8 + T cells express higher levels of P2X7R compared to B6-WT mice (Er-Lukowiak et al., 2020).

Cardiac-specific P2X4R KO mice P2rx4$^{tm1.1Ngc}$ show more serious heart failure (Yang et al., 2014).

P2rx4^{tm1Dgen} (mutation in exon 2) mice display abnormal macrophage physiology (Brône et al., 2007).

P2rx4$^{tm1b(EUCOMM)Wtsi}$ mice show decreased mean corpuscular volume and abnormal clavicle morphology (International Mouse Phenotyping Consortium).

Transgenic mouse models that overexpress human P2X4Rs show better contraction performance and raised contractility of cardiomyocytes in the intact heart model (Hu et al., 2001) and remarkably delayed progression of heart failure and extended life expectancy in the cardiomyopathy model (Yang et al., 2004).

Transgenic hP2X4R overexpressing mice display elevated myocyte contractility (Shen et al., 2006).

There are several mouse models available that express fluorescent reporter proteins. The transgenic Tg(P2rx4-tdTomato)1Khakh mice express soluble tdTomato (Xu et al., 2016).

Conditional P2X4R-mCherry expressing mouse model P2rx4mCherryIN (Cre activity dependent genetic swapping of the P2RX4 internalization motif by the fluorescent mCherry) reveals learning and memory deficits and anxiolytic effects (Bertin et al., 2021).

Outside mammals, P2X4 receptors are mostly related to nervous and immune system functions. In chicken, P2X4R regulates chondrogenesis (Fodor et al., 2009) and in a teleost fish *Paralichthys olivaceus*, P2X4R participates in the innate immune response (Li et al., 2015).

1.6 P2X5 receptor

The human P2X5 gene (ID: 5026, ENSG00000083454) is 2060 bp long, has 12 exons and encodes a protein of 444 AA (NCBI, Cunningham et al., 2022). It is located on chromosome 17p13.3 reverse strand (Cunningham et al., 2022). Orthologues of P2RX5 have been detected in 486 species (NCBI).

The human P2RX5 is usually missing the typical P2RX exon 10 coding TM2 and codes only 422 AA long protein with a misfolded subunit. The full-length gene (coding 444 AA long protein) is present in about 10% of humans (detected only in black Americans so far). Rat and mouse P2X5Rs are formed fully (455 AA) but still respond weakly to ATP (North, 2002, King, 2022).

Co-immunoprecipitation studies have shown that rat P2X5R subunits might co-assemble with P2X1R, P2X2R, P2X3R, P2X4R, and P2X6R subunits (Torres et al., 1999). For heterotrimeric P2X1/5 and P2X2/5 receptors, it is determined that their pharmacological profile differs from homomeric P2X1 and P2X2 receptors (North, 2002, Illes et al., 2021).

P2X5 receptor functions as a non-selective, slowly desensitizing cation channel (PCa/PNa = 1,5) when stimulated by ATP. Unlike other P2XR family members, P2X5Rs are permeable to chloride ions as well (PCl/PNa = 0,5). Otherwise, the operational profile of the full-length human P2X5R is similar to the profile of rat P2X2R (Bo et al., 2003a).

P2X5 receptors have a supporting function in the inflammatory response, including regulating pro-inflammatory cytokines (Interleukins (ILs) 1β, 6, 17a and TNF-sf11) expression (Kim et al., 2017, Kim et al., 2018). mRNA of the typical human isoform (hP2X5A) has been found plentifully in the B cells, T cells, and NK cells of peripheral blood, lymphoid tissues, and normal and malignant progenitor cells. It is highly described in lymphoid tissues (thymus, tonsils, spleen, and bone marrow) (de Rijke et al., 2005, Overes et al., 2008).

Data about the P2X5 receptor involvement in cancer are controversial. P2X5Rs are densely expressed on human squamous and basal cell carcinomas and their expression is negatively correlated with the tumour aggressiveness (Greig et al., 2003). Overexpression of the P2X5 receptor is detected in several hematologic tumours (Norde et al., 2009). In colon cancer, it has an unfavourable prognostic value (Gao et al., 2018).

P2rx5-/- mice osteoclasts have deficits in maturation and inflammasome activation under inflammatory conditions (Kim et al., 2017).

P2rx5^{tm1Lex} (mutation in exon 1) mice have abnormal immune cell numbers (increased NK cell number, decreased CD8+ and αβ T cell numbers), problems with learning, and increased exploratory behaviour (European Mouse Mutant Archive).

KO mice P2rx7$^{tm1b(EUCOMM)Hmgu}$ show abnormal gait, reduced exploration in new environments, preweaning lethality, and incomplete penetrance (International Mouse Phenotyping Consortium).

1.7 P2X6 receptor

The human P2X6 gene (ID: 9127, ENSG00000099957) is 2727 bp long, has 15 exons and encodes a protein of 441 AA. It is located on chromosome 22p11.21 forward strand (NCBI, Cunningham et al., 2022). Orthologues of P2RX6 have been detected in 274 species (NCBI).

This P2XR subunit is missing 9 AA that are part of the left flipper motive and thus essential for receptor activation (Wang et al., 2017).

P2X6R is a non-selective slowly desensitizing cationic channel that needs to be fully glycosylated to act as a homotrimer (Jones et al., 2004). It forms heterotrimers with P2X2R and P2X4R subunits (Royle et al., 2002, Torres et al., 1999).

P2X6R is the least investigated receptor in the family. Only two research groups have been successful in characterizing the functional rat P2X6Rs (Collo et al., 1996, Jones et al., 2004).

As the P2X6R subunit glycosylation might be more effective in native cells in contrast to expression systems, there might be more functional receptors in vivo. P2X6Rs have been found in the human thymocytes, atrium, midbrain, kidney, and urinary bladder (Jones et al., 2004).

P2X6 receptor overexpression is associated with the poor prognosis and progression of renal cancer (Gong et al., 2019). The P2X6 receptor has also been proposed to play a role in breast cancer (Chadet et al., 2014). On the other hand, cell-line experiments show that in human bladder cancer, high P2X6R expression might be an indicator of a good prognosis (Dietrich et al., 2022).

In mouse hippocampal neurons, non-glycosylated P2X6R was detected in the nucleus where it cooperated with splicing factor 3A1 to decrease the mRNA splicing (Diaz-Hernández et al. 2015).

P2rx6^{tm1Dgen} mice show a pain phenotype with an increased thermal nociceptive threshold and decreased susceptibility to pharmacologically induced seizures (de Baaij et al., 2016).

1.8 P2X7 receptor

The human P2X7 gene (ID: 5027, ENSG00000089041) is 5113 bp long, has 13 exons and encodes a protein of 595 AA. It is located on chromosome 12p24.31 forward strand close to the hP2X4 gene (NCBI, Cunningham et al., 2022). Orthologues of P2RX7 have been detected in 272 species (NCBI).

Several P2X7R isoforms obtained from alternative splicing have been identified in rodents and also in humans (Bartlett et al., 2014). Some of these variants are functional, for example, rat and mouse variant „k“ and human variant P2X7BR (Schwarz et al., 2012, Carluccio et al., 2019).

Crystal structures of P2X7Rs have been determined for intracellularly truncated giant panda P2X7R (Karasawa, Kawate, 2016) and chicken P2X7R (Kasuya et al., 2017b) and also for the full-length rat P2X7R (McCarthy et al., 2019).

As no other P2XR family members, P2X7 receptors have a cytoplasmic domain, which contains 18 AA long cysteine-rich (C-cys) region at the end of TM2 called C-cys anchor, and 120 residues in the C-terminus named cytoplasmic ballast (Figure 8). C-cys anchor connects TM2 to the cytoplasmic cap and regulates receptor function by mediating the interactions of the P2X7 receptor and membrane phospholipid rafts. Palmitoylation of the C-cys anchor prevents receptor desensitization and allows to initiate the apoptosis in the presence of a high concentration of eATP. The cytoplasmic ballast has a unique globular, wedge-shaped fold. The ballast does not participate in receptor gating but is involved in the modulation of P2X7R's capability of pore dilation and induction of apoptosis by membrane blebbing, caspases activation, and initiation of cytolytic signal transduction (Suprenant et al., 1996, Wilson et al., 2002, Cheewatrakoolpong et al., 2005, Gonnord et al., 2009, Adinolfi et al., 2010, Costa-Junior et al., 2011, McCarthy et al., 2019). It contains a guanosine nucleotide-binding site and a zinc-ion complex (Figure 8). Functions of these features are still unknown (McCarthy et al., 2019). The ballast domain

may act as a connection between P2X7R and intracellular signalling proteins (Adinolfi et al., 2010) indicating that P2X7R might act not only as an ion channel but also as a metabotropic receptor (Ugur, Ugur, 2019).

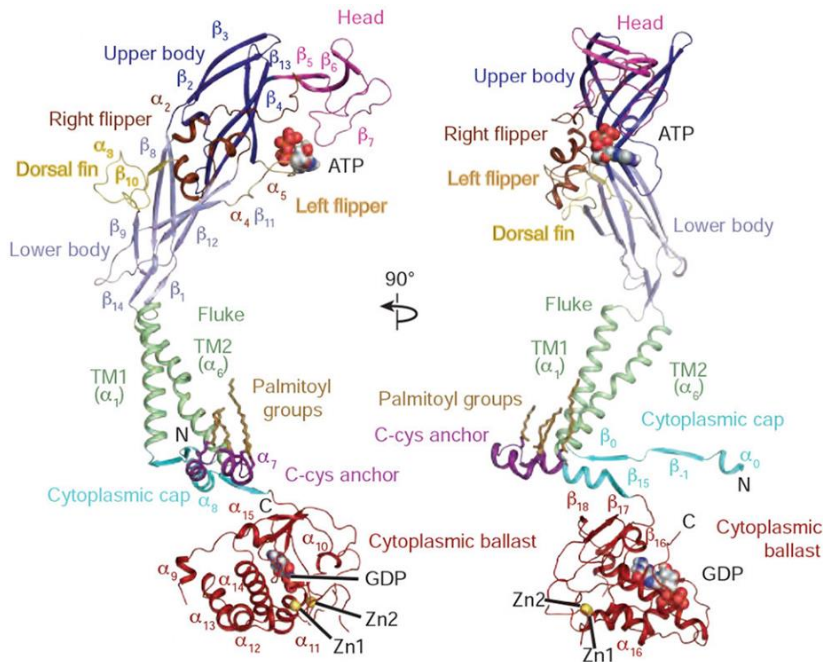

Figure 8. Structure of open state rP2X7 receptor subunit in ribbon representation in orthogonal views. Major domains are shown in different colours. From (McCarthy et al., 2019) with permission.

P2X4 and P2X7 receptors might form functional heteromers as their cooperation has been detected in macrophages. Whether these heteromers really form or whether they are important participants in pain pathology is yet unknown (Perez-Florez et al., 2015).

In the P2XR family, P2X7R demonstrates the lowest sensitivity to ATP (Sadovnick et al., 2017). But at inflammation sites and in cancer, the concentrations of eATP can increase to the level needed to activate the P2X7Rs (Di Virgilio et al., 2018a). Besides, some factors of inflammation can act as PAMs and reduce the ATP threshold for activating P2X7 receptors (Di Virgilio et al., 2018c).

P2X7Rs are able to form large pores allowing the passing of large cationic molecules, however, at a much lower speed than of the small molecules like Ca^{2+}, K^{+}, and Na^{2+} (Di Virgilio et al., 2018c).

As the P2X7 receptor is the most extensively investigated subtype of the P2X receptor family, it is also most studied as a drug target (Illes et al., 2021). Several selective, potent, and mainly allosteric antagonists have been described (Gelin et al., 2020). Some of them are sulfonate dye Brilliant Blue G (Geraghty et al., 2017), AZD9056 that was used in clinical studies for rheumatoid arthritis treatment but with low effectiveness (Keystone et al., 2012), and JNJ-54175446, which has been evaluated in clinical studies for the treatment of major depression disorders (Bhattacharya, Ceusters, 2020, Recourt et al., 2023). There are also a few PAMs of P2X7R activation like LL-37 (Tomasinsig et al., 2008), polymyxin B (Ferrari et al., 2004), tenidap (Sanz et al., 1998), clemastine (Norenberg et al., 2011), and ginsenosides (Helliwell et al., 2015).

Some types of cells, like platelets and leukocytes, contain large amounts of intracellular P2X7Rs within various intracellular compartments (Gu et al., 2000, Burnstock 2015). These receptors do not translocate to the plasma membrane when the membrane P2X7Rs are activated but can do so after extended receptor activation (Smart et al., 2002, Connon et al., 2003). In monocytes, intracellular P2X7 receptors might contribute to high cell surface expression in their differentiation process to macrophages (Gudipaty et al., 2001).

P2X7 receptors are broadly expressed in lymphoid and myeloid immune cells and at low levels in platelets (Di Virgilio et al., 2017). Among immune cells, dendritic cells express P2X7Rs at the highest level. In these cells, P2X7 receptors are involved in several immune responses like antigen presentation, probably being the key factor of the DAMP-dependent stimulatory route (Mutini et al., 1999, Di Virgilio et al., 2017).

eATP's activation of P2X7R leads to pore formation in macrophages and monocytes and provokes a pro-inflammatory reaction. This involves cytokine secretion (especially IL-1β and IL-18), B and T cell proliferation, regulation of phagocytosis, and promoting the chemotaxis (Solle et al., 2001, Feske et al., 2012, Junger, 2011, Di Virgilio et al., 2017). In macrophages, P2X7 receptors are also involved in the killing of several intracellular pathogens like mycobacteria (Lammas et al., 1997), *Chlamydia trachomatis* (Coutinho-Silva, 2003), and *Plasmodium chabaudi* (Salles et al., 2017).

Nevertheless, the best-described immune response of P2X7Rs is the activation of the cryopyrin inflammasome. Under pathological conditions, ATP release can induce sustained P2X7 receptor activation, with NLRP3 inflammasome assembly and pro-inflammatory cytokine release (Di Virgilio et al., 2017, Adinolfi et al., 2017, Calzaferri et al., 2020) (Figure 9).

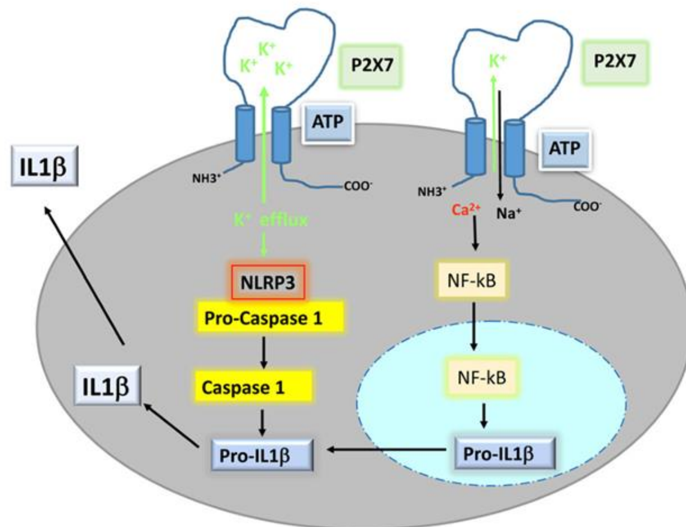

Figure 9. P2X7R activated inflammatory pathways. Left: P2X7Rs activation promotes the release of IL-1β by K^+ efflux and the formation of macropores. This leads to the formation of the inflammasome complex that performs the cleaving of pro-caspase 1 into biologically active caspase-1. As a result, pro-IL-1β is processed into mature IL-1β and released out of the cell. Right: Activation of P2X7R also stimulates the increase of intracellular Ca^{2+} which activates nuclear factor kappa B (NF-κB). NF-kB translocates into the nucleus and evokes pro-IL-1β transcription. From (Calzaferri et al., 2020) with permission.

In addition, P2X7R activation is involved in the release of some other inflammation mediators like eicosanoids leukotriene B4, prostaglandin E2, and thromboxane A2. Their production is induced by the interactions of P2X7Rs and cathelicidin peptide LL-37. Activation of P2X7R leads to cyclo-oxygenase 1 (COX1) and cyclo-oxygenase 2 (COX2) activity that catalyzes production of prostaglandins and thromboxane which are involved in fever and inflammatory pain induction (Barbera-Cremades et al., 2012, Anrather et al., 2011, Chotjumlong et al., 2013, Toki et al., 2015, Lin et al., 2016, Alves et al., 2013).

P2X7Rs are essential for forming long-lasting CD8+ memory cells (Borges da Silva et al., 2018) and differentiation of T helper 17 cells (Fan et al., 2016). In human neutrophils, P2X7R receptors are needed to fight the bacterial infection of *Streptococcus pneumoniae* (Karmakar et al., 2016). Some Natural killer cell (NK) subtypes have high P2X7R expression necessary for energy metabolism and fitness of these cells. In the T follicular CD4+ cells of the gut, P2X7 receptors mediate communication with B cells (Perruzza et al., 2017). The coagulation pathway in macrophages and dendritic cells is activated after their P2X7R activation leads to the tissue factor release (Baroni et al., 2007).

In viral infections, P2X7 receptors have contrasting roles as they are host-protecting in some but infection-promoting in others. P2X7Rs help to decrease viral load in Dengue virus-2 infection (Correa et al., 2016) but worsen adenovirus and hepatitis B infections by quickening the damage of host cells (Lee et al., 2012, Taylor, Han, 2010). P2X7 receptors might have a central role in HIV pathogenesis as their activation leads to the exocytosis of HIV-1 virions from macrophages and P2X7R antagonist A-438079 prevents release of the virion. Besides, nucleotide reverse transcriptase inhibitors that are part of the antiretroviral therapy, effectively inhibit P2X7 receptors in vivo and in vitro models (Swartz et al., 2015, Graziano et al., 2015, Fowler et al., 2014).

P2X7 receptors also participate in the exposal of several cell surface markers and the release of membrane-derived microvesicles and exosomes (Sluyter, 2017). P2X7R activation stimulates the release of vascular endothelial growth factor (VEGF) and promotes angiogenesis in vivo, giving it an important role also in the growth and metastatization of cancers (Adinolfi et al., 2012, Di Virgilio et al., 2018a). Many human and mouse tumours express elevated levels of P2X7Rs, and in some of them the expression correlates with the progression (Lara et al., 2020). Administration of selective P2X7R inhibitors suppresses the metastatization and growth of several P2X7R-expressing tumours (Di Virgilio, 2012, Di Virgilio, Adinolfi, 2017, Di Virgilio et al., 2018a, Lara et al., 2020). On the other hand, P2X7R expression on macrophages and lymphocytes is essential for a successful anti-cancer immune response (Ghiringhelli et al., 2009, Adinolfi et al., 2015).

In the CNS, P2X7 receptors are mostly expressed in microglia, where they are believed to function similarly as in the immune system by activating the discharge of inflammatory cytokines (Sadovnick et al., 2017). The interaction between neurons and astrocytes is primarily mediated through ATP release from these cells (Ferrero, 2009). Astrocytes and oligodendrocytes also express P2X7Rs, only at lower levels, while neurons seem to be lacking these receptors (Illes et al., 2017). The effects of P2X7R expression on neurons are indirect and mediated by glial signalling molecules (Illes et al., 2019a).

Even though neurodegenerative diseases like MS, amyotrophic lateral sclerosis, AD, and Parkinson’s disease have distinct causes, they all include a similar neuroinflammatory component where the ATP concentrations are high and activate P2X7Rs of glial cells (Figure 10). Such neuroinflammatory condition is also induced during acute CNS injuries, such as ischemia, trauma, and epilepsy seizures (Burnstock et al., 2011, Yiangou et al.,

2006). A leading reason behind prolonged epileptic seizures called "status epilepticus" is elevated level of ATP release (Engel et al., 2016). A neurotoxic molecule amyloid- β that is produced in the course of AD promotes the release of ATP from damaged cells of CNS through P2X7R stimulation (Sanz et al., 2009). P2X7Rs are also upregulated in monocytes and lymphocytes in patients suffering from neuropathic pain (Luchting et al., 2016).

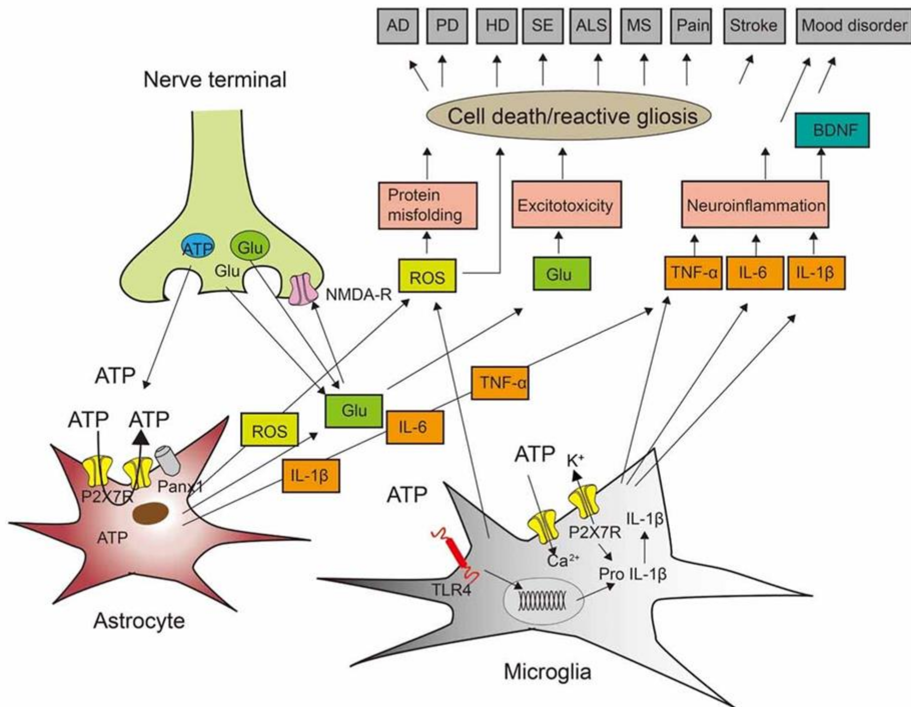

Figure 10. P2X7 receptors are involved in the interactions of astrocytes, microglia, and neurons in neurodegenerative disorders and psychiatric illnesses. Glutamate (Glu) and ATP are neuro- and gliotransmitters secreted from nerve terminals, astrocytes, and microglia. ATP can be released by exocytosis from neurons and astrocytes but also through the P2X7R or pannexin-1 (Panx-1) pores from astrocytes. K^+ efflux from the P2X7R channel is the main stimulus for the NLRP3 inflammasome activation but also lipopolysaccharide (LPS) stimulation of toll-like receptor TLR4R may have a role in some cases. Inflammasome mediates cleavage of pro-IL-1β into IL-1β and boosts its release. Presynaptic N-methyl-D-aspartate (NMDA) receptors contribute to the secretion of glutamate which is an excitotoxin in high concentrations. Microglial cells release reactive oxygen species (ROS) that induce protein misfolding. Pro-inflammatory cytokines IL-1β, IL-6, and TNF-α cause neuroinflammation that stimulates the release of BDNF. AD – Alzheimer's disease, PD - Parkinson's disease, HD – Huntington's disease, SE – status epilepticus, ALS – amyotrophic lateral sclerosis, MS – multiple sclerosis. From (Zhao et al., 2021) with permission.

The neuro-inflammatory processes that cause tissue damage are the key components in MS pathogenesis (Naegele, Martin, 2014, Domercq, Matute, 2019). P2X7 expression is increased in the optic nerve oligodendrocytes of MS patients compared to control samples in both – mRNA and protein levels (Matute et al., 2007). In progressive MS, P2X7R expression is raised in parenchymal astrocytes (Amadio et al., 2017). Concordant with the prior, during EAE, P2X7Rs are overexpressed in astrocytes and their expression

is associated with early astrogliosis (Grygorowicz et al., 2016). Furthermore, treatment with P2X7R antagonists improves recovery in the course of chronic EAE (Matute et al., 2007). Experiments with different strains of P2RX7 KO mice show controversial results – EAE symptoms are reduced in some cases (Sharp et al., 2008) but exacerbated in others (Chen, Brosnan, 2006).

Increased expression of P2X7 receptors has been detected in autoimmune diseases like systemic lupus erythematosus, immune-related glomerulonephritis, and rheumatoid arthritis. All these conditions include immune-mediated tissue damage (Di Virgilio, Giuliani, 2016, Turner et al., 2007, Al-Shukaili et al., 2008).

P2X7 receptors are broadly (but at different levels) expressed also outside of the immune system and the CNS. For example, they can be found in vascular endothelial cells, fibroblasts, osteoblasts, osteoclasts, keratinocytes, intestinal epithelial cells, hepatocytes, skeletal and vascular muscle cells, corneal cells, and retinal ganglion cells (Bartlett et al., 2014, Sluyter, 2017).

Prolonged P2X7R activation can evoke membrane blebbing and apoptotic or necrotic cell death (Wilson et al., 2002, Ferrari et al., 1999, Adinolfi et al., 2005, Auger et al., 2005, Kong et al., 2005, Delarasse et al., 2009) dependent on the cell type. In macrophages, bacterial products induce pyroptosis through P2X7R activation (Dubyak, 2012, Yang et al., 2015). Yet, P2X7 receptors can also provoke growth and promote survival (Baricordi et al., 1999, Adinolfi et al., 2009).

In the absence of ATP, the P2X7 receptor regulates apoptotic cell removal, which has an essential part in early human neurogenesis (Lovelace et al., 2015).

In addition, P2X7 receptors are involved in proteolytic cleavage of proteins on plasma membrane like CD23, L-Selectin (Gu et al., 1998), CD27 (Moon et al., 2006), TNFα (Suzuki et al., 2004), interleukin-6 receptor (Garbers et al., 2011), matrix metalloproteinase-9 (Gu, Wiley, 2006), and the amyloid precursor protein (Delarasse et al., 2011).

In some cells, P2X7Rs have been observed in lipid rafts. These are stiff regions of the plasma membrane that have accumulations of sphingolipids and cholesterol (Garcia-Marcos et al., 2009). P2X7Rs have been found in lipid rafts in rat submandibular glands (Garcia-Marcos et al., 2006a) murine osteoblasts (Gangadharan et al., 2015), lymphoma (Bannas et al., 2005), and lung epithelial cells (Barth et al., 2007). Activation of P2X7 receptors associated with lipid rafts might stimulate lipid-signalling pathways including phospholipase A2 and neutral sphingomyelinase (Garcia-Marcos et al., 2006b). Cholesterol can regulate the functioning of P2X7 receptors on the plasma membrane, especially within lipid rafts (Sluyter, 2017).

A number of missense, intronic, and nonsynonymous SNPs have been found in the P2X7 gene. Some coding region SNPs cause gain or loss of the receptor function. For example, SNP rs2230912 with mutation Gln460Arg has been proposed to be involved in major depression and bipolar disorder (Illes et al., 2019b). Gain-of-function SNPs His155Tyr (489C>T) and Ala348Thr (1068G>A) rise the predisposition for rheumatoid arthritis (Portales-Cervantes et al., 2012, Al-Shukaili et al., 2011).

Both P2RX4 and P2RX7 functional and non-functional gene variants have been indicated as possible factors to modulate MS predisposition (Sadovnick et al., 2017). The P2X7 Gly150Arg + P2X4 Tyr315Cys haplotype induces impaired phagocytosis due to the reduction in P2X7R scavenger function and is a risk factor for the primary progressive type of MS (Gu, Wiley, 2018).

P2RX7 deletion is beneficial in several inflammatory and pathophysiological conditions. At least seven P2RX7-/- mouse lines have been produced that display flaws in immune system function and cytokine release besides reduced inflammatory and neuropathic pain (Illes et al., 2021).

P2rx7^{tm1Gab} mice (AA 506-532 replaced by the insertion of a neomycin selection cassette; the 13b isoform expressed in low level in the brain, salivary gland, and spleen) have abnormal mast cell physiology and alterations in bone formation (Arandjelovic et al., 2012, Ke et al., 2003).

P2rx7^{tm1Lex} mice (A 1496 bp fragment encoding exons 2 and replaced with a lacZ/neo selection cassette) show antidepressant-like phenotype and have abnormal macrophage physiology (Basso et al., 2009).

T cells of P2RX7 KO mice P2rx7^{tm1Ipch} (disruption of exon 1 by insertion of a LacZ gene in-frame with the initiation codon and neomycin selection cassette; without any deletion in the p2rx7 sequence; the 13a isoform is expressed) preserve high levels of P2X7R activity (Kaczmarek-Hajek et al., 2012). In these animals, inflammatory and neuropathic hypersensitivity is entirely absent for both thermal and mechanical stimuli, although normal nociceptive processing is existent (Chessell et al., 2005).

KO mice P2rx7$^{tm1a(EUCOMM)Wtsi}$ have reduced circulating glycerol levels compared to WT mice and KO mice P2rx7$^{tm1b(EUCOMM)Wtsi}$ show an abnormal eye morphology and cataract phenotype (International Mouse Phenotyping Consortium).

Floxed humanized P2RX7 knock-in mice P2rx7$^{tm1.1(P2RX7)Jde}$ (murine exon 2 replaced by the human P2RX7 cDNA comprising exons 2-13) show abnormal physiology of primary hippocampal cells and sleep disturbances (Metzger et al., 2017).

A knocked-in mouse line P2rx7$^{tm1(EGFP_CreERT2)Wtsi}$ generated by the EUCOMMTOOLS program is expressing enhanced green fluorescent protein (EGFP) together with inducible Cre recombinase (CreERT2) that is controlled by the P2RX7 promoter. Unfortunately, the mouse line does not perform as expected having the P2X7R expression in neurons but not in microglia, astrocytes, and oligodendrocytes due to the incorrect cassette insertion (Urbina Treviño, 2021).

Transgenic reporter mouse line Tg(RP24-114E20-P2X7-His-StrepEGFP)Ani has a sequence of EGFP inserted into the P2RX7 C-terminus that results in a fused P2X7R-EGFP protein. The EGFP tag does not change the P2X7 receptor function but the receptor is remarkably over-expressed in this mouse line (Kaczmarek-Hajek et al., 2018).

In another transgenic reporter mouse line Tg(P2rx7-EGFP)FY174Gsat, the EGFP cassette accompanied with polyA signal is inserted downstream of P2RX7 exon 1. In this mouse line, the EGFP is soluble. Unexpectedly, these mice have overexpression of P2X4R and P2X7R with generally atypical expression patterns (Ramírez-Fernández et al., 2020).

1.9 Interactions between P2X4 and P2X7 receptors

The probable interactions between P2X4 and P2X7 receptors have been broadly studied.

Yet, there is still no definitive proof of whether P2X4R and P2X7R interact also as heterotrimers at the plasma membrane. Some electrophysiological data is pointing out that rat P2X4R and P2X7R subunits are able to form heteromeric receptors as P2X7R was co-immunoprecipitated together with P2X4R (Guo et al., 2007). A study of murine pancreatic acinar cells and HEK293 showed that P2X4R and P2X7R form heterotrimers and these heteromers have electrophysiological and pharmacological properties differing from homotrimeric receptors (Casas-Pruneda et al., 2009). Another study used human P2RX4, human P2RX7 or both human P2RX4 and P2RX7 transfected Xenopus

oocytes and found that P2X4R and P2X7R co-express in the oocytes and are assembled as heterotrimers but do not produce a new electrophysiological phenotype (Schneider et al., 2017). Contrarily, four studies, using mouse or rat P2RX4 and P2RX7, could not find evidence of P2X4R and P2X7R co-assembling and producing P2X4/P2X7 heterotrimeric receptors and concluded that interactions of P2X4R and P2X7R occur because of the association of homomers (Nicke 2008, Boumechache et al., 2009, Antonio et al., 2011, Trang et al., 2020). Possibly, a small percentage of P2X4R and P2X7R subunits form heterotrimeric receptors that are not easy to detect.

Several research groups have shown functional interactions occurring between P2X4 and P2X7 receptors. These studies demonstrate that P2X4R and P2X7R co-expression endorses P2X7R-dependent death of macrophages and P2X4R potentiates P2X7R-dependent inflammasome activation that leads to raised release of IL-1b and IL-18 (Kawano et al., 2012, Sakaki et al., 2013). P2X4R activation can also trigger P2X7R upregulation (Zech et al., 2016). In general, these studies imply that P2X4R positively controls P2X7R-dependent Ca^{2+} influxes and promotes inflammation through P2X7R-dependent maturation of pro-inflammatory cytokines.

2 Aims of the Thesis

The aim of this thesis was to collect new insights and confirm known data about the biology of P2X4 and P2X7 receptors in evolutionary, physiological, inflammatory, and autoimmunological contexts. This included:

- Investigation of the evolutionary origin of P2X7 receptor.
- Production and validation of mononuclear antibodies against human P2X4 receptor to provide additional tools to study the presence and function of this receptor in various tissues.
- Studying the P2X4 receptor expression in human peripheral blood leukocytes.
- Exploration of the role of P2X4 and P2X7 gene expression in the context of multiple sclerosis and its animal model experimental autoimmune encephalomyelitis. Finding possible sex-dependent patterns.

3 Materials and methods

Detailed descriptions of materials and methods are provided in the publications. Following is the overview of the methods:

- Identification and analysis of counterparts of the intracytoplasmic domain of P2X7 receptor (publication I)
- Gene linkage analysis (publication I)
- Molecular modelling of the structure of P2X7 receptor homologs (publication I)
- Cell culture (publication II)
- Transfection of HEK293 cells (publication II)
- Development of anti-human P2X4 receptor monoclonal antibodies (publication II)
- Confocal microscopy (publication II)
- Isolation of blood cells (publications II, III)
- Immunostaining of cells (publications II, III)
- Flow cytometry (publications II, III)
- Statistical data analysis (publications II, III)
- Induction of experimental autoimmune encephalomyelitis in mice (publication III)
- Dissection of mice and tissue extraction (publication III)
- RNA extraction (publication III)
- cDNA synthesis (publication III)
- Quantitative PCR (qPCR) (publication III)

4 Results and discussion

4.1 Evolutionary origin of the P2X7 receptor (Publication I)

Genes of P2X receptor family have been recognized across eukaryotes, for example in plants, amoebas, fungi, and the Metazoa. These receptors are ATP-activated and have common well-conserved structural elements. Although the extracellular domains of P2XR family members are highly similar, the long intracellular region of P2X7R cannot be found in other P2X receptors (Hou, Cao, 2016, Fountain, 2013).

Therefore, it was decided to examine the evolutionary origin of the P2X7 receptor and especially its intracytoplasmic sequence and the conserved motifs in it.

4.1.1 The Zn-coordinating cysteine-based domain of P2X7 gene is conserved across vertebrates

Each human P2RX sequence contains a standard and highly conserved P2RX motif (Interpro IPR001429) that is encoded by exons 1–10. In contrast, the C-terminal regions have varied numbers of exons, different exon junction positions and variable sequences. Exon 12 is not conserved across the P2RX family members and only P2RX5 and P2RX7 have the 13th exon – only a 13 AA coding in P2RX5 and a long 170 AA coding in P2RX7 (McCarthy et al., 2019).

The P2RX7 sequences were compared across vertebrates to find conserved regions. The P2RX motif was found to be highly conserved. However, the exon 11 3' side and the exon 13 5' side are not well conserved among vertebrates. Thus, the conserved motifs and residues were sought. In exon 13, which encodes the so-called "ballast domain" (McCarthy et al., 2019), Zn-coordinating cysteines are generally very well conserved from fish to mammals. Yet, the 5′ end of the exon 13 is highly variable, in sequence as well as in length (Publication I, Figure 1).

As a result, a highly conserved region in exon 13 containing most of the Zn-coordinating cysteines in human P2RX7 was defined. The consensus sequences of the cysteine clusters are PxWCxCx2C, LCCRx3GxCITTS/T, and (L/I/V)PSC(C/S)x3IRx2(F/Y)Px5Y(S/T)G. The region was named „Zn-coordinating cysteine-based domain“ (ZCD) and it includes seven/eight conserved C residues (Publication I, Figure 1) but does not contain the C-cys anchor motif.

4.1.2 The ZCD of P2X7 gene is an ancient module present across all main divisions of Metazoa but found only in a few other proteins

To get more functional insight into the ZCD, its combination with other domains was analysed across vertebrate proteins.

Phylogenetic analyses detected three clusters of proteins containing ZCD: classic P2X7R identified in vertebrates from bony fish to mammals, nanor-like proteins that contain only the ZCD and a short N-terminal region, and three similar Xenopus proteins without P2X-like domain which form a separate group (Publication I, Figure 4).

Then, the ZCD sequence was searched for in proteins of other Metazoa groups. It was mainly found in relatively short proteins somewhat similar to nanor, with no other conserved domains and without TM region, contrary to P2X7R. ZCD-containing proteins were detected in sponges, cnidarians, echinoderms, arthropods, nematodes, and molluscs (Publication I, Figure 5). Linkage analysis identified a conserved ancestral association of ZCD-containing genes with other markers that shows their common origin

(Publication I, Figure 6). These data support the ZCD conservation across the sequences of Metazoa and suggest it might establish the primordial pattern of the ballast domain.

ZCD-like proteins were not detected in bacteria, plants, or fungi. Therefore, this domain seems to be an original invention of Metazoa like several other domains (Frederic et al., 2013).

Recently, a DNA transposon family *KolobokP* was identified where Kol0 encodes a KolX protein, which has a c-terminal part similar to the cytoplasmic ballast of P2X7R (Kojima, Bao, 2022). So, the ballast domain might be captured from a transposon sequence.

4.1.3 P2X7 gene originates from the fusion of a P2X4-like gene and a Zn-coordinating cysteine-based domain

P2X4 and P2X7 genes have close genomic locations and encode very similar membrane receptors (Suurväli et al., 2017). This demonstrates that these genes were presumably produced by local gene duplication. An ancient domain that contained a conserved cysteine-based Zn-coordinating motif could have been combined to a P2RX4-like sequence after the divergence of cartilaginous fish from other jawed vertebrates. Indeed, no typical P2RX7 with a ballast could be found in cartilaginous fish species (Publication I, Figure 8).

The ballast domain of P2X7R contains a high-affinity GDP binding site (McCarthy et al., 2019). The residues that interact with GDP in rat P2X7R (R_{546}, H_{547}, R_{574}, R_{578}, K_{583}) are not all conserved in P2X7 receptors of other species. Therefore, it is unknown whether fish P2X7 receptors, nanor-like proteins, and other ZCD-containing proteins do bind GDP.

The ballast domain and P2RX4-like sequence were connected by a region in which evolved the C-cys anchor that is crucial for the P2X7R properties of desensitization (McCarthy et al., 2019). The result was the unique purinergic receptor P2X7R essential in immunity and inflammation.

4.2 Production and validation of monoclonal antibodies against human P2X4 receptor (Publication II)

P2X4 receptors are involved in several inflammatory pathologies and neurodegenerative diseases. Still, their functions and expression have not been thoroughly characterized. At the beginning of this project, there was an absence of antibodies (Abs) targeting these receptors. Thus, it was decided to produce monoclonal antibodies (mAbs) against the human P2X4R.

In bacteria, a protein consisting of the extracellular part of the P2X4 receptor fused to a 6xHis-tag was produced. After purification, the protein was used for mouse immunization. The final selection contained four candidate hybridomas: two IgM/kappa named mAb8 and mAb29 and two IgG2b/kappa named mAb19 and mAb27.

In western blot analyses on P2X4R-expressing HEK cells, none of the four antibodies detected P2X4R, demonstrating that they do not recognize the denatured form of the protein and thus target the native conformation (mAb19 and mAb27 in Publication II, Figure 1A, left panel).

Immunoprecipitation (IP) analyses showed that mAb19 and mAb27 immunoprecipitated P2X4R from the lysates of human P2X4R expressing HEK cells (Publication II, Figure 1A, right panel). mAb27 and mAb29 were also tested on mouse P2X4R expressing cells and confirmed to also immunoprecipitate mouse P2X4R (Publication II, Figure 1B). Therefore,

mAb27, mAb19, and mA29 specifically recognize human P2X4R and mAb27 and mAb29 also bind to the mouse P2X4R.

Hereafter, the work focused on mAb27 (IgG2b) and mAb29 (IgM).

To verify that the mAbs bind to the mouse P2X4R, P2RX4 KO and WT C57Bl/6 mice were used. These mice were injected with thioglycolate intraperitoneally. Then peritoneal cells were extracted, labelled with fluorescein isothiocyanate (FITC) –conjugated mAb27 or isotype control, and analysed with the flow cytometer. In WT mice, a considerable amount of cells in all specified gates (Publication II, Figure 2A) were recognized by mAb27. However, in P2RX4 KO mice, mAb27 staining did not differ from the isotype control (Publication II, Figure 2B). So, the mAb27 specifically stains mouse P2X4R in flow cytometry experiments. A similar result was obtained using mAb29.

In immunohistochemistry experiments, mAb27 stained the human P2X4R overexpressing HEK cells, but not the HEK cells that were transfected with the empty vector (Publication II, Figure 3A). Similar results were obtained with the other 3 mAbs. mAb27 also stained glial cells and neurons of rat hippocampus cell culture (Publication II, Figure 3C) that are known for their high P2X4R expression (Sim et al., 2006). Therefore, mAb27 can also recognize the rat P2X4R. Identical results were obtained with mAb29.

Flow cytometry analyses demonstrated that mAb27-FITC considerably stains HEK cells that express mouse P2X4R (Publication II, Figure 3B, left panel) but with less efficiency than HEK cells that express human P2X4R (Publication II, Figure 3B, right panel). Analyses with other 3 mAbs gave similar results.

Furthermore, mAb27 was used to stain human immortal bronchial epithelial cells and human prostate cancer cell line. The results were compared to the staining of two commercial (Abnova and Santa Cruz) anti-P2X4R antibodies. All the Abs had similar staining patterns (Publication II, Figure 4) confirming the specificity of the mAb27.

These mAbs that are specific for the human P2X4R and cross-react with the murine P2X4R, serve as novel tools helping to determine this receptor's presence and function in various tissues.

Other available non-commercial antibodies against P2X4 receptors:

- Mouse IgM mAb anti-P2X4R – recognizes and immunoprecipitates rat P2X4R and does not cross-react with other P2X receptors (Bo et al., 2003b).
- Mouse mAb anti-P2X4R – recognizes rat P2X4R, immunoprecipitates its extracellular domain (Igawa et al., 2013).
- IgG#151 LO – recognizes and inhibits human P2X4R, does not cross-react with other P2X receptors (Williams et al., 2019).
- IgG#191 and IgG#191-Bbbt0626 – recognize and inhibit mouse P2X4R and reduce mechanical hyperalgesia (Williams et al., 2019).
- Nodu 225 mAb Rat anti-P2X4R and Nodu 246 mAb Rat anti-P2X4R – recognize mouse P2X4R, immunoprecipitate mouse but not human P2X4R (Bergmann et al., 2019).
- Nodu 19 mAb Rat anti-P2X4R and Nodu 344 mAb Rat anti-P2X4R – recognize human P2X4R (Bergmann et al., 2019).
- Nanobodies Nb 262 and Nb 301 – recognize human P2X4R (Bergmann et al., 2019).
- Nanobody Nb 325 – recognizes mouse P2X4R (Bergmann et al., 2019).
- Nanobody Nb 318 – recognizes human and rat P2X4R (Bergmann et al., 2019).
- Nanobody Nb 258 – recognizes human and mouse P2X4R (Bergmann et al., 2019).
- Nanobodies Nb 271 and Nb 284 – recognize human, mouse, and rat P2X4R (Bergmann et al., 2019).

4.3 P2X4 receptor expression in cancer and immune cells (Publication II)

4.3.1 P2X4 receptor is mainly located in intracellular compartments of mouse astrocytes, glioma and microglial cells but is also expressed on the surface of mouse glioma and astrocytoma cells

In neural cells, expression of P2X4Rs has previously been indicated in glia, microglia, and astrocytes (Dou et al., 2012, Verkhratsky et al., 2009, Zhang et al., 2007). The produced mAbs conjugated with FITC were used to examine the P2X4Rs cellular location in corresponding mouse tumour cell lines: GL261 (glioma), BV2 (microglia), and ALT (astrocytoma). All the cell lines were P2X4R positive (mAb27 staining in Publication II, Figure 5). Microglial cells displayed an intense accumulation of P2X4Rs in internal compartments (Publication II, Figure 5H, 5I) that are probably lysosomes as described in HEK293 and COS1 cells (Huang et al., 2014). Glioma and astrocytoma cells showed also high expression in internal compartments but they had significantly higher P2X4R surface expression compared to microglia (Publication II, Figure 5B-5I).

4.3.2 Human peripheral blood leukocyte subsets express P2X4 receptor in different levels

The expression of P2X4Rs on leukocytes (CD45+ cells) was determined in the peripheral blood (PB) of seven healthy human donors. Peripheral blood leukocytes (PBLs) were stained with anti-P2X4R mAb27-FITC and lineage-specific Abs (CD45, CD3, CD14, CD20) and analysed with the flow cytometer to determine the P2X4R expression on the main subsets of leukocytes (Publication II, Figure 6).

Among leukocytes, T cells (CD3+ cells) had very low P2X4R expression (Publication II, Figure 6D), B cells (CD20+ cells) were slightly positive (Publication II, Figure 6E), and monocytes (CD14+ cells) and granulocytes (CD14+/- cells) had intermediate P2X4R expression levels (Publication II, Figure 6F-6G). However, a distinct subset of PBL that expressed P2X4Rs at high levels was detected (Publication II, Figure 6D-6F). This subpopulation of cells was among the most granular cells and expressed greater levels of P2X4Rs than other granulocytes (Publication II, Figure 6G). From the phenotype (large, granular, P2X4Rhigh, CD45+, CD13-, CD14-) and frequency of these cells, it was hypothesized that these cells could be eosinophils.

4.3.3 Human eosinophils in peripheral blood and tissues express high levels of P2X4 receptor

There are 2-3 times more eosinophils in the PB of allergic patients compared to non-allergic (Metcalfe et al., 2016). Therefore, the PBLs of six allergic human donors were stained with anti-P2X4R mAb27-FITC, anti-CD45, and anti-Siglec8 antibodies and analysed with the flow cytometer. The subset of P2X4Rhigh and vastly granular cells also expressed a great level of Siglec8 that is considered a marker for eosinophils (Publication II, Figure 7) and a low level of CD123 that distinguishes them from the CD123-positive basophils. This indicates that a strong P2X4R surface expression is a suitable marker for Siglec8+ cells of human PBL. The proportions of large granular cells with high P2X4R surface expression are similar to the proportions of Siglec8-high cells in PBLs of allergic patients (Publication II, Table 1).

Lymphoid and myeloid cells that express low or intermediate P2X4R levels express lower Siglec8 levels (Publication II, Figure 7D). Siglec8low P2X4R-med cells are large

granular cells that have medium CD45 levels and correspond generally to neutrophils. Siglec8-low P2X4R-med/low cells are smaller cells expressing CD45 in high levels and corresponding to lymphocytes and monocytes (Publication II, Figure 7D-7F).

Gallbladder cryosections of a chronic calculous cholecystitis patient were co-stained with anti-P2X4R mAb27 and anti-Siglec8. Confocal microscopy of these sections showed specific co-staining of Siglec8 and P2X4R (Publication II, Figure 8), demonstrating that tissue eosinophils express abundantly both of these markers. Hemotoxylin/eosin staining was used to establish the eosinophil density in the tissue and it confirmed that the anti-P2X4R mAb27 can be used to mark eosinophils in tissues with inflammation.

Eosinophils are mature cytotoxic effector cells that implement immunomodulatory functions and maintain immune homeostasis. They combat bacterial, viral, fungal, and parasitic infections (Ramirez et al., 2018, Davoine, Lacy, 2014) and participate in generating tissue damage in the course of infections, autoimmune diseases, and asthma. Eosinophil differentiation is regulated by inflammatory stimuli like chemokine CCL11/eotaxin and interleukins IL3 and IL5. After maturation, eosinophils are excreted into the blood and infiltrate lymph organs like the spleen, thymus, and lymph nodes (Hogan et al., 2008, Berek, 2016). As P2X4R reacts to eATP at micromolar concentrations, this receptor might be used in eosinophils for ATP-induced activation.

Eosinophils are activated in several pathologies like asthma and immune responses against parasites (Rosenberg et al., 2013). P2X4Rs strengthen allergic responses (Zech et al., 2016, Chen et al., 2016) and thus, it is logical that eosinophils express high levels of P2X4Rs.

Still, the immune functions of eosinophils are not completely understood. One reason for this is the absence of cell lines and specific surface markers. As a novel human eosinophil surface marker, P2X4R might assist in the research of their biology. The importance is highlighted by the fact that Siglec8 cannot be used as a human eosinophil marker in in vivo or in vitro culture experiments as its interactions result in eosinophil apoptosis and mast cell mediator release inhibition (Kiwamoto et al., 2012).

4.3.4 P2X4 receptor expression in leukocytes is higher in males

P2X4R functions differently in the brain microglial cells of males and females. In male mice, increased expression of P2X4 receptor is necessary for pain hypersensitivity but in female mice, lymphocytes may be involved instead of microglia (Tsuda et al., 2003, Sorge et al., 2015). In female mice, PNI causes a P2X4R expression rise in the spinal dorsal horn but the rise is much lower than in male mice (Taves et al., 2016).

So, P2X4R expression on PBLs was compared between men and women and also between male and female mice. When all CD45+ leucocytes in the blood were considered, men had a higher percentage of P2X4R-expressing cells compared to women (Publication II, Figure 9A). Analogous experiments with mouse PBLs showed similar results – male mice having more P2X4R positive cells than female mice (Publication II, Figure 9B). Such a sexual dimorphism might appear to be an important factor in pathological processes.

In general, these results indicate that human and mouse subsets of leukocytes express a substantial amount of P2X4Rs and suggest that P2X4 receptors might be involved in their activation.

4.4 Expression of P2X4 and P2X7 genes in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (Publication III)

Multiple sclerosis is a heterogeneous and complex chronic autoimmune disease of the CNS. The main characteristic of the disease is the presence of multifocal inflammatory lesions that finally lead to axonal damage and gradual neurological deterioration (Hauser, Goodin, 2005). It is the most common cause of progressive neurological disability in young adults (Friese et al., 2014). About 2 500 000 people worldwide and 1500 people in Estonia suffer from this disease. Most often, MS starts between 20 and 50 years of age, and women are more often affected than men. Half of the patients need assistance with mobility within 20 years after diagnosis, and half of all patients eventually suffer from significant cognitive deficits. MS lowers the patient's lifespan by five to ten years. As for many other autoimmune diseases, statistics indicate that the incidence of MS is rising, especially in the northern hemisphere (Sawcer et al., 2014, Koriem, 2016, Gross-Paju et al., 2009).

The name multiple sclerosis refers to the scars (lesions) that form in the brain and spinal cord and comes from the Latin word *sclera*. The key event of MS occurs when the autoreactive cells migrate across the blood-brain barrier (BBB). In the CNS, these cells target the protective myelin coating that surrounds axons and helps neurons to transmit electrical signals. The disease progression involves the loss of oligodendrocytes, cells that are responsible for generating and maintaining the myelin coating. Clinical aspects of MS vary remarkably among the patients as they depend on the areas of the brain that are affected by the disease, likewise on the severity of the damage. Principally, MS patients may experience partial or entire loss of functions controlled by the spinal cord or brain. In the most prevailing form of the disease, relapses are followed by remissions. During relapses, symptoms manifest or worsen. In the course of remission, patients recover completely or partially. (Compston, Coles, 2008, Miljkovic, Spasojevic, 2013, Domercq et al., 2018). The exact cause of multiple sclerosis has remained unknown, but genetic predisposition and various environmental risk factors are assumed to play a role (Koriem, 2016).

There is no known cure for MS so far. Still, existing treatments attempt to decrease the recurrence and severity of relapses, shorten the relapse periods, ease the symptoms, stimulate tissue repair, and prevent disability resulting from the disease progression (Miljkovic, Spasojevic, 2013). Nevertheless, no medication completely restricts or reverses neurological degradation (Reich et al., 2018). Only immune system reconstruction by autologous hematopoietic stem cell transplantation after immune ablation has shown a promising effect (Massey et al., 2018).

Early diagnosis and treatment rise the possibility of a better outcome (Reich et al., 2018). With this in mind, identifying novel biomarkers for multiple sclerosis is highly necessary. Such markers could become helpful for early diagnosis of MS, monitoring the progression of the disease, and evaluating the impact of treatment (Tomioka, Matsui, 2014).

The prevailing experimental animal model for MS studies is experimental autoimmune encephalomyelitis, an artificially induced condition in mice. This model simulates the immuno- and neuropathological processes of MS, like brain inflammation, demyelination, axonal loss, and gliosis (Constantinescu et al., 2011).

The purinergic receptors P2X4R and P2X7R are both upregulated in the CNS during MS (Amadio et al., 2017, Vazquez- Villoldo et al., 2014). It has been shown in post-mortem studies, that the P2X7 receptor is more expressed in the microglia of MS patients than in non-MS controls (Yiangou et al., 2006). P2RX4 and P2RX7 genetic variants have been demonstrated to influence MS predisposition (Sadovnick et al., 2017).

The expression of P2RX4 and P2RX7 genes in peripheral blood mononuclear cells (PBMC) of healthy donors and patients with MS receiving different disease-modifying drugs was measured. For analysis, all patients were divided into three groups according to their current treatment: patients treated with Interferon-β (IFNβ) drugs, patients treated with glatiramer acetate (GA), and patients not treated (NT) at the moment they donated blood. As MS occurs among women more often but the disease progression is generally faster in men, the mechanisms may be partly diverse. Therefore, also sex-specific differences were tested to find out whether these potential biomarkers could be equally used in both biological sexes.

4.4.1 Interferon-β treatment elevates P2X4 gene expression

Comparing a merged MS data set (all treated and non-treated MS patients together) with healthy controls (HC), showed that P2RX4 expression was significantly raised in MS patients (Publication III, Figure 1A). Even 7 samples with the highest P2RX4 expression belong to MS patients (Publication III, Figure 1A, 1B). But when comparing all treatment groups separately, a considerable difference was detected only between patients treated with IFNβ and HCs (Publication III, Figure 1B). Thus, IFNβ treatment rather than MS upregulates P2RX4 expression. P2X4 receptors are up-regulated by IFNβ in microglia (Inoue, 2009). So, it is possible that IFNβ has a similar effect in PBMC.

Still, a tendency toward increased P2RX4 expression was noticeable in other MS patient groups compared to HCs (Publication III, Figure 1B). The raised P2RX4 expression may turn out to be significant in a larger sample group. It is also possible that the raise of P2RX4 expression is not significant in PBMC but rather in CNS tissues. P2RX4 has been shown to be up-regulated in the optic nerve of MS patients and also in the spinal cord microglia in a rat EAE model (Sadovnick et al., 2017).

4.4.2 P2X7 gene is up-regulated in the peripheral blood mononuclear cells of multiple sclerosis patients

It has been indicated that altered expression of P2X7 is linked to neurogenerative diseases, including MS (Matute et al., 2007, Sperlágh, Illes, 2014). Analogous comparison of merged MS patients with HC for P2RX7 revealed a similar pattern as for P2RX4. MS patients had increased P2RX7 expression compared to HC (Publication III, Figure 1C). Also this time, the highest expressions were observed in the MS group (Publication III, Figure 1C). But contrary to P2RX4, P2RX7 had significantly higher expression in NT and GA-treated patients compared to the HC group (Publication III, Figure 1D). No considerable up-regulation of P2RX7 was detected in IFNβ-treated patients compared to HCs, suggesting that IFNβ treatment might prevent MS-promoted P2RX7 induction. It has been shown that antagonizing P2X7 receptors reduces clinical signs of EAE (Matute et al., 2007, Grygorowicz et al., 2018). So, the inhibition of P2RX7 expression may be one of the mechanisms of action of IFNβ treatment. No reducing effect of GA on P2RX7 expression was observed, even though it has been reported in vivo and in vitro in monocytes (Caragnano et al., 2012).

4.4.3 P2X4 gene expression is significantly up-regulated only in male patients treated with interferon-β

P2RX4 expression was considerably increased only in IFNβ-treated MS patients. When the groups were further analysed based on sex, surprisingly, the pattern was seen exclusively in men. IFNβ-treated male MS patients had significantly higher P2RX4 expression compared to NT males and the male HC group (Publication III, Figure 4B). No such increase was observed in IFNβ-treated women. In IFNβ-treated female patients, the P2RX4 expression median value was even a little lower than in female GA or NT patients (Publication III, Figure 4C). However, a trend of a modest (but not statistically supported) P2RX4 up-regulation in all female MS patient groups was seen compared to the HC group (Publication III, Figure 4C).

The sex-dependent regulation of P2RX4 expression may lead to different responses to MS treatment in women and men. This would not be the first expression of P2X4 receptors that has sex-based differences. Pain hypersensitivity is known to be mediated by P2X4 receptors in male mice but by adaptive immune cells without P2X4 receptors participation in female mice (Sorge et al., 2015). In addition, BDNF loss in microglia suppresses allodynia after PNI only in male mice and does not have the same effect in female mice (Tsuda, Inoue, 2016). The exact reasons for these sex-dependent expressions may be complex and are not known.

4.4.4 The increased P2X7 gene expression is observed mainly in female multiple sclerosis patients

Non-treated MS patients had significantly increased P2RX7 expression compared to HCs only among women (Publication III, Figure 4D). P2RX7 expression levels were similar in all male groups (NT, IFNβ-treated, GA-treated and HCs) (Publication III, Figure 4E). This differential response based on the sex of the patients might indicate that in PBMC there are distinct inflammatory pathways in men and women that need further studies. Besides, P2RX7 had increased expression in GA-treated female MS patients compared to the HC group (Publication III, Figure 4F) demonstrating that GA did not suppress the P2RX7 induction in women as did the IFNβ treatment. Therefore, P2RX7 expression might be useful as a marker of IFNβ treatment response in women.

A sex-specific mechanism of the P2X7 receptor has been discovered also in a carrageenan synovitis rat model experiment where P2X7R activation was essential for inflammation and hyperalgesia development in all rats but P2X7R blocking had significantly better anti-inflammatory and antihyperalgesic effects in female rats (Teixeira et al., 2017). Again, the precise reasons for the sex-specific expressions have remained unclear.

4.4.5 RGS16 is required for the up-regulation of P2X4 and P2X7 genes in the mouse spinal cord by experimental autoimmune encephalomyelitis

Regulator of G protein signalling 16 (RGS16) is part of a family that down-modulates biochemical pathways downstream of G-protein-coupled receptors. Different RGS proteins regulate different receptors, yet their specificities may overlap. RGS16 restricts proinflammatory responses and suppresses T cell migration by modulating the G-protein-coupled receptors signalling (Lippert et al., 2003, Suurväli et al., 2015). The preliminary analyses at the Immunology laboratory of Tallinn University of Technology were suggesting that the presence of RGS16 may affect P2X4R and P2X7R expression in MS model EAE.

In the mouse brain and spinal cord experiment, the mice were divided into groups based on their genetic background (WT and RGS16KO mice) and according to their participation in EAE induction (EAE-induced mice and control mice).

The analyses of P2RX4 and P2RX7 expression after EAE induction confirmed the fact that EAE mainly affects the spinal cord and not so much the brain (Guo, Schluesener, 2005). It was best observed by comparing the brain and spinal cord P2RX4 and P2RX7 expression values of WT mice. The P2RX4 and P2RX7 expression values were remarkably higher in the spinal cords of EAE mice compared to the spinal cords of the control group mice and the brains of both, the EAE and the control group, mice (Figure 11).

There was no such increase in RGS16KO mice (Publication III, Figure 6A, 6B). This striking discovery indicates that in the MS mouse model, induction of P2RX4 and P2RX7 is RGS16-dependent. The effect could be mediated by its role in inhibiting the T cell migration to the brain (Estes et al., 2004). Besides, the RGS16-controlled chemokine receptor 4 (CCR4) expression in dendritic cells is essential to the development of EAE (Shankar et al., 2012, Poppensieker et al., 2012).

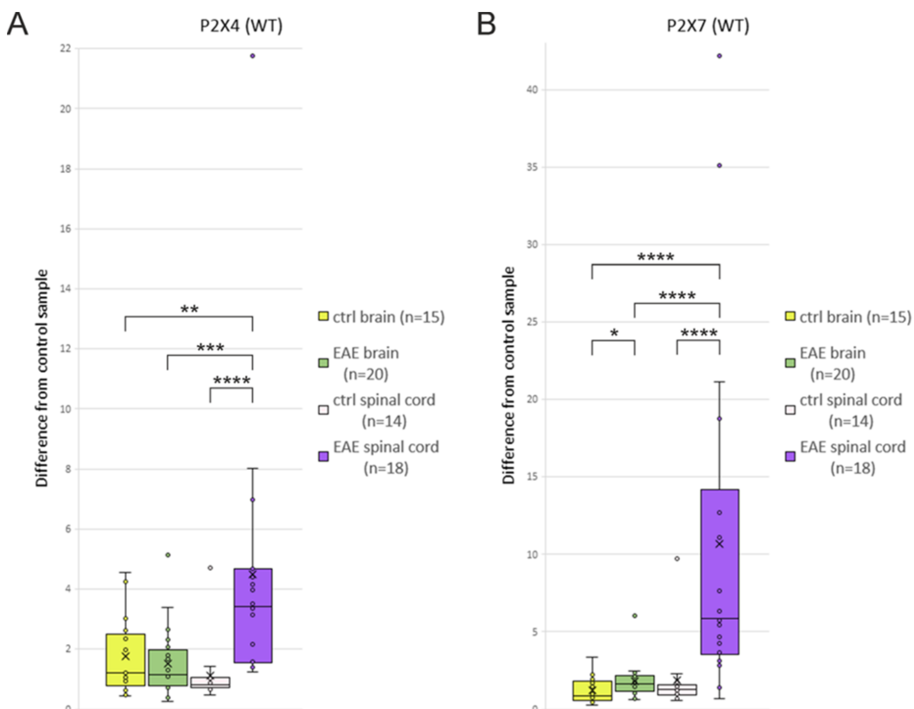

Figure 11. Relative P2X4 and P2X7 gene expression in WT mouse brains and spinal cords. A and B: yellow – control mouse brains, green – EAE mouse brains, pink – control mouse spinal cords, violet – EAE mouse spinal cords. A: P2RX4 expression level is increased in WT mouse spinal cords after EAE induction compared to control mouse spinal cords (p<0,0001). B: P2RX7 expression level is remarkably increased in WT mouse spinal cords after EAE induction compared to control mouse spinal cords (p<0,0001) and moderately increased in brains (p=0,0427).
*Y-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. * - p-value < 0,05, ** - p-value <0,01, *** - p-value <0,001, **** - p-value <0,0001.*

4.4.6 P2X4 gene expression in wild-type mouse brains and the control mechanism of RGS16 over P2X4 receptor expression are sex-dependent

In addition, the WT mouse groups were analysed based on sex. This showed that female mice had higher P2RX4 expression levels in the brain compared to male mice. Female EAE mice had greater P2RX4 expression in the brain compared to both – male control group mice and male EAE mice. In addition, female control group mice had a significantly elevated P2RX4 expression in the brain compared to male EAE mice, the difference was observed but was not considered statistically significant compared to male control group mice (probably because of the small size of the group) (Figure 12A).

As EAE did not affect P2RX4 expression significantly in mouse brains, the control and EAE mice were analysed together. This way the difference between sexes came into sight even better. But the P2RX4 expression levels were not significantly higher in the brains of female RGS16KO mice compared to male mice (Figure 12B). So, the sex-specific difference existed only among WT mice.

Such a sex-dependent difference was not detected in the P2RX7 expression (data not shown).

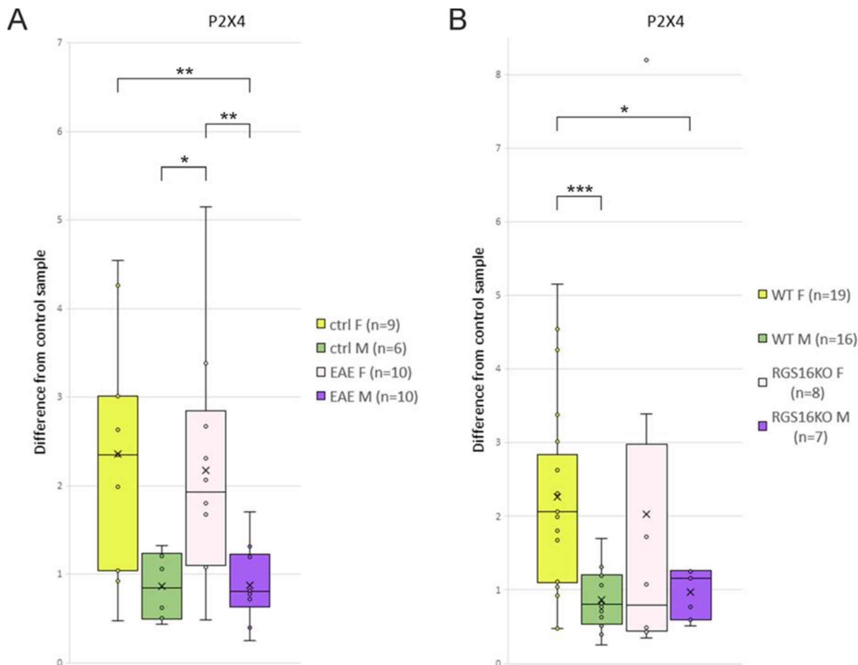

*Figure 12. Relative gender-dependent P2X4 gene expression in mouse brains. A: WT mouse brains: yellow – female control mice, green – male control mice, pink – female EAE mice, violet – male EAE mice. B: mouse brains (EAE induced and control mice together): yellow – female WT mice, green – male WT mice, pink – female RGS16KO mice, violet – male RGS16KO mice. A. Female WT mice have higher P2RX4 expression in the brain compared to male mice. The effect is more evident in EAE-induced mice (p=0,0225 compared to male control mice and p=0,0064 compared to male EAE mice). B. Only female WT mice have higher P2RX4 expression in the brain compared to male mice (p=0,0004 compared to male WT mice and p=0,0352 compared to male RGS16KO mice). Y-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. * - p-value < 0,05, ** - p-value <0,01, *** - p-value <0,001.*

P2X4R expression on the surface of WT and RGS16KO mouse blood cells was analysed by flow cytometry. The P2X4R expression level was similar in WT and RGS16KO females but it was considerably lower in RGS16KO males compared to WT (Publication III, Figure 6C). Thus, RGS16 was mandatory for P2X4R expression on blood cells in male but not in female mice indicating the sex-dependent control mechanism of RGS16 over P2X4R expression. Further studies should address if this mechanism might be relevant for these genes also in autoimmune neurological disorders.

To conclude, P2RX4 and P2RX7 induction in the spinal cord depended on RGS16 in both sexes but P2X4R expression on PBMC on healthy mice required RGS16 only in males. Therefore, part of the RGS16-regulated pathway of P2X receptors expression is sex-dependent. RGS16 is connected to the regulation of circadian rhythm and anti-viral response (Doi et al., 2011). Both of these functions may be at least partially sex-dependently controlled as circadian disruption induces MS (Hedström et al., 2011) that is more common in women (Harbo et al., 2013) and immune responses to viruses are generally stronger in the female sex (Klein, Flanagan, 2016). So, it would be beneficial to investigate further the sex-associated mechanisms of RGS16 and its connections to purinergic receptors and neuroinflammation.

5 Main conclusions

- The P2X7 purinoreceptor probably originated from the fusion of a P2X4-like gene and a Zn-coordinating cysteine-based domain coding exon during the early evolution of tetrapods and bony fish common ancestors.
- Production and validation of anti-P2X4 receptor monoclonal antibodies provides additional tools to study the presence and function of this receptor in various tissues.
- Eosinophils express the highest level of P2X4 receptor on their cell surface among human peripheral blood leukocytes indicating that ATP-dependent activation might have an essential role in the biology of eosinophils in healthy and pathological conditions.
- P2X4 and P2X7 genes display sex-dependent expression in peripheral blood mononuclear cells of multiple sclerosis patients that are associated with the disease and also with its treatments. Additional studies should verify if such sex-dependent regulations also appear in the central nervous system and have a direct impact on the pathological mechanisms of multiple sclerosis. Besides, different multiple sclerosis treatment options may affect men and women in different ways through purinergic signalling.
- The RGS16 protein is required for the up-regulation of P2X4 and P2X7 genes in the mouse spinal cord by experimental autoimmune encephalomyelitis. The joint impact of these genes on inflammation should be further characterized in the multiple sclerosis context. P2X4 gene expression in the mouse brain and the control mechanism of RGS16 over P2X4 protein expression are sex-dependent.

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Abstract

P2X4 and P2X7 receptors in evolution, physiology, inflammation, and autoimmunity

P2X receptors (including P2X4 and P2X7 receptors) are trimeric ligand-gated cation channels that are activated after the binding of extracellular ATP and participate in numerous biological processes.

P2X4 receptors are widely expressed in most of the tissues in the human body. P2X4 receptors mediate pain and inflammation and modulate cardiac and lung functions. They are implicated in metabolic and neurodevelopmental disorders, neuro-inflammation, auto-immune diseases, and numerous other pathologies. This makes P2X4 receptors potential therapeutic targets in multiple pathologies in which they are involved.

P2X7 receptors are present on various cell types including blood, stem, neural, glial, bone, exocrine, ocular, dental, muscle, endothelial, skin, and renal cells. They are involved in immune reactions and inflammation, they induce numerous cell-specific downstream events, including cell proliferation and death, pro-inflammatory molecule release, phagocytosis, and metabolic events. Therefore, these receptors have crucial roles in health and disease and are also therapeutic targets with much potential in the pathologies in which they are implicated.

With this in mind, all additional knowledge about these receptors is of great importance.

This thesis is based on three publications that aimed to collect new insights and confirm known data about the biology of P2X4 and P2X7 receptors in evolutionary, physiological, inflammatory, and autoimmunological contexts.

It was found that P2X7 gene probably originated from the fusion of a P2X4-like gene and a Zn-coordinating cysteine-based domain coding exon during the early evolution of tetrapods and bony fish common ancestors. Anti-P2X4 receptor monoclonal antibodies were produced and validated to provide additional tools to study the presence and function of this receptor in various tissues. It was shown that eosinophils express the highest level of P2X4 receptor on their cell surface among human peripheral blood leukocytes indicating that ATP-dependent activation might have an essential role in the biology of eosinophils in healthy and pathological conditions.

Sex-dependent expression of P2X4 and P2X7 genes in peripheral blood mononuclear cells of multiple sclerosis patients was detected. The expression changes were caused by the disease but also by its treatment with Interferon-β based drugs and glatiramer acetate. Additional studies should verify if such sex-dependent regulations also appear in the central nervous system and have a direct impact on the pathological mechanisms of multiple sclerosis. It seems that different multiple sclerosis treatment options may affect men and women in different ways through purinergic signalling. It was determined that RGS16 is required for the up-regulation of P2X4 and P2X7 genes in the mouse spinal cord by experimental autoimmune encephalomyelitis. The joint impact of these genes on inflammation should be further characterized in the multiple sclerosis context. P2X4 gene expression in wild-type mouse brains and the control mechanism of RGS16 over P2X4 receptor expression was shown to be sex-dependent.

Lühikokkuvõte

P2X4 ja P2X7 retseptorid evolutsiooni, füsioloogia, põletiku ja autoimmuunsuse kontekstis

Puriinergilised P2X retseptorid (kaasa arvatud P2X4 ja P2X7 retseptorid) on trimeersed ATP poolt aktiveeritavad katioonkanalid, mis osalevad mitmetes bioloogiliste protsessides.

P2X4 retseptoreid leidub inimese keha enamikes kudedes. Nad vahendavad valu ja põletiku protsesse, mõjutavad südame ja kopsu funktsioone, osalevad metaboolsetes ja neuronaalse arengu häiretes, närvikoe põletikes, autoimmuunhaigustes ja paljudes teistes patoloogilistes seisundites. Seetõttu on P2X4 retseptorid olulised ravimiarenduse sihtmärgid.

P2X7 retseptoreid leidub paljudes erinevates rakkudes, nagu näiteks vere-, tüvi-, närvi-, gliia-, luu-, eksokriin-, silma-, hamba-, lihas-, endoteeli-, naha- ja neerurakkudes. Nad võtavad osa immuunreaktsioonidest ja põletikuprotsessidest, kutsuvad esile mitmeid rakupõhiseid muutuseid – rakkude paljunemist, surma, põletikumolekulide vabastamist rakust, fagotsütoosi ja metaboolseid nihkeid. Seega on neil retseptoritel oluline roll nii füsiloogilistes kui patoloogilistes seisundites ja nemadki on olulised ravimiarenduse sihtmärgid.

Need põhjused muudavad P2X4 ja P2X7 retseptorite kohta lisanduva teabe äärmiselt oluliseks.

Käesolev doktoritöö põhineb kolmel publikatsioonil, mille raames koguti uusi teadmisi ja kinnitati juba teadaolevaid fakte P2X4 ja P2X7 retseptorite bioloogiast uurides neid retseptoreid evolutsiooni, füsioloogia, põletiku ja autoimmuunsuse kontekstis.

Töö käigus avastati, et P2X7 retseptor tekkis tõanäoliselt P2X4 retseptorile sarnase eellase ja tsinki siduva tsüsteiine sisaldava domeeni liitumisel neljajalgsete ja luukalade eellaste evolutsiooni käigus. Toodeti ja valideeriti P2X4 retseptori vastased monoklonaalsed antikehad, mis on üheks vahendiks, millega uurida selle retseptori olemasolu ja funktsioone kudedes. Näidati, et perifeerse vere leukotsüütide hulgas ekspresseerivad P2X4 retseptorit raku pinnal kõige kõrgemal tasemel eosinofiilid. See võib tähendada, et ATP aktivatsioon omab eosinofiilide bioloogias olulist rolli nii füsioloogilises kui patoloogilises kontekstis.

Leiti, et P2X4 ja P2X7 geenide ekspressioon hulgiskleroosi patsientide perifeerse vere mononukleaarsetes rakkudes on soospetsiifiline. Sooliseid erinevusi leidus nii haiguse enda kui ka selle ravimite interferoon-β ja glatirameeratsetaadi põhjustatud muutuste hulgas. Järgnevad uuringud peaksid kindlaks tegema, kas soospetsiifilisi erinevusi esineb ka kesknärvisüsteemis ja kas need erinevused mõjutavad hulgiskleroosi patoloogilisi mehhanisme. Lisaks avastati, et P2X4 ja P2X7 geenide üles reguleerimiseks hiirte seljaajus hulgiskleroosi loommudeli eksperimentaalse autoimmuunse entsefalomüeliidi korral on vajalik RGS16 geen. Nende geenide koosmõju hulgiskleroosi põletikuprotsessis vajab edasist täpsemat uurimist. Näidati, et P2X4 geeni ekspressioon metsik-tüüpi hiiret peaajudes ja RGS16 geeni kontrollmehhanism P2X4 retseptori üle on samuti soospetsiifilised.

Appendix

Publication I

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Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates

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P2X purinergic receptors are extracellular ATP-gated ion channel receptors present on the cell plasma membrane. P2X receptors have been found in Metazoa, fungi, amoebas, and in plants. In mammals, P2X7 is expressed by a large number of cell types and is involved in inflammation and immunity. Remarkably, P2X7 does not desensitize as other P2X do, a feature linked to a "C-cysteine anchor" intra-cytoplasmic motif encoded by exon 11. Another specific feature of P2X7 is its C-terminal cytoplasmic ballast domain (exon 13) which contains a zinc (Zn) coordinating cysteine motif and a GDP-binding region. To determine the origin of P2X7, we analyzed and compared sequences and protein motifs of the C-terminal intra-cytoplasmic region across all main groups of Metazoa. We identified proteins with typical ballast domains, sharing a remarkably conserved Zn-coordinating cysteine motif. Apart from vertebrates, these ballast domains were not associated with a typical P2X architecture. These results strongly suggest that P2X7 resulted from the fusion of a P2X gene, highly similar to P2X4, with an exon encoding a ballast domain. Our work brings new evidence on the origin of the P2X7 purinergic receptor and identifies the Zn-coordinating cysteine domain as the fundamental feature of the ancient ballast fold.

Keywords: purinercic receptors, P2X7, evolution, ballast domain, NANOR-like proteins

INTRODUCTION

The P2X7 receptor is the seventh member of the P2X receptor family of ATP-gated cation channels. Brief activation of P2X7R with extracellular ATP in its tetra-anionic form, ATP^{4-}, opens cation-specific ion channels. P2X7 activation requires higher ATP concentrations (0.5 to 1 mM) than for other members of the P2XR family; required concentrations are nanomolar for P2X1 and P2X3, and micromolar for P2X2 and P2X4. In addition, P2X7R does not desensitize while the other P2X receptors like P2X1 and P2X3 desensitize rapidly or like P2X4 for which the desensitization is not as fast. Furthermore, prolonged ligation of P2X7 results in the formation of non-selective pores in the plasma membrane, permeable to molecules up to 900 Da.

Prolonged ATP ligation of P2X7 can trigger membrane blebbing (1) and cell death by apoptosis (2) or lysis/necrosis (3–6) depending on the cell type. In several cell types, for example in

macrophages activated by bacterial products, the main cell death induced via P2X7R stimulation is pyroptosis [(7–9), reviewed in (10)]. However, several studies have shown that P2X7 is able to stimulate growth or promote survival (11, 12). Besides its involvement in cell death or proliferation, P2X7 triggers several biochemical pathways, leading to rapid release of mature IL-1β and IL-18 from macrophages (13, 14), killing of various intracellular pathogens in macrophages (15, 16), and proteolytic cleavage of plasma membrane proteins such as L-Selectin, CD23, TNFα, CD27, matrix metalloproteinase-9, interleukin-6 receptor, and the amyloid precursor protein (17–22). The role of P2X7 in inflammation and infectious diseases has been the subject of numerous studies and has been reviewed thoroughly (10, 23).

One striking feature of P2X7 is its ability to open a non-selective ≪ macropore ≫ after repetitive or prolonged stimulation by ATP. The nature of this non-selective pore has been the subject of numerous studies and remains controversial [reviewed in (24)]. It was hypothesized that P2X7 is able to dilate and form the pore or that non-selective pore formation requires additional molecules such as connexin 43 (25), pannexin-1 (26), or anoctamin 6 (27). However, Karasawa et al. have recently incorporated highly purified panda P2X7 into liposomes and found that ATP stimulation triggered the non-selective pore formation in the absence of other proteins. In addition, a cysteine rich motif, containing C362 and C363, is required for the non-selective macropore opening. These cysteines are palmitoylated, and their mutation to serine abolishes P2X7 capacity to form the macropore (28). The main conclusion of this work is consistent with studies showing that P2X7 stimulation triggers the formation of a non-selective macropore in macrophages from pannexin-1 or connexin 43 knock-out mice (29, 30).

The first crystallographic structure of a P2X receptor showed that zebrafish P2X4 is organized as a homotrimer of P2X4 subunits (31). Crystal structures of a truncated panda P2X7 in the presence of five different antagonists were later reported. They bind to the same hydrophobic pocket away from the ATP binding site acting as allosteric non-competitive inhibitors (32). A major breakthrough was recently achieved by McCarthy et al. (33) who published the first complete structure of the rat P2X7 receptor obtained by single-particle cryoelectron microscopy. The structure of the carboxy-terminal portion of the P2X7 receptor, which is unique to this P2X, defines a novel fold called ≪ ballast ≫ which contains a dinuclear Zn ion complex and a pocket containing a guanosine nucleotide.

P2X genes have been identified across eukaryotes, for example in Metazoa, fungi, amoebas, and plants (34). These receptors share well-conserved structural elements and are activated by ATP (35). Among Metazoa, most species have one or two P2X genes. However, they have been apparently lost in some groups such as insects and nematodes. Jawed vertebrates generally possess seven conserved types of P2X, including P2X7. P2X7 and P2X4 genes are closely linked and encode highly similar membrane receptors (36). While the extracellular domains of all P2X receptors are highly similar, it is important to note that the long intracytoplasmic region of P2X7 was not found in any other P2X, either in vertebrates or other species. Little is known about the function of P2X7 in non-mammalian species. However, P2X7 identified in Ayu (*Plecoglossus altivelis*, an Asian salmoniform), is induced by infection and is involved in ATP dependent cell death, phagocytosis, and bactericidal activity of macrophages (37, 38). Importantly, after transfection in HEK293 cells, seabream or zebrafish P2X7 receptors were unable to induce the maturation and secretion of human or fish IL-1β. However, the chimeric P2X7 receptor composed of the extracellular domain of the seabream P2X7 linked to the intracellular region of the rat P2X7 triggers the maturation and release of both types of IL-1β. These experiments pointed to functional differences between intracellular parts of the rat and fish receptors (39).

In this work, we focused on the carboxy-terminal, intracytoplasmic sequence of P2X7 receptor and its conserved motifs including Zn-coordinating set of Cysteines and the GDP binding domain identified in McCarthy et al. (33). We looked for proteins comprising related domains within vertebrates and beyond. We identified the primordial module from which the P2X7 carboxy-terminal region originated and found its representatives across the main groups of Metazoa.

MATERIALS AND METHODS

Identification and Analysis of Counterparts of the Intracytoplasmic Domain of P2X7

Available EST indices and genome databases were mined using TBLASTN and human or rat P2X7 intracytoplasmic sequences as queries. Searches in EST databases were mainly performed at http://www.ncbi.nlm.nih.gov/. Blast queries on complete genomes were sent to http://www.ensembl.org, http://www.ncbi.nlm.nih.gov/ and http://reefgenomics.org/blast. When relevant genomic regions were identified, potential exons were identified by comparison with known sequences, consensus nucleotide sequences were translated and ORF were compared to predicted protein models. Multiple alignments were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), to analyse the conservation of key residues identified previously in the P2X7 ballast domain. Putative domains and motifs were analyzed based on literature and sequence analysis using Interproscan and Smart programs. Phylogenetic analyses were performed using MEGA version 7 (40).

Linkage Analysis

The next five markers were studied upstream and downstream of each gene containing domains homologous to P2X7 ballast. Sets of paralogs and syntenic homologs of these markers were identified combining the phylogenetic relationships available at Ensembl Metazoa (http://metazoa.ensembl.org/index.html), Genomicus Metazoa (http://www.genomicus.biologie.ens.fr/genomicus-metazoa-30.01/cgi-bin/search.pl) and direct tblastn queries on relevant genomes. Their location relative to homologous ZCD-containing genes within the same species or across species was analyzed to look for conserved synteny set.

Molecular Modeling of the Structure of P2X7 Homologs

Protein structure homology-modeling was performed using the SWISS-MODEL program, accessible via the ExPASy web server

(https://swissmodel.expasy.org/), using as template the structure of the rat P2X7 receptor obtained by single-particle cryoelectron microscopy [PDB ID: 6u9v (33)]. The relevant domains of the models were extracted and compared using Pymol (available at https://pymol.org/2/).

RESULTS

P2X7 C-Terminal Region Comprises a Conserved Zn-Coordinating Cysteine Based Domain

All human P2X sequences contain a typical P2X motif (Interpro IPR001429) encoded by exons 1–10 (**Figures S1A,B**). In this region, exon junctions are highly conserved between the different P2X. In contrast, sequences of P2X C-terminal regions are very variable, with various number of exons and different positions of exon junctions. Thus, exon 12 is not related between P2X, and only P2X5 and P2X7 have a 13th exon: a short one for P2X5 encoding 13 aa, and a long one for P2X7 encoding a 170 aa peptide with multiple conserved cysteines which were shown to be in a tetrahedral geometry likely coordinating a dinuclear Zn ion complex [**Figure S1B** (33)]. We then compared P2X7 sequences across vertebrates, from fin fish to mammals. While we could not find any typical P2X7 in agnathans or in chondrichthyans (sharks and rays), bony fish species have typical P2X7, as well as all studied tetrapods. The P2X motif was highly conserved in these sequences (**Figure S1A**). In contrast, the 3′ end of exon11 and the 5′ end of exon 13 are not well-conserved across vertebrate P2X7. Interestingly, the palmitoylation site [the "C-cys anchor" from McCarthy et al. (33)] located in exon11 in rat P2X7 (motif **S**N**CC**RSHIYPW**C**K**CC**QP**C**) is not conserved across vertebrates (**Figure 1**). This motif is present across mammals (both in eutherians and marsupials, **Figure S2**), although not fully conserved in the elephant. The most conserved part is the initial SxxCC motif, which is also present in some reptiles/birds as well as in Xenopus, while the end of the motif is lost in these species **Figure 1**. In teleost fish, a unique cysteine is conserved at the position of the human C-Cys anchor, but in a different context (LIGTGCYSK). In the spotted gar, which belongs to a basal branch of the fish lineage, the motif is different (FITTYLYPRCCAR), suggesting that the one found in teleosts may have evolved secondarily. In brief, only one C of the exon 11 motif can be found from mammals to fish, and might constitute a conserved palmitoylated site. Exon12 is relatively well-conserved, but does not contain cysteine. In exon 13, encoding the so-called "ballast domain" (33), Zn-coordinating cysteines are overall extremely well-conserved in P2X7 from fish to mammals (**Figure 1**). However, the 5′ side of the exon is highly variable, in length as well as in sequence. This region does not contain any position with a residue present in all species analyzed in **Figure 1** and **Figure S1A**. It is particularly long in cyprinids as observed both in zebrafish (dare) and goldfish (not shown), in EST as well as in genomic sequences. The region encoded by the 3′ end of exon 13 of rat P2X7 also contains a high-affinity guanosine nucleotide binding site (R_{546}-H_{547},R_{574}xxR_{578}xxxxxK_{583}) (33). Interestingly, the R_{546}-H_{547} motif is well-conserved across vertebrates except frogs (**Figure 1**), and R_{578} is present in most analyzed species. We then used SwissModel to produce structural models of this region from zebrafish P2X7, using the cryo-EM structure of the rat P2X7 (33) as a template. Superimposition of this model with the structure of the human GDP-binding region shows a very good fit (**Figures 2A,B**), with the conserved residues standing in similar positions. It is important to note that the region between the R_{546}-H_{547} and the $R_{574}R_{578}K_{583}$ motifs is overall well-conserved, with a WRF motif always present across vertebrate P2X7 (**Figure 1**). Thus, although residues coordinating GDP in rat are not all conserved in fish, the structure of this part of the protein may remain compatible with GDP binding.

Based on these data, we therefore defined a highly conserved region located in exon13 which contains most of the Zn-coordinating cysteines in human P2X7. The three clusters of cysteines have the following consensus sequences: (1) the first one contains the motif PxW**C**x**C**x$_2$**C**, (2) the second one L**CC**Rx3Gx**C**ITTS/T (3) the last motif is composed of (L/I/V)PS**C**(**C**/S)x$_3$IRx$_2$(F/Y)Px$_5$Y(S/T)G. This region—we name "Zn-coordinating cysteine based domain" (ZCD) hereafter—contains seven/eight conserved C residues (**Figure 1**) but does not comprise the C-cys anchor motif.

P2X7 ZCD Is Found in Only a Few Other Proteins Within Vertebrates

To get more functional insights about P2X7 C-terminal region, we first looked for its association with other domains across vertebrate proteomes. To this purpose, we first performed blast searches using the human P2X7 ZCD as bait. We mainly detected P2X7 proteins in all tested tetrapods and in most bony fish genomes. However, in zebrafish, two additional proteins were detected, which contains only ZCD: nnr (nanor; ENSDARG00000058917; chromosome 15:1589899) and nanor-b (ENSDARG00000076264; chromosome 22:4797625). These proteins also showed a very well-conserved ZCD motif, as found in P2X7 (**Figure 3**). Nanor genes have Ensembl orthologs in several other fish species, in the coelacanth and in an agnathan, the hagfish. It seems that nanor-like genes are duplicated in most species in which they are present (**Figure S3**). Considering their genomic context, only two markers were conserved close to nanor-like paralogs: one between medaka and mangrove rivulus, and one between electric eel and medaka. Sequences with a significant level of similarity were found close to nanor genes in other fish species, but they were not true orthologs (**Figure S3**). Thus, nanor and related genes apparently do not belong to a synteny block conserved across vertebrate groups, not even across bony fish. No conserved synteny was detected between nanor and p2X7 genes either. Further blast searches identified also additional sequences with ZCD in cartilaginous fish: in a ray (*Raja erinacea;* GH269666), and in the elephant shark (*Callorhinchus milii*; ENSCMIG0000001795).

The ZCD present in all these proteins actually defines a Panther family (PTHR36981). While blast search did not find any obvious counterpart of nanor sequences in tetrapods,

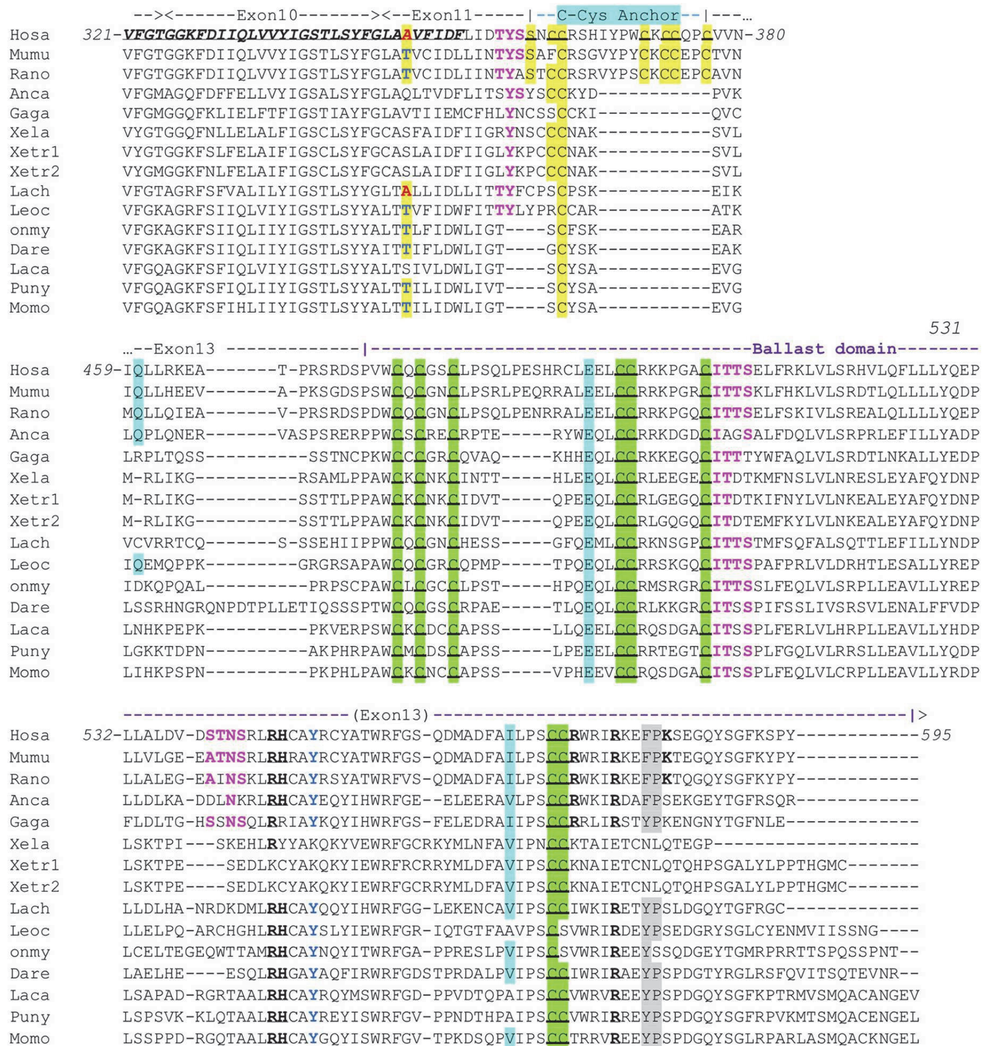

FIGURE 1 | Conserved motifs in P2X7 C-terminus from representative vertebrate species. Exon limits and functional domains are indicated above sequence alignment. Positions within the human P2X7 protein are indicated. The second TM domain is in bold italic and underlined. Key residues of the C-Cyst Anchor are boxed and represented in black on yellow background (33). Zn-coordinating cysteines which define ZCD are boxed and highlighted in green (33). Key residues of the GDP binding motif are in bold underlined (33). Conserved Y_{550} is in blue (41, 42), F/$Y_{581}P_{582}$ are highlighted on gray background (43), β arrestin binding sites [T_{357}YSS, I_{507}TTS, A_{540}TNS (42)] are in purple and bold. Residues Q460, E496, and I568 are highlighted on light blue background. Their importance is based on the identification of three loss of function polymorphisms of the human P2X7 gene, Q460R, E496A, and I568N (44). Interestingly, the A348 residue (in red on yellow background) is replaced by a T in several mammal and fish species (in blue on yellow background), indicating it constitutes an ancestral variation. In human, the A348T substitution is associated to a major gain of function (45). Sequences: Mammals: Human, *Homo sapiens* (hosa, ENSG00000089041); Mouse, *Mus musculus* [mumu (ENSMUSG00000029468)]; Rat, *Rattus norvegicus* (rano, ENSRNOG00000001296). Reptiles and birds: Carolina anole, *Anolis carolinensis* (anca, ENSACAG00000022072); Chicken, *Gallus gallus* (gaga, ENSGALG00000003863). Amphibians: Clawed frog, *Xenopus laevis* (xela, NP_001082196); *Xenopus tropicalis* (xetr1: ENSXETG00000001030, xetr2: ENSXETG00000001030) Crossopterygians: Coelacanth, *Latimeria chalumnae* (lach, XP_005989088). Bony fishes: Spotted gar, *Lepisosteus ocelatus* (leoc, ENSLOCG00000006925); Rainbow trout *Oncorhynchus mykiss* (Onmy; XP_021461359); Zebrafish, *Danio rerio* (dare, ENSDARG00000042440); Barramundi perch, *Lates calcarifer* (laca, ENSLCAG00010007049); Makobe Island cichlid, *Pundamilia nyererei* (puny, ENSPNYG00000007433); Sunfish, *Mola mola* (Momo, ENSMMOG00000014797).

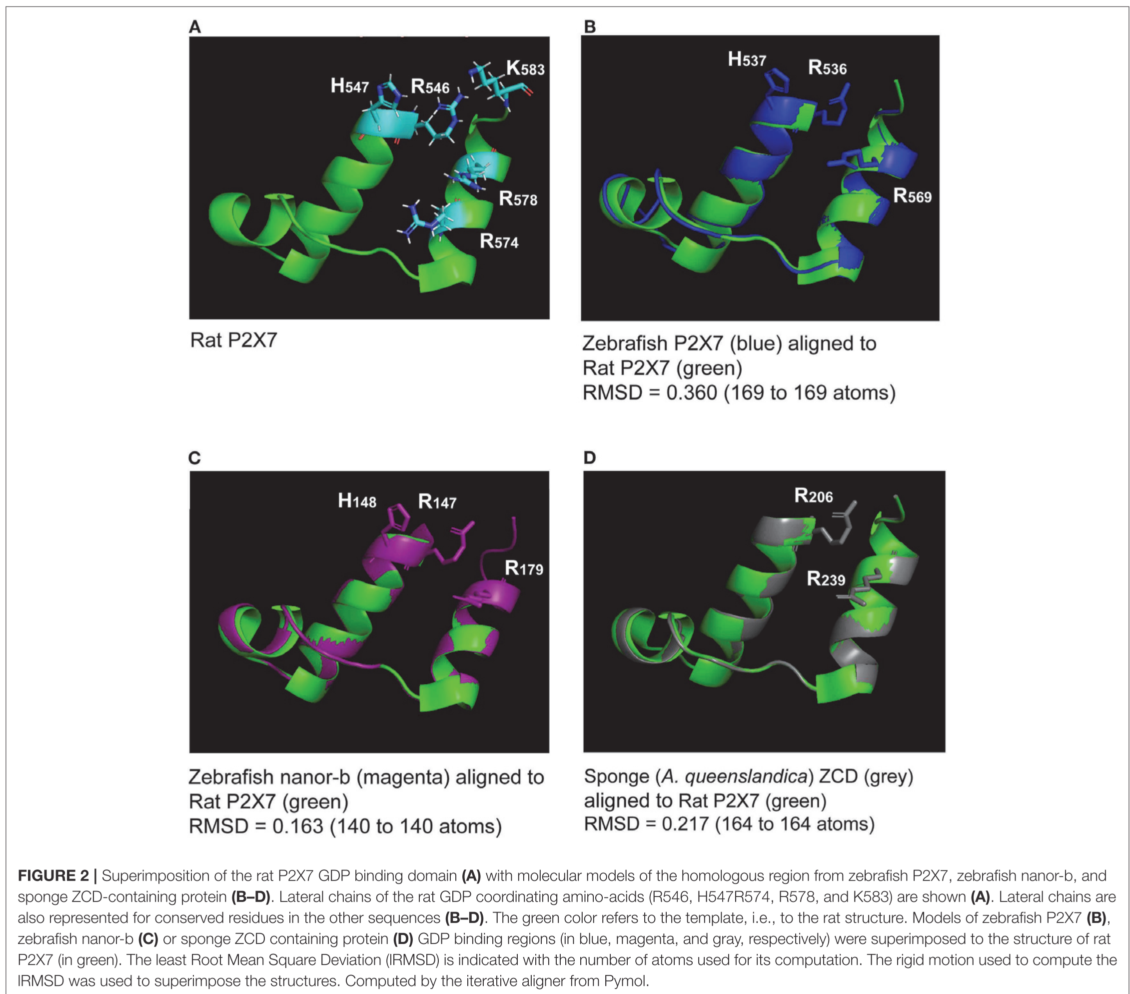

FIGURE 2 | Superimposition of the rat P2X7 GDP binding domain **(A)** with molecular models of the homologous region from zebrafish P2X7, zebrafish nanor-b, and sponge ZCD-containing protein **(B–D)**. Lateral chains of the rat GDP coordinating amino-acids (R546, H547R574, R578, and K583) are shown **(A)**. Lateral chains are also represented for conserved residues in the other sequences **(B–D)**. The green color refers to the template, i.e., to the rat structure. Models of zebrafish P2X7 **(B)**, zebrafish nanor-b **(C)** or sponge ZCD containing protein **(D)** GDP binding regions (in blue, magenta, and gray, respectively) were superimposed to the structure of rat P2X7 (in green). The least Root Mean Square Deviation (lRMSD) is indicated with the number of atoms used for its computation. The rigid motion used to compute the lRMSD was used to superimpose the structures. Computed by the iterative aligner from Pymol.

three sequences from Xenopus belong to the Panther family PTHR36981 (**Figure 3**). Phylogenetic analyses identified three clusters of ZCD-containing proteins: (1) typical P2X7 found from bony fish to mammals, (2) nanor-like proteins, which contain only the Zn-coordinating domain and a short additional N-ter region, and (3) three Xenopus proteins of similar structure, without P2X-like domain, which form a distinct group (**Figure 4**). Although these frog sequences do not contain an N-terminal P2X motif, they are not highly similar to fish nanor sequences, and are in fact more distant from them than the hagfish nanor-like sequence.

Key residues for GDP binding identified in rat P2X7 are not all conserved in nanor-like proteins. Residues homologous to rat R546H547 and R578 are quasi-conserved, H being sometimes replaced by Y (**Figure 3**). In contrast, rat R574 and K583 which are not present in amphibians and fish P2X7, are also absent in nanor sequences. However, superposition of the Swissmodel of the region from zebrafish nanor-b with its counterpart in rat P2X7 suggests that these structures may be rather similar, allowing GDP binding (**Figure 2C**).

Altogether, these data show that the ZCD seen in vertebrate P2X7 is also found in a few shorter proteins containing no other domains, in species belonging to Agnathans, Chondrichtyans, bony fish and tetrapods (in Amphibians). However, these genes are apparently absent in amniotes.

The P2X7 ZCD Is an Ancient Module Present Across All Main Divisions of Metazoa

We then looked for ZCD in proteins from other groups of Metazoa. Within deuterostomians, such sequences were

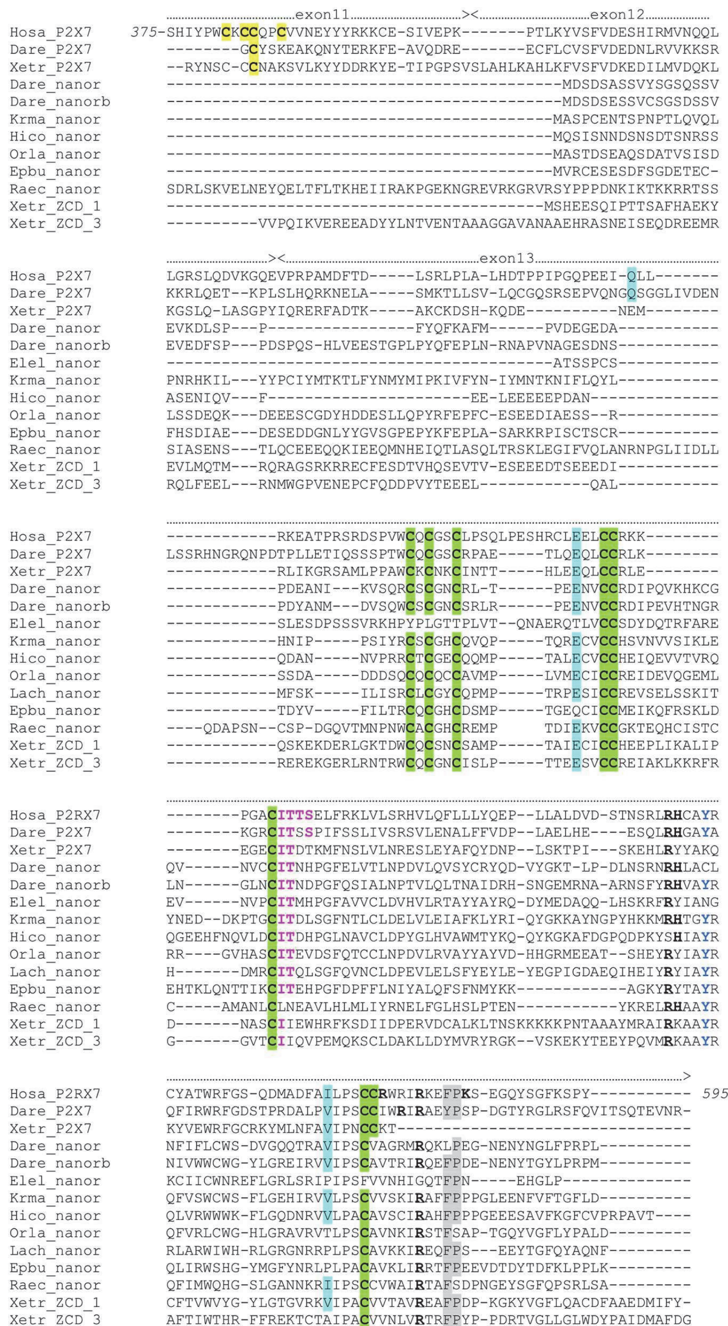

FIGURE 3 | Multiple alignment of P2X7 Cter region with nanor proteins, and frog ZCD-containing proteins. Exon limits and functional domains are indicated above sequence alignment. Positions within the human P2X7 protein are indicated. Key residues of the C Cyst Anchor are boxed and represented in black on yellow

(Continued)

FIGURE 3 | background in P2X7 sequences. Zn-coordinating cysteines which define ZCD are boxed and highlighted in green. Key residues of the GDP binding motif are in bold underlined. Conserved Y_{550} is in blue, F/$Y_{581}P_{582}$ are highlighted on gray background, the β arrestin binding site I_{507}TTS, is in purple and bold. Residues Q460, E496 and I568 are highlighted on blue background. Their importance stems from the identification of three loss of function polymorphisms of the human P2X7 gene: Q460R, E496A, and I568N (44). Sequences: Hosa_P2X7, human (ENSG00000089041); Leoc_P2X7, spotted gar (ENSLOCG00000006925); Dare_P2X7, zebrafish (ENSDARG00000042440); Xetr_P2X7, Tropical clawed frog (ENSXETG00000032525), Dare_nanor, zebrafish (ENSDARG00000058917); Dare_nanor-b, zebrafish (ENSDARG00000076264); Elel_nanor, Electric eel (ENSEEEG00000011457); Krma_nanor, mangrove rivulus (ENSKMAG00000012512); Hico_nanor, tiger tail seahorse (ENSHCOG00000018472); Orla_nanor, japanese medaka (ENSEBUG00000006500); Lach_nanor, coelacanth (ENSLACG00000022457); Epbu_nanor-like, Hagfish (ENSEBUG00000006500); Raec_nanor, little skate (GH269666); Xetr_Palm_ 1, Tropical clawed frog, A0A1B8XY14; Xetr_Palm_3, Xenopus, EL845305.

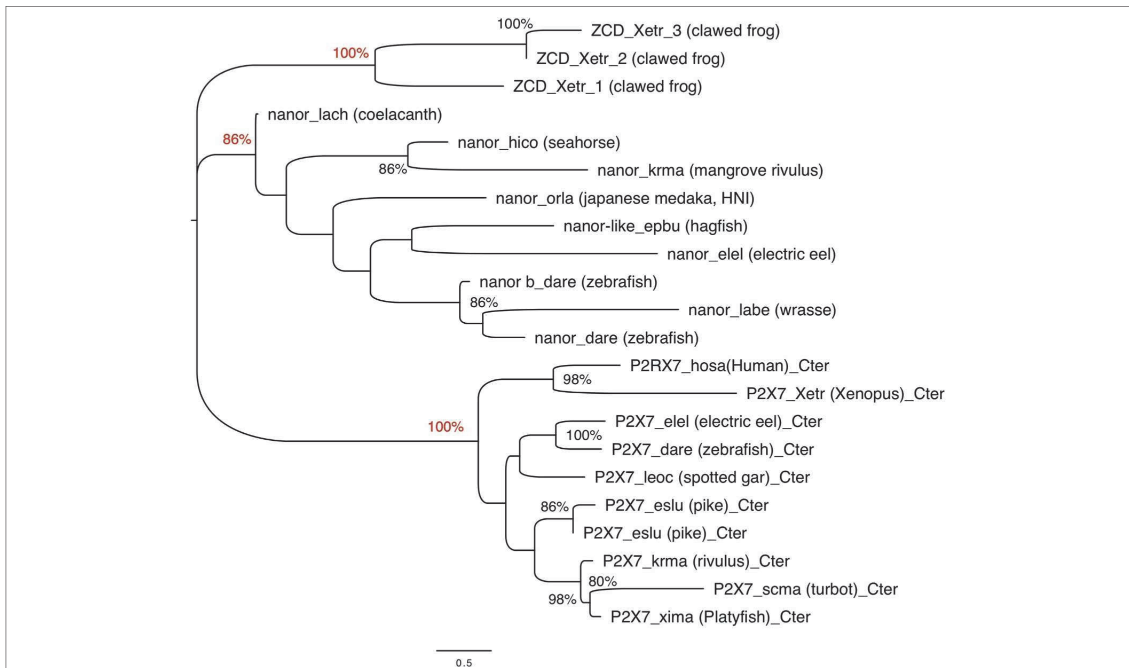

FIGURE 4 | Phylogenetic analysis of P2X7 and nanor-like sequences from vertebrates. The optimal tree (Neighbor-Joining method, 100 bootstrap) is shown. Bootstrap values higher that 80% are indicated at nodes, and key positions are in red. The distances were computed by Mega6 using the JTT matrix-based method and are in the units of the number of aa substitutions per site. Sequences are as follows: nanor_dare (zebrafish, ENSDARG00000058917); nanor b_dare (zebrafish,ENSDARG00000076264); nanor_labe (ballan wrasse,ENSLBEG00000021957); nanor_elel (Electric eel, ENSEEEG00000011457); nanor-like_epbu (Hagfish, ENSEBUG00000006500);nanor_orla (japanese medaka, ENSEBUG00000006500); nanor_krma (mangrove rivulus, ENSKMAG00000012512); nanor_hico (tiger tail seahorse; ENSHCOG00000018472); nanor_lach (coelacanth, ENSLACG00000022457); ZCD-containing protein 1,ZCD_xetr_1, A0A1B8XY14; ZCD-containing protein 2, ZCD_xetr_2, F6R9E3; ZCD-containing protein 3, ZCD_xetr_3, EL845305; P2X7_hosa, human (ENSG00000089041); P2X7_xetr, xenopus tropicalis (ENSXETG00000032525), P2X7_leoc, spotted gar (ENSLOCG00000006925); P2X7_dare (zebrafish) (ENSDARG00000042440); P2X7_elel, Electric eel (ENSCMIG00000006667);P2X7_eslu, pike (ENSELUG00000001869); P2X7_eslu, pike (ENSELUG00000018695); P2X7_krma, mangrove rivulus(ENSKMAG00000003524); P2X7_scma turbot (ENSSMAG00000016255); P2X7_xima platyfish (ENSXMAG00000016836).

found in an echinoderm, the sea urchin *Strongylocentrotus purpuratus*, and in the acorn worm *Saccoglossus*. All Zn-coordinating cysteines found in P2X7 and nanor-like sequences were conserved in these proteins (**Figure 5**). In protostomes, no such domain could be detected from *C. elegans* or *Drosophila* databases. However, ZCD was found in proteins from the oyster *Crassostrea gigas* and from the limpet *Lottia gigantea,* indicating that it is present in mollusks. Among Ecdysozoa, the motif was found in arthropods–in the mite *Ixodes scapularis* and in a true bug (*Myzus persicae*)—as well as in nematods *Haemonchus contortus* and *Ancylostoma caninum* (**Figure 5**). Surprisingly, it may be absent from holometabole insects since we could not find it in dipterans (flyes), hymenopterans (ants, bees and wasps) or coleopterans (beetles). ZCD was also found in a sponge (*Amphimedon queenslandica*) and in a number of Cnidaria (**Figure 5**), but not in *Trichoplax adhaerens* (a placozoan) or in *Mnemiopsis leidyi* (a ctenophore). All these ZCD containing proteins belong to the Panther family PTHR36981, as nanor and nanor-like proteins. The Zn-coordinating cysteines were remarkably conserved in all these sequences. In contrast,

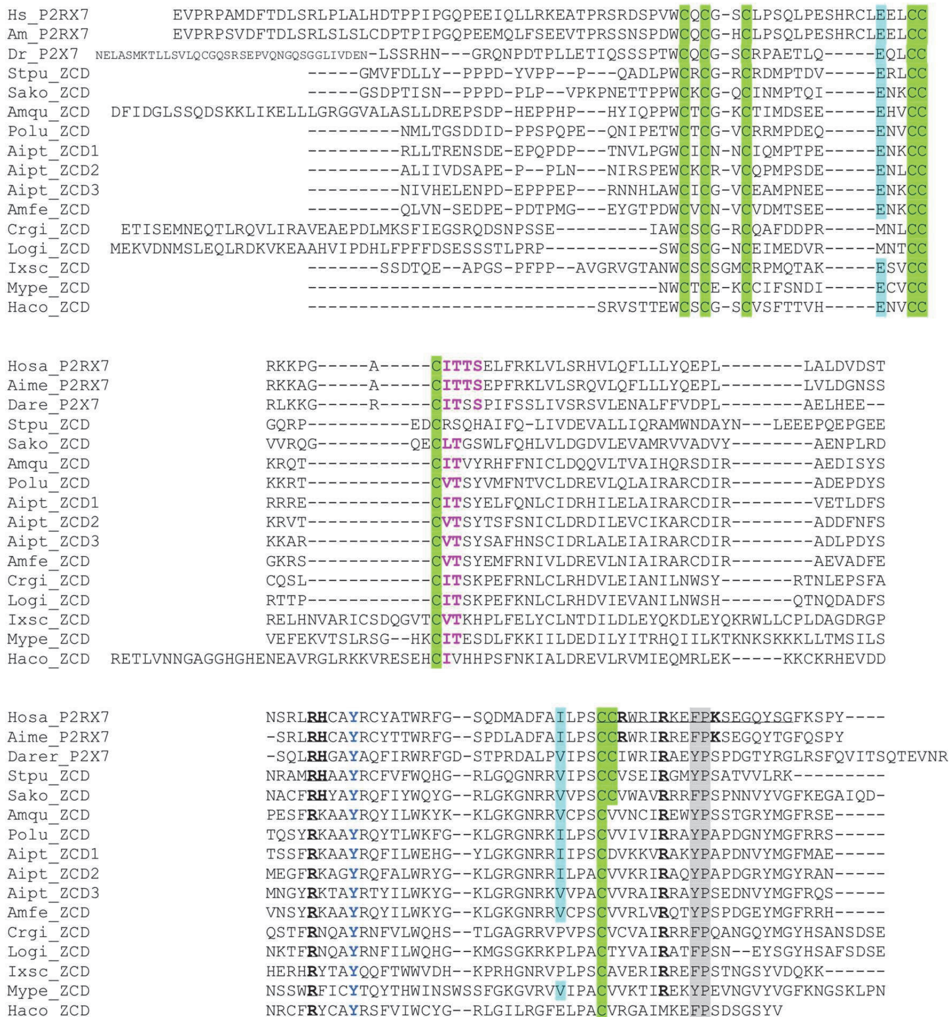

FIGURE 5 | Multiple alignment of ZCD domains across Metazoa. Zn-coordinating cysteines which define ZCD are boxed and represented in black on green background. Key residues of the GDP binding motif are in bold underlined. Conserved Y_{550} is in blue, $F/Y_{581}P_{582}$ are highlighted on gray background, the β arrestin binding site I_{507}TTS is in purple and bold. Residues E496 and I568 are highlighted on blue background. Their importance stems from the identification of two loss of function polymorphisms of the human P2X7 gene: E496A and I568N (44). Sequences: from Deuterostomes [Hosa_P2X7, human (ENSG00000089041); Rano_P2X7, rat (ENSRNOG00000001296); Aime_P2X7, panda (ENSAMEG00000014102); Dare_P2X7, zebrafish (ENSDARG00000042440); Stpu_ZCD, sea urchin (SPU_000568); Sako_ZCD, accorn worm (XP_002733146)]; from Protostomes [Crgi_ZCD, oyster (Cg_CGI_10024238); Logi_ZCD, Lottia (LotgiG163261); Ixsc_ZCD, mite (XP_029850345), Mype_ZCD, a hemiptere insect (XP_022182386); Haco_ZCD, a *Haemonchus* nematode (VDO68347)]; and from basal groups of Metazoa [Amqu_ZCD, sponge (Aq_Aqu2.1.01189); Polu_ZCD, *Porites lutea* (Pl_ut2.m8.33056, see http://reefgenomics.org/blast/#result) (Cnidaria); Aipt_ZCD, *Aiptasia* sp., AIPGENE13307 (Cnidaria) and Amfe_ZCD, *Amplexidiscus fenestrafer* (scaffold 1288, see http://reefgenomics.org/blast/#result) (Cnidaria)].

among residues coordinating the binding of GDP in rat, only R546 and R578 were conserved. As for zebrafish P2X7 and NANOR-B, we superimposed the structure of this region from rat P2X7 to a model build from the sponge ZCD. **Figure 2D** shows that the conserved R residues stand in similar configuration in the model and in the rat structure. Although H547, R554, and K583 were not found in invertebrates, the region comprises several highly conserved positions including: A549, Y550, Y/F553, W559 I/V568, P570, and C572 (**Figure 5**). Our data therefore suggest that the structure of this region in the sponge ZCD may be conserved and may also bind a GDP.

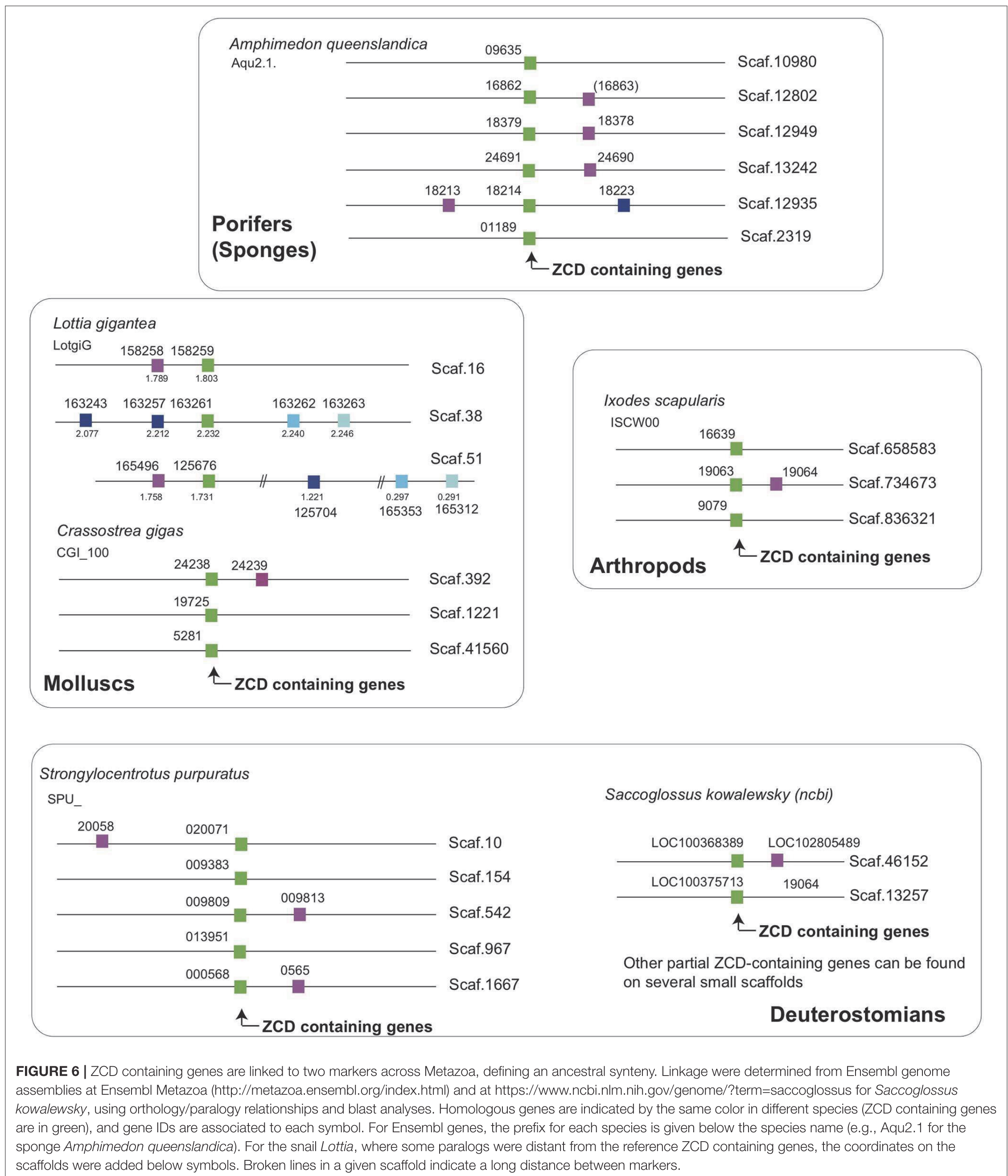

FIGURE 6 | ZCD containing genes are linked to two markers across Metazoa, defining an ancestral synteny. Linkage were determined from Ensembl genome assemblies at Ensembl Metazoa (http://metazoa.ensembl.org/index.html) and at https://www.ncbi.nlm.nih.gov/genome/?term=saccoglossus for *Saccoglossus kowalewsky*, using orthology/paralogy relationships and blast analyses. Homologous genes are indicated by the same color in different species (ZCD containing genes are in green), and gene IDs are associated to each symbol. For Ensembl genes, the prefix for each species is given below the species name (e.g., Aqu2.1 for the sponge *Amphimedon queenslandica*). For the snail *Lottia*, where some paralogs were distant from the reference ZCD containing genes, the coordinates on the scaffolds were added below symbols. Broken lines in a given scaffold indicate a long distance between markers.

As noted above for vertebrates, only one or a few (<10) ZCD-containing genes were found in each species. ZCD was typically found in relatively short proteins without other conserved domains and without transmembrane region, in contrast to P2X7. The only exceptions were found in the mite *Ixodes scapularis* in which two ORF comprised, respectively, a

TolA (XP_029850345) or TAHP(XP_029850348) domain at the N-terminus, with the Zn-coordinating cysteines region at the C-terminus. The ZCD domain is always found at the C-terminus of the protein, while the N-terminal parts were of variable length and without obvious homologs.

Interestingly, two markers located close to ZCD-containing genes from the sponge *Amphimedon queenslandica* had orthologs in the neighborhood of genes encoding ZCD in molluscs, arthropods and echinoderms (**Figure 6**). These linkages identify an ancestral association of ZCD containing genes with other markers that support a unique common origin.

Overall, phylogenetic analysis of ZCD containing protein sequences identified groups corresponding to the taxonomic divisions (sponges and cnidarians, mollusks, arthropods). Interestingly, zebrafish nanor-b clustered with arthropods sequences, and not with ZCD from deuterostomians, or with ZCD from vertebrate P2X7 (**Figure 7**).

The P2X7 Cytoplasmic Tail: LPS Binding Domain, TNFR Domain, and Others

In human and murine P2X7 sequences, Denlinger et al. (46) reported that the end of exon 13 contains a motif [positions 573–590] similar to the LPS binding domain of the LPS binding protein (LBP) and of the bactericidal/permeability-increasing protein (BPI). They showed that the corresponding peptide bind LPS *in vitro*, and could block LPS-mediated activation of ERK kinases in RAW 264.7 macrophages (46). Thus, these authors proposed that the F, W, and G and the basic conserved residues were critical for the function of the receptor. To study the evolution of this motif, we aligned sequences of human P2X7, LBP, and BPI with fish P2X7, LBP, and BPI, and with other ZCD (**Figure 5**). While the consensus proposed by Denlinger et al. based on human and murine P2X7, LBP, and BPI is not fully consistent with fish sequences, our alignment indicates that a **WRIR**x(5)**G** consensus is conserved across P2X7 sequences, but not in the other ZCD containing proteins. In rat, this putative LPS binding region overlaps with residues of the high-affinity guanosine nucleotide binding site: R_{546}-H_{547}, and R_{574}-R_{578}-K_{583}. As across vertebrate P2X7, R_{546} is remarkably conserved across ZCD containing proteins, but the other residues involved in GDP binding in rat are not found in those sequences (**Figure S4**). The presence of a LPS binding domain in the ballast domain is not obviously supported by recent structural data (47), and direct experiments with proteins from multiple species would have to be done to clarify this point.

In addition to the ZCD canonical cysteine based motif, two other motifs were strikingly conserved across Metazoa: the Y_{550} [position from human P2X7 (41, 42)], and the $F/Y_{581}P_{582}$ [positions from human P2X7 (43); **Figures 1**, **3**, and **5**]. Interestingly, tyrosine phosphorylation of HSP90 was

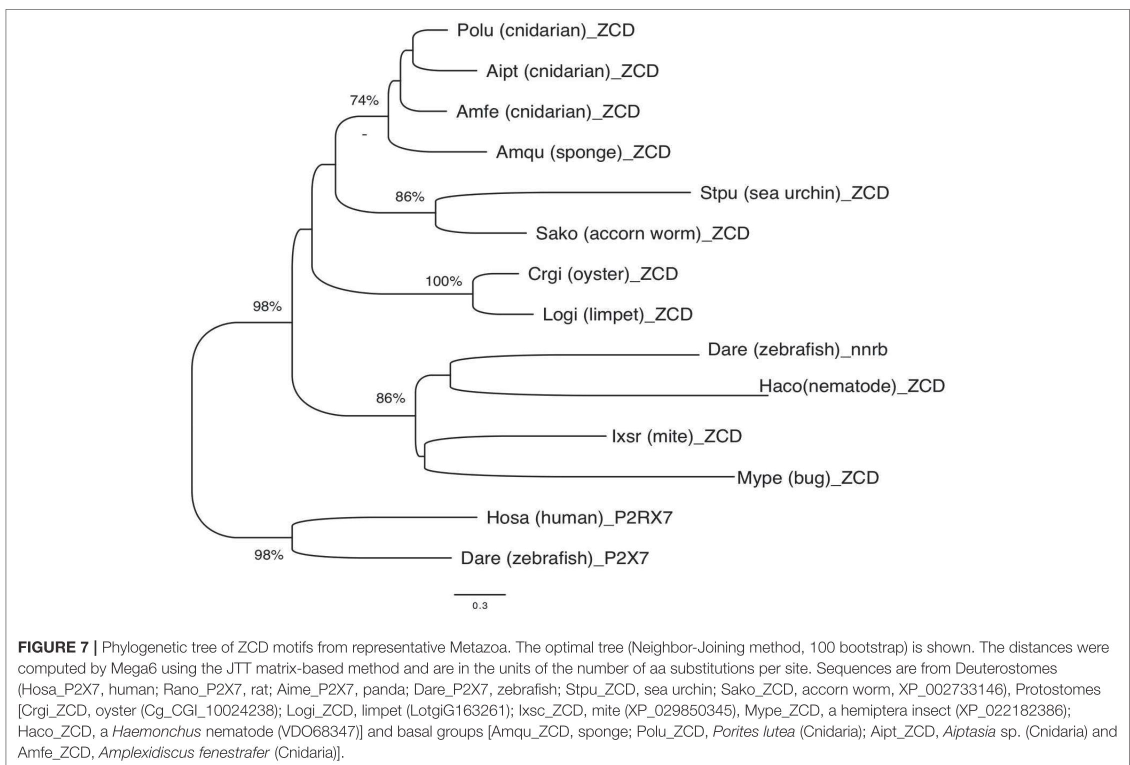

FIGURE 7 | Phylogenetic tree of ZCD motifs from representative Metazoa. The optimal tree (Neighbor-Joining method, 100 bootstrap) is shown. The distances were computed by Mega6 using the JTT matrix-based method and are in the units of the number of aa substitutions per site. Sequences are from Deuterostomes (Hosa_P2X7, human; Rano_P2X7, rat; Aime_P2X7, panda; Dare_P2X7, zebrafish; Stpu_ZCD, sea urchin; Sako_ZCD, accorn worm, XP_002733146), Protostomes [Crgi_ZCD, oyster (Cg_CGI_10024238); Logi_ZCD, limpet (LotgiG163261); Ixsc_ZCD, mite (XP_029850345), Mype_ZCD, a hemiptera insect (XP_022182386); Haco_ZCD, a *Haemonchus* nematode (VDO68347)] and basal groups [Amqu_ZCD, sponge; Polu_ZCD, *Porites lutea* (Cnidaria); Aipt_ZCD, *Aiptasia* sp. (Cnidaria) and Amfe_ZCD, *Amplexidiscus fenestrafer* (Cnidaria)].

significantly increased when this protein was associated to the P2X7 mutant Y_{550} F, compared to the wild-type complex (41). In contrast, β arrestin binding sites [T_{357}YSS, I_{507}TTS, A_{540}TNS in human P2X7 (42)] were not conserved: only I_{507}TTS was found beyond mammals, but it was not present in nanor-like proteins or in other ZCD outside deuterostomians.

Other motifs were proposed [reviewed in (48)]. They include a potential Src homology 3 (SH3) Domain binding region somewhat similar to the death domain of TNFR, with a PxxP motif located on the 5'side of exon 13 (position 441) (46). These motifs are not conserved in other ZCD containing proteins and their presence is not supported by the recent report of the rat P2X7 structure.

Interestingly, several single nucleotide polymorphisms of the human P2X7 gene have been identified [reviewed in (44)]. Three loss of function polymorphisms have been discovered in the human exon 13: Q460R, E496A, and I568N. We compared these variable sites across human P2X7 haplotypes with the corresponding sites in sequences found in multiple species, to get insights into their level of evolutionary conservation.

Homozygous E496A substitution leads to a significant reduction of P2X7 function in multiple leucocytic cells, with loss of P2X7-dependent non-selective pore formation (49). As seen in **Figure 1**, E496 is strictly conserved in all vertebrate P2X7 sequences emphasizing the importance of this residue for P2X7 signaling (49). In addition, E496 is also conserved in nanor and frog ZCD (**Figure 3**) as well as in ZCD across Metazoa (**Figure 5**). Intriguingly, E496A is a relatively frequent substitution in human P2X7 (45).

I568 is also an important amino-acid because its mutation to N (I568N) inhibits P2X7 plasma membrane expression and normal trafficking (50). I568 forms a di-leucine trafficking/sorting motif (-LL or -IL) (51). This residue is conserved or replaced by another hydrophobic residue, valine, in most P2X7 vertebrate sequences (**Figure 1**) as well as in ZCD across Metazoa (**Figure 5**). In human, this substitution is uncommon, being found in 2–3% of the Caucasian population (50). Altogether, this suggests that I568 is highly conserved across species and within the human population.

Finally, the association of the human Q460R polymorphism with bipolar and depressive disorders remains highly controversial as discussed in Stokes et al. (45). Residue Q460 is not well-conserved in vertebrate P2X7 sequences and ZCD domains across Metazoa.

Altogether, our data underscore the conservation of the ZCD across Metazoa sequences similar to P2X7 exon 13, and suggest that it may constitute the primordial pattern of the so-called ballast domain.

DISCUSSION

Our data indicate that an ancient domain containing a conserved Zn-coordinating cysteine-based motif has been captured by a P2X4-like sequence during vertebrate evolution—possibly after the divergence of agnathans and jawed vertebrates and before the divergence between bony fish and tetrapods. Our data provide an evolutionary perspective about the variation and functional importance of the intracellular C-terminal part typical of the purinergic receptor P2X7.

Origins of P2X7 ZCD

Our data show that the ZCD motif present in the last exon of P2X7 is an ancient domain already present in the proteome of Sponges and Cnidaria (Sea anemones, jellyfish, and corals). The cysteine based motif coordinating two Zn ions (33) is strikingly conserved not only in all P2X7, but also in all other ZCD sequences we found from sponges to fish across the main groups of Metazoa: sponges, cnidaria, arthropods and nematodes, molluscs, and deuterostomians (echinoderms, acorn worms, and vertebrates). This signature is designed as PTHR36981 in the PANTHER (Protein ANalysis THrough Evolutionary Relationships) protein Classification System, and comprise P2X7, NANOR-like proteins, and unnamed proteins from multiple groups of Metazoa. This Panther ID (http://www.pantherdb.org/panther/family.do?clsAccession=PTHR36981) was not associated to a biological function. Besides P2X7, the only ZCD containing gene for which functional information is available is the zebrafish *nnr* (*nanor*), a zygotic gene expressed at the midblastula transition: the presence of a myristoylation site and Zn-coordinating motifs in NANOR led to the hypothesis of a role in transcription regulation (52). We were unable to detect this domain in fungi, plants, or bacteria. It was not found either in *Monosiga brevicollis,* a choanoflagellate; these protozoans are similar to the choanocytes of sponges, and constitute close relatives of Metazoa. Hence, ZCD seems to be a generic invention of Metazoa like a number of other domains (53).

ZCD are mostly present in relatively short proteins which do not contain other known domains, with the exception of P2X7 and two other proteins from the mite *Ixodes scapularis*. Although sequences of ZCD containing genes seems to be partial in many genomes and should be confirmed in future assemblies, several paralogs were generally found in most species, as in the snail *Lottia*. We could not find well-conserved synteny blocks shared by all ZCD containing genes. P2X7 is located in a relatively stable genomic context across jawed vertebrates. In contrast, nanor-like genes found in many bony fish and in hagfish do not appear to be part of a conserved synteny block. Interestingly, ZCD containing genes were found in association with two closely linked markers across invertebrates, from sponges to mollusks and sea urchin. Overall, our observations indicate that this association is probably the ancestral configuration, which was disrupted by later recombination and duplications. While our observations suggest that ZCD domains have been duplicated multiple times during evolution, they apparently did not expand into large multigenic families. Interestingly, these proteins seem to have been lost in entire groups of animals, such as holometabole insects as well as in smaller taxonomic groups; also, nanor-like genes were apparently absent from many species of bony fish. Besides, they were not seen in basal phyla such as placozoa and ctenophores.

Are ZCD Conserved GDP-Binding Domains?

The recent report by McCarthy and colleagues demonstrates that P2X7 cytoplasmic ballast domain contains a high-affinity guanosine binding site (33). Our data raise the issue of the conservation of this site in ZCD/ballast-like domains, which we found across Metazoa. The residues interacting with GDP in rat P2X7 ($R_{546}H_{547}R_{574}R_{578}K_{583}$) are not all conserved in P2X7 of other species: while $R_{546}H_{547}$ R_{578} are generally present except in frog sequences, the two other residues are lost outside mammals. In other ZCD, only $R_{546}H_{547}$ and R_{578} were conserved in sea urchin and in the accorn worm *Saccoglossus*, and only R_{546and} R_{578} beyond deuterostomians. Interestingly, molecular models of this region suggests that the two alpha helix in which these key residues are located are overall conserved in all ZCD, as well as the position of the two conserved Arginine. Further work will be required to demonstrate that the ZCD/ballast like domains of fish P2X7, nanor-like proteins, and other ZCD containing proteins from non-vertebrates do indeed bind GDP. This would represent a significant addition to the repertoire of membrane guanine nucleotide binding proteins involved in signal transduction.

Evolution of the Poly-Palmitoylated Motif

In rat P2X7, at least four cysteines (C362, C363, C374, and C377) and one serine (S360) are palmitoylated in the region 360–377 named C-cys anchor by McCarthy et al. (33). This 18-AA region starts where the second TM region enters the cytoplasm, and anchors the protein to the intracellular face of the plasma membrane. Importantly, the palmitoylated residues within this region are required to maintain a specific property of P2X7, the absence of desensitization after ATP stimulation. This characteristic, which is not observed for other P2X, does not involve the ballast domain. Indeed, when transfected into Xenopus oocytes, a P2X7 lacking the ballast domains displays ATP binding curves, ion selectivity, and lack of desensitization comparable to P2X7 WT (33). In contrast, P2X7-ΔC-cys lacking the C-cys anchor - or P2X7 in which each palmitoylated residues was mutated to alanine–desensitized very quickly after ATP stimulation (33). Interestingly, our comparison of P2X7 sequences across vertebrates show that the C-cys-anchor, encoded by exon 11, is not highly conserved since the number of cysteines is variable and reduced to only one in several fish species. Interestingly, zebrafish or seabream

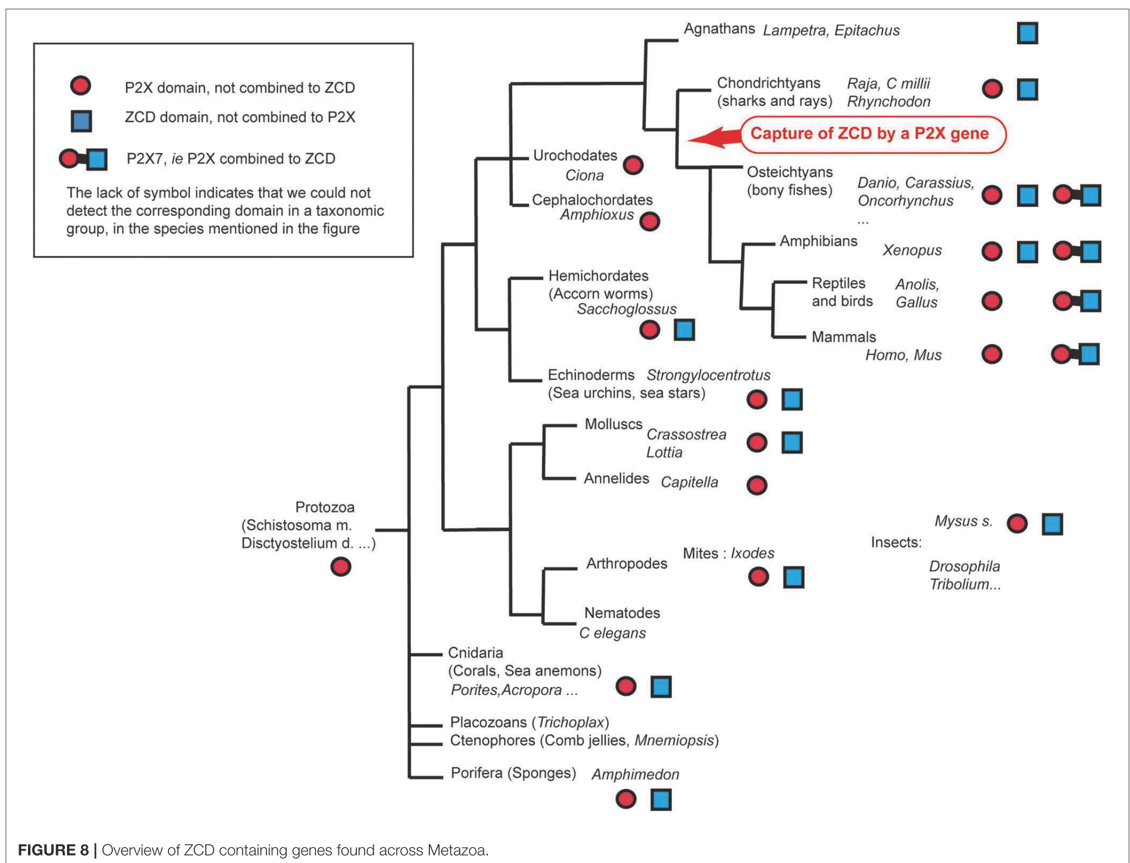

FIGURE 8 | Overview of ZCD containing genes found across Metazoa.

P2X7 receptors transfected in HEK293 cells were unable to desensitize after stimulation by ATP or BzATP, as observed for mammalian P2X7 (39). Furthermore, this lack of desensitization was also found when seabream P2X7 constitutively expressed by a seabream fibroblast line was stimulated (39). These results strongly suggest that the palmitoylation of a unique cysteine can be sufficient to maintain a lack of desensitization of the fish P2X7. Alternatively, palmitoylation of the serine and threonine residues located upstream of the cysteine in fish P2X7 sequences could create a poly-palmitoylation anchor and lead to lack of desensitization. Overall, our data support the idea that palmitoylation and lack of desensitization, which constitute specific features of P2X7, have been added to this receptor independently of the capture of the ancient ballast domain.

CONCLUSION

P2X7 is found across vertebrates from bony fish to mammals combining a P2X domain, a putative C-cys anchor and a ballast domain while the other P2X receptors lack the two last features. The conserved genomic co-location of P2X7 and P2X4 genes, with highly similar P2X domain sequences, indicate that they were likely produced by local gene duplication of a unique ancestral gene. Our work suggests that P2X7 originated from the fusion of a P2X4-like gene and a ZCD coding exon during the early evolution of bony fish and tetrapods common ancestors (**Figure 8**). These domains were connected by a region in which the C-cys anchor critical for the P2X7 properties of desensitization evolved, to produce this unique purinergic receptor critically involved in immunity and inflammation. Our data are important because they demonstrate that the new GDP binding ballast domain identified by McCarthy et al. (33) originates in an ancient family of proteins present across all Metazoa.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AR, OS, SR, JK, and PB conceived the project, designed experiments and approaches, and edited the manuscript. AR, SR, JK, and PB performed primary data analysis. AR, JK, and PB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00113/full#supplementary-material

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Conflict of Interest: The 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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Publication II

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Human Peripheral Blood Eosinophils Express High Levels of the Purinergic Receptor P2X4

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Extracellular nucleotides are important mediators of cell activation and trigger multiple responses via membrane receptors known as purinergic receptors (P2). P2X receptors are ligand-gated ion channels, activated by extracellular ATP. P2X4 is one of the most sensitive purinergic receptors, that is typically expressed by neurons, microglia, and some epithelial and endothelial cells. P2X4 mediates neuropathic pain via brain-derived neurotrophic factor and is also involved in inflammation in response to high ATP release. It is therefore involved in multiple inflammatory pathologies as well as neurodegenerative diseases. We have produced monoclonal antibodies (mAb) directed against this important human P2X4 receptor. Focusing on two mAbs, we showed that they also recognize mouse and rat P2X4. We demonstrated that these mAbs can be used in flow cytometry, immunoprecipitation, and immunohistochemistry, but not in Western blot assays, indicating that they target conformational epitopes. We also characterized the expression of P2X4 receptor on mouse and human peripheral blood lymphocytes (PBL). We showed that P2X4 is expressed at the surface of several leukocyte cell types, with the highest expression level on eosinophils, making them potentially sensitive to adenosine triphosphate (ATP). P2X4 is expressed by leucocytes, in human and mouse, with a significant gender difference, males having higher surface expression levels than females. Our findings reveal that PBL express significant levels of P2X4 receptor, and suggest an important role of this receptor in leukocyte activation by ATP, particularly in P2X4^{high} expressing eosinophils.

Keywords: P2X4 purinergic receptor, monoclonal antibody, eosinophils, PBL marker, gender difference

INTRODUCTION

The purinergic receptors comprise two different groups of receptors, P2Y and P2X. P2X receptors (1–3) are trimeric ATP-gated ion channels (4) which play a key role in neurotransmission, inflammation, and in a variety of other physiological processes. They are encoded by seven genes (*p2rx1 to p2rx7*), corresponding to seven different protein subunits. When ATP is released in a

non-regulated manner, it can initiate inflammation and further induce and amplify cell-mediated immunity through P2X receptors (5). Thus, ATP is considered as a danger-associated signal, released from cells during damage, hypoxia, or another cellular stress (6, 7). Extracellular ATP is quickly degraded into ADP, then AMP and finally adenosine by ectonucleotidases present at the plasma membrane of many cells. In chronically inflamed tissues, both extracellular ATP and adenosine may be present at high concentrations for extended periods (8), which suggests a possible role for purinergic signaling in chronic inflammation.

ATP, in its tetra-anionic form, ATP^{4-}, can be recognized by myeloid cells via cell surface P2X7 and P2X4 receptors, which leads to $Ca2^{+}$ and Na^{+} influx. While these two receptors are very similar and located close to each other on human chromosome 12, they differ from each other by their ATP binding affinity (from a micromolar range for P2X4 to a millimolar range for P2X7).

The functions of P2X4 are best known in the nervous system [reviewed in (9)]. In particular, P2X4 upregulation in spinal microglia is critical for pain hypersensitivity (allodynia) after peripheral nerve injury (10–12). P2X4 receptors are also involved in alcohol preference (13–15). P2X4 signaling in the CNS is inhibited by ethanol through complex mechanisms leading to altered behavior and promoting the development of alcohol use disorders (16). A broad-spectrum antiparasitic drug named Ivermectin can inhibit this effect of ethanol on P2X4, probably by antagonizing ethanol binding to the purinergic receptor (17, 18).

Data regarding P2X4 involvement in inflammation and immunity are scarce. A potential role of P2X4 in airways pathologies has been reported (19–21). Purinergic signaling has also been linked to allergic asthma (19). Also, in animal models of allergic asthma, elevated levels of P2X4 have been detected in the inflammatory cells of broncho-alveolar lavages (20, 21). While the implication of P2X7 in inflammation has been extensively studied [reviewed in (19, 22)], the characterization of the expression and function of P2X4 in immune cells has been hindered by the lack of Abs directed against this receptor in mouse and human. We therefore undertook the production of mAbs against the human P2X4.

We have produced four monoclonal antibodies (mAb) that recognize human P2X4 specifically. We focused our studies on two of these mAbs [mAb27 (IgG2b) and mAb29 (IgM)], and found that they cross-react with mouse and rat P2X4. Using these mAbs, we characterized the expression profile of P2X4 by human and mouse PBLs. We show that human eosinophils express the highest level of P2X4, while myeloid cells are also positive but express lower amounts of this receptor. Eosinophils are extremely versatile effector cells that damage tissues or modulate the activity of other cells. Eosinophils in homeostasis represent about 1% of circulating leucocytes however that number can rise to 3–5% in inflammatory conditions. In healthy individuals, beside bone marrow and lungs, eosinophils are found in various tissues: ovary, uterus, thymus, spleen and lower gastrointestinal tract. The principal chemotaxins mediating eosinophil recruitment are eotaxins, which attract eosinophils to the gastrointestinal tract, thymus, and uterus and to other organs in disease conditions (23). It has been shown that during chronic inflammation, eosinophils locate close to nerves (24) where activated eosinophils induce nerve damage, neuropeptide release, or altered nerve growth (25). Contact between eosinophils and nerves is one of the causes of airway hypersensitivity in asthma (26). Eosinophils have been considered to be effector cells that are engaged mainly in allergic reactions and in response to parasites. Recently, the role of eosinophils in immunoregulation and homeostasis in the steady state has become more and more evident. A subset of eosinophils ($CD16^{high}$, 1–5% of all eosinophils) have immune regulatory and protective functions since they interact with several immune cell types including dendritic cells and Th1/Th17 lymphocytes (27). The subsets of regulatory eosinophils express the immunoregulatory protein galectin-10, that functions as a T cell—suppressive molecule (28).

In this work, we characterized mAbs specifically recognizing hP2X4, which cross-react with murine P2X4. Our data show that P2X4 is highly expressed by human eosinophils. The P2X4 expression by PBL shows a surprising difference between sexes, males having higher surface levels than females. Our findings pave the way for future studies of the P2X4 role in leukocyte activation by extracellular ATP in normal or pathological conditions.

MATERIALS AND METHODS

Ethics Statement

Animal handling and maintenance were performed according to the interdisciplinary principles and guidelines for the use of animals in research, testing and education (FELASA) prepared by Ad Hoc Committee on Animal Research (The New York Academy of Sciences, New York, NY, USA). The animal experiments described in this study were authorized by the Ethical and Animal Welfare Committee of Estonia (Tartu University, ERC nr 181T-1). The P2X4 KO mice were generated by Sim et al. (29). Human blood samples used in the current study were obtained from healthy donors in accordance with the principles of the Helsinki Declaration of 1975 and subsequent amendments by the World Medical Assembly. Permission No. 160 was issued to Sirje Rüütel Boudinot on 18.02.2013 by the Ethics Review Committee (ERC) on Human Research of the National Institute for Health Development, Tallinn. The patients provided informed consent for data and biological substance collection for usage in clinical research.

Cell Culture

THP-1 cells (human acute monocytic leukemia cells) obtained from the American Type Cell Culture Collection were cultured in RPMI 1640 GlutaMAX^{TM} medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest SAS, Nuaillé, France). ALT (mouse astrocytoma), GL261 (mouse glioma), BV2 (mouse microglial cell), and HEK293 (Human Embryonic Kidney 293 cells obtained from Prof Priit Kogerman, TTU, Estonia) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Biowest SAS, Nuaillé, France).

The human prostate carcinoma cell line DU145 was purchased from ATCC (American Type Culture Collection, Manassas,

VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), with 10% inactivated FBS, penicillin/streptomycin, 1 mM sodium pyruvate. Immortal human bronchial epithelial cells (16HBE14o–) were incubated as described in Cozens et al. (30). In brief, pre-coated conditions were used. Coating was done with bovine serum albumin (BSA, Sigma Aldrich) (0.01 mg/ml), collagen I from bovine 0.03 mg/ml (Corning 354231), human fibronectin 0.01 mg/ml (Corning 354008). Cells were grown in Minimum Essential Medium Eagle with L-glutamine (Lonza, BE12-611F), the medium was supplemented with 10% inactivated FBS, and penicillin-streptomycin. Cell viability in cultures was routinely checked. 16HBE14o– cells were kindly provided by Professor Dieter C. Gruenert, University of California San Francisco (UCSF).

Stable Transfection of HEK293 Cells

The human or mouse P2X4 receptor-mCherry plasmids (EX-A1754-M56 and EX-Mm24590-M56) were purchased from GeneCopeia and used to tranfect HEK293 cells.

HEK293 cells were transfected using Lipofectamine LTX and Plus^{TM} reagent (Invitrogen) according to the manufacturer's instructions. Stable cell lines were selected in G418 medium and were cultured in DMEM supplemented with 10% FBS and containing 1 mg/ml of G418. Several cell lines expressing the human P2X4 receptor-mCherry or the mouse P2X4 receptor-mCherry were analyzed by flow cytometry. Strongly positive cell lines were frozen down and used for immunoprecipitation experiments.

Development of Anti-human P2X4 Receptor Monoclonal Antibodies

Mouse mAbs were generated by standard hybridoma technology. C57Bl/6 mice (2 mice, 15 week old females) were immunized intraperitoneally with 40 μg of purified P2X4 HIS-Ectodomain (ECD W50-I339, 289aa not including the HIS-tag) in PBS on days 0, 21, and 42, and boosted without adjuvant on day 59. Spleen and bone marrow cells were then isolated and fused with Sp2/0 myeloma cells 4 days later. Hybridomas producing mAbs against hP2X4 ECD were identified by ELISA, and cloned by the limiting dilution method (31). A detailed description of the protocol is provided in **Supplemental Methods**. After a second screening based on ELISA and FACS analysis, hybridomas were cultured in 10% FBS DMEM medium. The isotype of mAbs was determined with the Mouse Immunoglobulin Isotyping ELISA Kit (BD Biosciences). IgGs were purified from the supernatant of clones 19 (IgG2b) and 27 (IgG2b) using Pierce Protein G Agarose (Thermofisher Scientific), and conjugated with fluorescein isothiocyanate (FITC, Thermo Fisher Scientific).

Confocal Microscopy

For visualization of P2X4 on cell lines, cells were fixed with 4% paraformaldehyde Immunostaining was performed using rabbit polyclonal P2X4 antibody [P2X4 (H-40) sc-28764, from Santa Cruz Biotechnology INC. Santa Cruz, CA], mouse polyclonal P2X4 antibody [P2RX4 MaxPab mouse polyclonal antibody (B01) from Abnova Taipei, Taiwan], and our mouse anti-human P2X4 mAb27 (IgG2a/κ). Staining with primary antibodies was performed for 1 h at room temperature. Anti-rabbit or anti-mouse IgG secondary antibodies conjugated with Alexa-Fluor 488 (Invitrogen at Eugene, Oregon, USA) were used. Samples were mounted in DAPI. Fixed cells were analyzed with a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a ×63 Plan-oil-immersion lens. An argon laser was excited at 488 nm and fluorescence image was recorded from 500 to 550 nm.

For imaging P2X4 expression by eosinophils in gall bladder from a patient with acute cholecystitis, the tissue was dissected after surgery, cryo-embedded, snap frozen, and stored at −25°C until preparation of cryosections (5 μm). Sections were air-dried, washed with TBS 3 times, and incubated in TBS + 0.2% BSA for blocking. Sections were stained with anti-hP2X4 mAb27-FITC (1:1,600; IgG2b), or mouse anti-h-Siglec-8-PE mAb (1:10; IgG1; from BioLegend clone 7C9). Nuclei were stained using Hoechst33342 (2 μg/ml, Sigma). Control staining was performed using a mouse anti-h CD3-FITC mAb (1:10; clone HIT3a RUO, Isotype IgG2a/, BD Pharmingen). Sections were incubated with Abs in TBS at room temperature for 30 min, washed twice, incubated with Hoechst for 10 min, then washed three times in TBS. Images were acquired on a Zeiss Axioskop2 (10× or a 20× objective) for **Figure 9A**, or a Zeiss LSM780 inverted confocal microscope at 63× for **Figures 9B,C**. Image analysis was performed using ImageJ (NIH).

Blood Sampling and Leukocyte Isolation

Human samples of venous blood (10 ml) were collected from allergic individuals or healthy controls by venipuncture in the arm into a sterile BD Vacutainer EDTA blood collection tube (BD#367844), at the West Tallinn Central Hospital or at the East Tallinn Central Hospital. Blood samples were kept at room temperature until staining. Regarding mouse PBL, blood samples were collected immediately after death; heparin (100 IU, LEO Pharma, Malmo, Sweden) was used as anticoagulant and cells were stained as described below.

Flow Cytometry Analysis

Extracellular Staining of Cell Lines

Cells were stained with FITC (fluorescein isothiocyanate) or PE (phycoerythrin) conjugated antibodies for 1 h on ice. One million cells were labeled in 100 μl of antibody solution. The following anti-human P2X4 antibodies were used: anti-hP2X4-mAb19-FITC, anti-hP2X4-mAb27-FITC, anti-hP2X4-mAb29 (IgM/κ, hybridoma supernatant or ascites), anti-hP2X4-mAb8 (IgM/κ, hybridoma supernatant or ascites). Secondary Abs were: FITC labeled Goat Anti-Mouse Ig (RUO, GMP, BD Biosciences, cat 349031); Alexa Fluor 488 labeled Goat anti-Mouse IgG, IgM(H+L) (Jackson ImmunoResearch); FITC labeled rat anti-mouse Igκ (clone 187.1 RUO, BD Biosciences, cat 550003). FITC labeled rat IgG2a/κ isotype control (eBR2a) was from eBioscience (cat 11-4321-42). Anti-mouse Fc receptor CD16/CD32 (eBioscience) was used to block Fc-receptors. Data were acquired using a CytoFLEX S (Beckman Coulter) (**Figure 2** and **Figure S6**) or a BD FACS Canto II (**Figure 3**).

Intracellular Staining of Cell Lines

Cells were resuspended in 1 ml of ice-cold Fixation-Permeabilization buffer (eBioscience) and were incubated 60 min in ice and in the dark. Thereafter, 2 ml permeabilization buffer were added and cells were pelleted at (400 x g) 10 min. The cell pellet was re-suspended in 90 µl of ice-cold permeabilization buffer. Reactive groups on cells were blocked with cold DMEM 4% FCS for 20 min. Mouse cells were first incubated with Fc block for 20 min, then stained with the relevant antibodies plus Fc block. Data were acquired using a BD FACS Canto II CS (**Figure 5**).

Extracellular Staining of Human PBL

Fluorochrome-labeled antibody mix was added to 50 µl of whole blood (about 2×10^6 leukocytes) and incubated 30 min at room temperature in the dark [for example, anti-P2X4-FITC mAb27, anti-Siglec-8-PE (Nordic BioSite), anti-CD3-PerCP/Cy5.5 SK7 (BD), anti-CD20 PE L27 (BD), anti-CD14-APC cloneMφP9 (BD), anti-CD45-APC-Cy7 2D1 (BD)]. After two washes in PBS, stained samples were treated with FACS lysing solution for 10 min in the dark (Becton Dickinson), which lyses erythrocytes under gentle hypotonic conditions while preserving the leucocytes. Cells were then analyzed by FACS after a final wash in PBS. Eosinophils were identified as: CD45+ Siglec-8+ SSC high. Flow cytometry was performed with BD FACS Canto II (**Figures 6**, **7**) and FACS data were analyzed using FlowJo, LLC.

Statistical Analysis

Tests were performed in Graphpad Prism 8. Experiments were performed at least with six individuals. Results were considered to be statistically significant for $p \leq 0.05$. A Shapiro–Wilkinson test indicated that data had normal distribution, so Welch's *t*-test was applied to test the significance of differences between groups. A Mann–Whitney test also supported a significant difference between male and female mice groups.

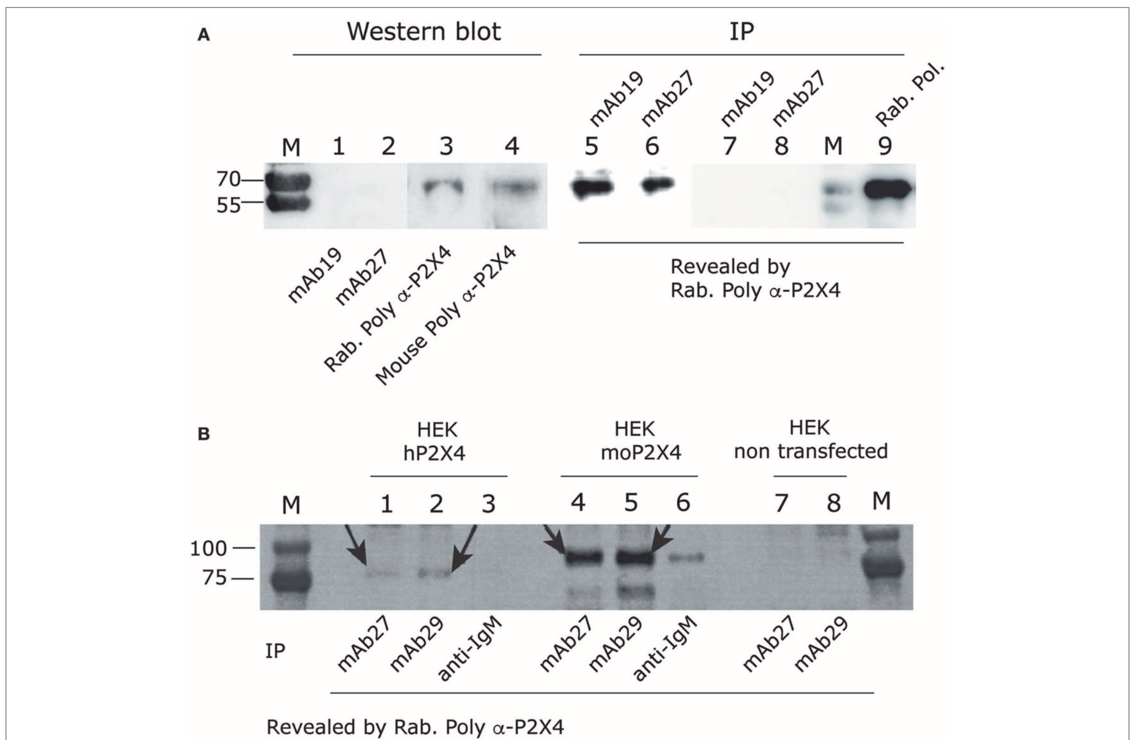

FIGURE 1 | Anti-hP2X4 recognizes hP2X4 in immunoprecipitation experiments, but not in western blot. **(A)** Left panel. Western blot using mAb19 (1), mAb27 (2), rabbit anti-rat-P2X4 polyclonal antibodies from Alomone (3) or our mouse anti-hP2X4 polyclonal antibodies (4), performed on total cell lysate of HEK293 cells transfected with hP2X4. Blots were revealed using anti-mouse and anti-rabbit HRP Abs. Right panel. hP2X4 was immuno-precipitated from the same lysate of HEK293 cells expressing human P2X4, using mAb19 (5) or mAb27 (6), and revealed by western blotting with rabbit anti-P2X4 polyclonal antibodies. Negative controls are shown in lanes 7 and 8: immunoprecipitation of non transfected HEK293 cell lysates with mAb19 (lane 7) and mAb27 (lane 8). Immunoprecipitation of P2X4 from cell lysate of HEK293 cells transfected with hP2X4 using rabbit anti-rat P2X4 polyclonal antibodies is shown in lane 9. **(B)** Immunoprecipitation assays using mAb27 (IgG2b; lanes 1, 4, 7) or mAb29 (IgM; lanes 2, 5, 8) or anti-IgM (lanes 3 and 6) from HEK cells overexpressing hP2X4-mcherry (lanes 1–3), mouse P2X4-mcherry (lanes 4–6), or non transfected (lanes 7, 8). P2X4 was revealed by western blotting with rabbit anti-P2X4 polyclonal antibodies. Arrows indicate hP2X4 or moP2X4 bands.

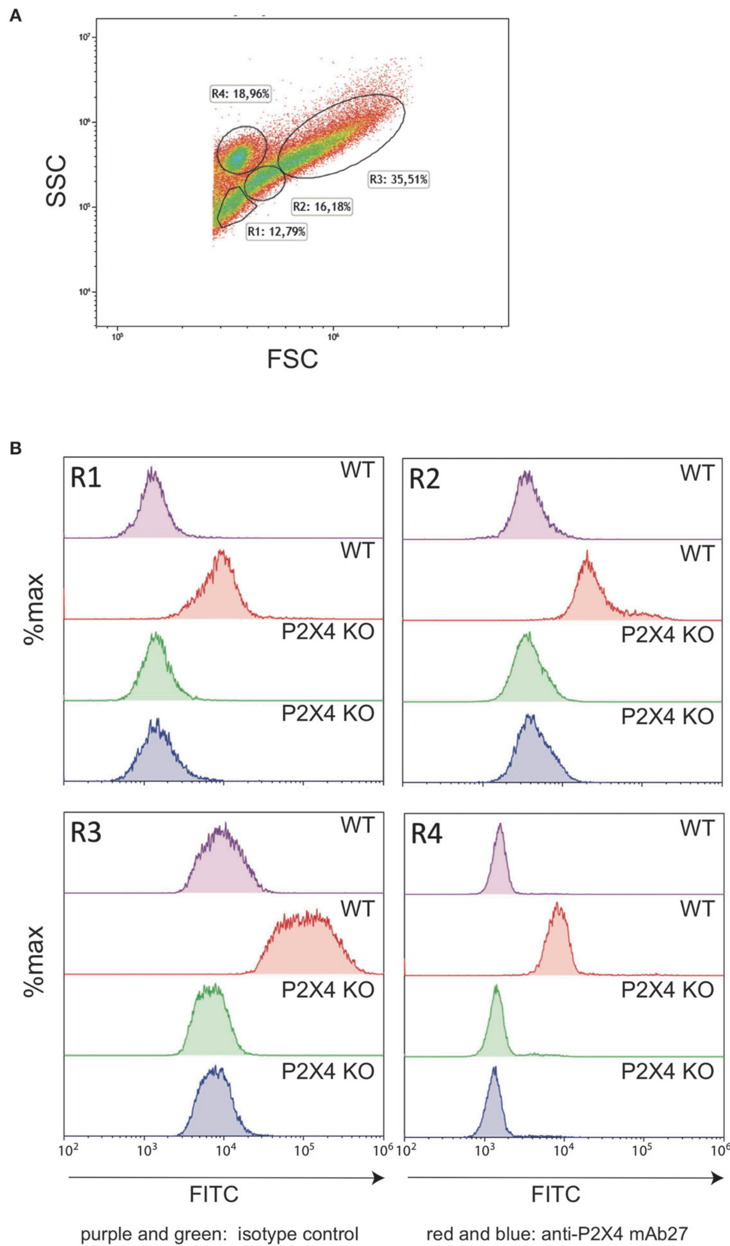

FIGURE 2 | Anti-hP2X4 mAb27 recognizes the mouse P2X4 specifically. **(A)** FSC/SSC representation of mouse peritoneal cells (C57BL/6 strain). Gates were defined as in Hermida et al. (32). Erythrocytes were gated out. During the preparation of cells, erythrocytes were not lysed to avoid ATP release, which would trigger P2X4. Each cell suspension corresponds to a pool from three different mice of each genetic background. **(B)** Comparison of wild type (WT) and P2X4 KO peritoneal cells from gates R1–R4 labeled with isotype control (purple, WT and green, P2X4 KO) or with mAb27 FITC (red, WT and blue, P2X4 KO).

Detailed protocols for hybridoma generation, ELISA, Immuno-precipitation, and western blotting, inhibition of mAb27 binding to the P2X4 receptor by unlabeled anti-hP2X4 mAbs, RNA preparation and RT-QPCRas well as staining of tissue sections are provided in **Supplemental Methods**.

RESULTS

Production of Four Monoclonal Antibodies Against Human P2X4

To generate mouse monoclonal antibodies against human P2X4, a protein comprising the extracellular part of this receptor fused to a 6HisTag was produced in bacteria. It was purified, refolded by dialysis, and used for mouse immunization. Four candidate hybridomas were finally selected, two IgG2b/kappa named mAb19 and mAb27 and two IgM/kappa named mAb29 and mAb8. To test the specificity of the mAbs, Western blot (WB) and immunoprecipitation (IP) analyses from HEK cells expressing the full-length human P2X4 were performed.

None of our four monoclonal antibodies detected P2X4 in Western blot experiments (as illustrated in **Figure 1A** for mAb19 and mAb27, left panel), indicating that they do not recognize the denaturated form of the protein and therefore target conformational epitopes. Similar results were obtained with mAb8 and mAb29 (IgM) (data not shown). In contrast, as shown in **Figure 1A** (right panel), mAb19 and mAb27 immunoprecipitated P2X4 from the lysates of transfected cells expressing human P2X4, as revealed by staining WB with the commercial rabbit anti-P2X4 extracellular part antibodies from Alomone (polyclonal, cat APR-024). We also asked whether our mAbs could immunoprecipitate both human and mouse P2X4 from lysates of HEK cells overexpressing these proteins (**Figure 1B**). We then used mAb27 (IgG2b) and mAb29 (IgM) for these experiments and showed that indeed these mAbs could immunoprecipitate both mouse and

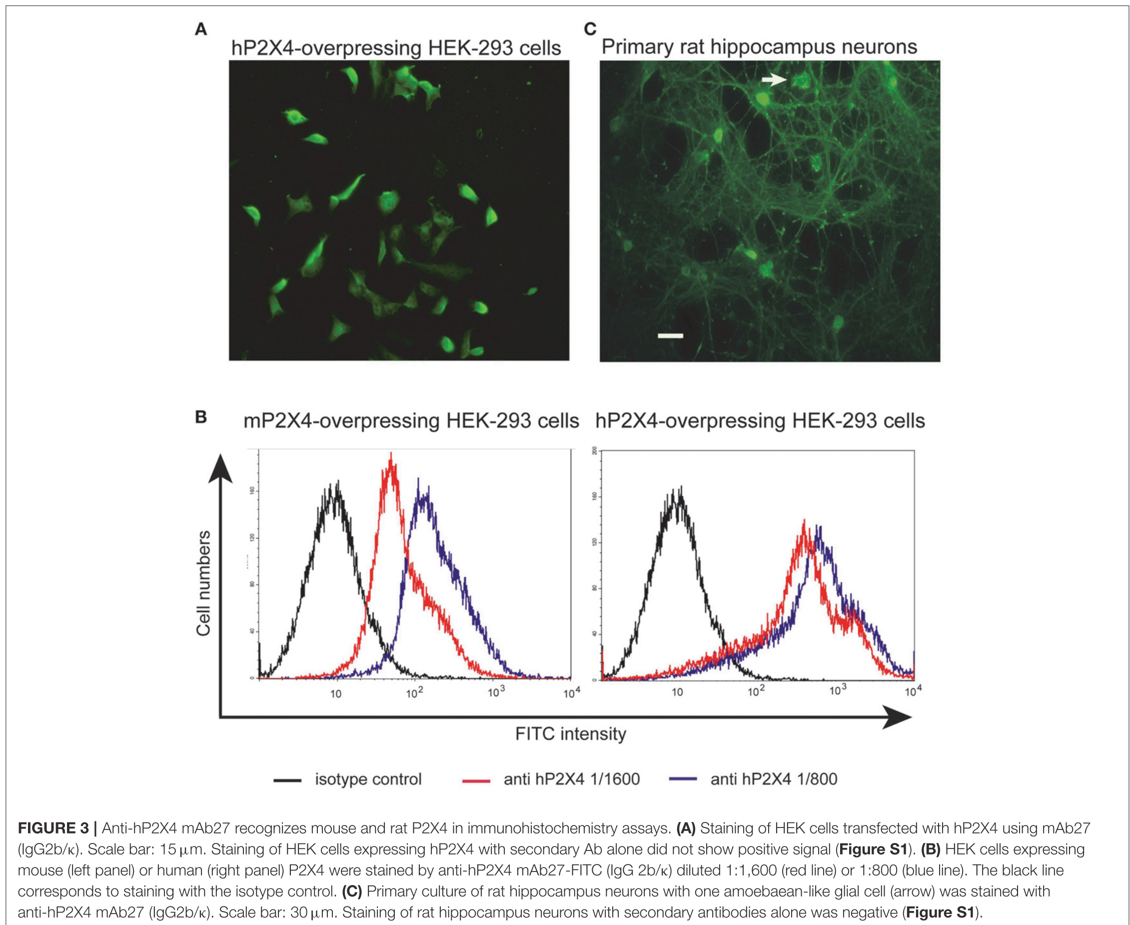

FIGURE 3 | Anti-hP2X4 mAb27 recognizes mouse and rat P2X4 in immunohistochemistry assays. **(A)** Staining of HEK cells transfected with hP2X4 using mAb27 (IgG2b/κ). Scale bar: 15 μm. Staining of HEK cells expressing hP2X4 with secondary Ab alone did not show positive signal (**Figure S1**). **(B)** HEK cells expressing mouse (left panel) or human (right panel) P2X4 were stained by anti-hP2X4 mAb27-FITC (IgG 2b/κ) diluted 1:1,600 (red line) or 1:800 (blue line). The black line corresponds to staining with the isotype control. **(C)** Primary culture of rat hippocampus neurons with one amoebaean-like glial cell (arrow) was stained with anti-hP2X4 mAb27 (IgG2b/κ). Scale bar: 30 μm. Staining of rat hippocampus neurons with secondary antibodies alone was negative (**Figure S1**).

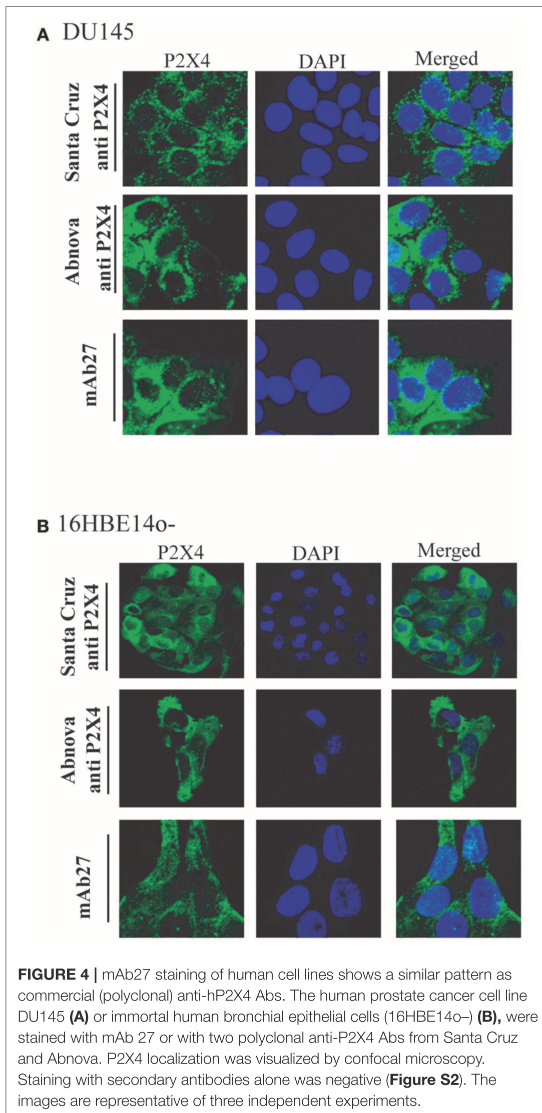

FIGURE 4 | mAb27 staining of human cell lines shows a similar pattern as commercial (polyclonal) anti-hP2X4 Abs. The human prostate cancer cell line DU145 **(A)** or immortal human bronchial epithelial cells (16HBE14o–) **(B),** were stained with mAb 27 or with two polyclonal anti-P2X4 Abs from Santa Cruz and Abnova. P2X4 localization was visualized by confocal microscopy. Staining with secondary antibodies alone was negative (**Figure S2**). The images are representative of three independent experiments.

human P2X4. As observed in **Figure 1B**, the human and mouse P2X4-mCherry proteins were found at an apparent MW of 81,800 and 89,500 Da, respectively. Thus, our data show that mAb27, mAb19, and mAb29 specifically recognize hP2X4 and that mAbs27 and 29 also bind the mouse receptor. We then mainly focused our studies on mAb27 (IgG2b) and mAb29 (IgM). Hereafter, we show experiments performed with mAb27, indicating when other mAbs were used in parallel.

To further confirm that these mAbs recognize the mouse P2X4 receptor, we injected thioglycolate i.p. in P2X4 KO and wild type C57Bl/6 mice. Peritoneal cells were collected and labeled with mAb27-FITC or with isotype control coupled to FITC. Flow cytometry analyses were performed on four main gates (**Figure 2A**). As shown in **Figure 2B**, in wild type mice a significant fraction of cells from all gates (R1–R4) were specifically recognized by mAb27. In contrast, in P2X4 KO mice mAb27 staining was not different from the isotype controls. Thus, these results demonstrate that mAb27 can be used to specifically stain murine P2X4 receptor in flow cytometry experiments. Consistent results were obtained with mAb29 (data not shown).

The anti-hP2X4 mAbs were further used for immunocytochemistry experiments. mAb27 stained HEK cells overexpressing the human P2X4, but not HEK cells transfected with the empty vector (**Figure 3A** and **Figure S1**). mAb19 (IgG2b), mAb29, and 8 (IgM) provided similar results, confirming the specificity of both IgG and IgM anti-hP2X4 mAbs (data not shown). HEK cells were also transfected with an expression plasmid for mouse P2X4; flow cytometry analyses showed that our anti-hP2X4 mAb27-FITC significantly stains cells expressing the mouse P2X4 receptor (**Figure 3B**, left panel) but less efficiently than cells expressing the human P2X4 receptor (**Figure 3B**, right panel). Consistent results were obtained with mAb19 (IgG2b), mAb29 (IgM), and mAb8 (IgM) (data not shown). In keeping with this, we used mAb27 to stain rat hippocampus cell cultures containing both P2X4^{+} neurons and glial cells (**Figure 3C**). Our data are consistent with the high expression of P2X4 in cell bodies of the hippocampus (29). As illustrated for the mAb27 clone (IgG2b) in **Figure 3C**, these experiments indicate that our anti-human P2X4 mAbs can also recognize the rat P2X4 receptor. Similar images were obtained with the IgM mAb29 (data not shown).

Additionally, a human prostate cancer cell line and human immortal bronchial epithelial cells were stained with the mAb27 and compared with the staining obtained with two other anti-P2X4 antibodies from Abnova and Santa Cruz. As shown in **Figure 4** and **Figure S2**, similar staining patterns were observed, further supporting the specificity of the mAb27.

P2X4 Is Expressed on the Surface of Mouse Astrocytoma and Glioma Cells but Accumulates Mainly in Intracellular Compartments of Mouse Microglial Cells

We then used our mAbs to study the cellular location of P2X4 in neural cells for which P2X4 expression has been previously demonstrated: astrocytes (33), glia and microglia (34, 35).

Non-activated mouse brain tumors corresponding to these cell types were stained with purified, FITC-coupled anti-P2X4 mAb27 (IgG2b): an astrocytoma (ALT), a glioma (GL261), and a microglial cell line (BV2). Typical results are shown in **Figure 5**. Similar results were obtained using mAb19 (data not shown). All cell lines were positive, but the staining revealed a higher surface expression of P2X4 for astrocytoma and glioma, compared to microglial cells which were stained after permeabilization only. BV2 cells showed a strong pattern of P2X4 accumulation in internal compartments, which may correspond to lysosomes as described in COS1 and HEK293 cells (36).

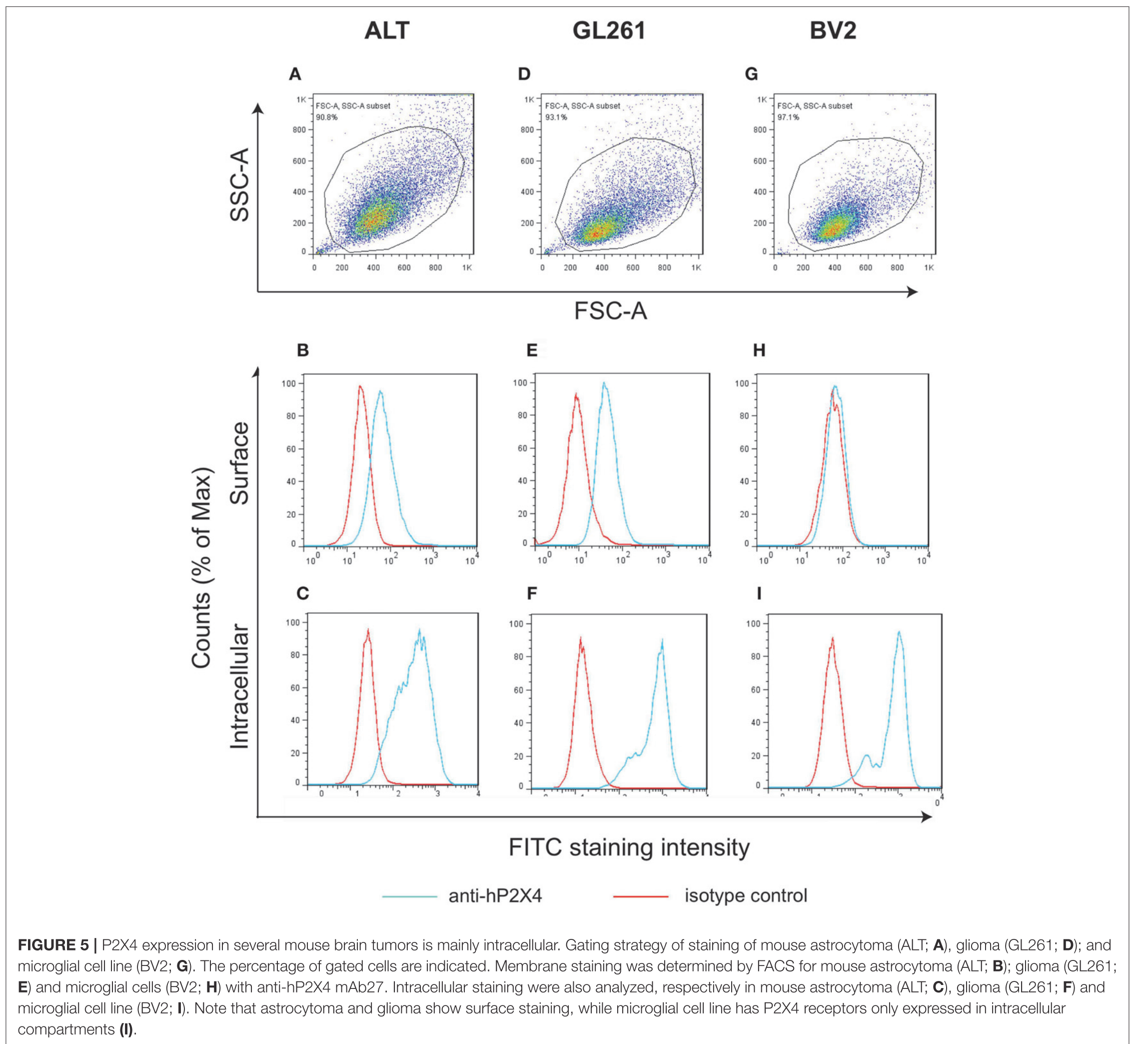

FIGURE 5 | P2X4 expression in several mouse brain tumors is mainly intracellular. Gating strategy of staining of mouse astrocytoma (ALT; **A**), glioma (GL261; **D**); and microglial cell line (BV2; **G**). The percentage of gated cells are indicated. Membrane staining was determined by FACS for mouse astrocytoma (ALT; **B**); glioma (GL261; **E**) and microglial cells (BV2; **H**) with anti-hP2X4 mAb27. Intracellular staining were also analyzed, respectively in mouse astrocytoma (ALT; **C**), glioma (GL261; **F**) and microglial cell line (BV2; **I**). Note that astrocytoma and glioma show surface staining, while microglial cell line has P2X4 receptors only expressed in intracellular compartments **(I)**.

P2X4 Is Expressed by Different Leukocyte Subsets From Human PBL

To investigate a possible role of P2X4 in immune cell activation, we then evaluated the P2X4 expression by leukocytes from peripheral blood of 7 healthy donors, defined as $CD45^{+}$cells. PBL were stained with purified, FITC-coupled anti-P2X4 mAb27, and with lineage-specific antibodies (CD45, CD20, CD3, CD14), and analyzed by flow cytometry to assess the expression of P2X4 by the main leukocyte subsets (**Figure 6**). Within $CD45^{+}$ cells, flow cytometry data showed that $CD3^{+}$ T cells expressed very low levels of P2X4 (**Figure 6D**), while $CD20^{+}$ B cells were slightly positive (**Figure 6E**). This was observed in all individuals studied but one, where P2X4 expression level was similar in B and T cells, at a very low level. In contrast, $CD14^{+}$ monocytes and $CD14^{+/-}$ granulocytes cells expressed intermediate levels of P2X4 (**Figures 6F,G**). Interestingly, a distinct subset expressing a high level of P2X4 appears in **Figures 6D–F**. As shown in **Figure 6G**, these cells were among the most granular cells in PBL, and expressed P2X4 receptor at a much higher level compared to other granulocytes. Overall, these observations identified a cell subset expressing high level of P2X4 that was distinct from T and B cell, monocytes, and the main fraction of granulocytes.

Human Eosinophils Express High Levels of P2X4

We then characterized a population of large, highly granular $P2X4^{high}CD45^{+}CD3^{-}CD14^{-}$ cells observed in human PBL by flow cytometry analyses. From their phenotype and

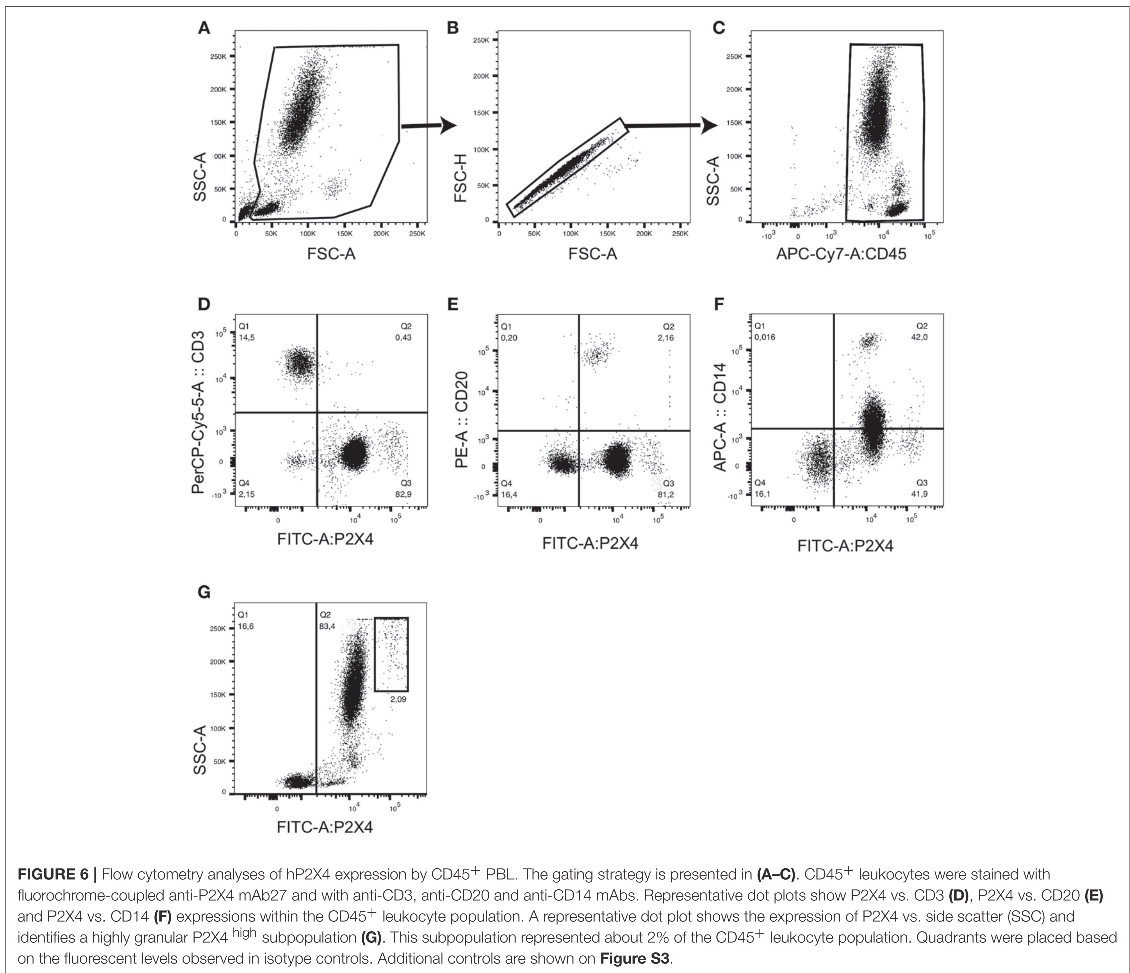

FIGURE 6 | Flow cytometry analyses of hP2X4 expression by $CD45^+$ PBL. The gating strategy is presented in **(A–C)**. $CD45^+$ leukocytes were stained with fluorochrome-coupled anti-P2X4 mAb27 and with anti-CD3, anti-CD20 and anti-CD14 mAbs. Representative dot plots show P2X4 vs. CD3 **(D)**, P2X4 vs. CD20 **(E)** and P2X4 vs. CD14 **(F)** expressions within the $CD45^+$ leukocyte population. A representative dot plot shows the expression of P2X4 vs. side scatter (SSC) and identifies a highly granular P2X4 ^{high} subpopulation **(G)**. This subpopulation represented about 2% of the $CD45^+$ leukocyte population. Quadrants were placed based on the fluorescent levels observed in isotype controls. Additional controls are shown on **Figure S3**.

frequency, we hypothesized that these cells might be eosinophils. Eosinophils are typically abundant among PBL of allergic patients. We therefore performed staining of human PBL from six allergic donors using anti-CD45, anti-P2X4 mAb27, and anti-Siglec8 mAbs, and analyzed these cells by flow cytometry. In **Figure 7**, representative dot plots show that the subpopulation of highly granular $P2X4^{high}$ cells corresponds to the cell subset expressing high level of the eosinophil marker Siglec-8. In comparison, lymphoid or myeloid cells expressing intermediate or low levels of P2X4 all express lower levels of Siglec-8 (**Figure 7D**): $P2X4^{med}Siglec8^{low}$ cells are granular, large cells with intermediate levels of CD45 and correspond mainly to neutrophils, while $P2X4^{med/low}Siglec8^{low}$ cells are smaller and express high levels of CD45, comprising monocytes and lymphocytes (**Figures 7D–F**). In addition, the $P2X4^{high}$ population of PBL from allergic patients expresses low levels of CD123 and is clearly distinct from the $CD123^+$ basophils (**Figure S4**).

Relative frequencies of $P2X4^{high}Siglec8^{high}$, all $P2X4^{high}$ and all $Siglec8^{high}$ cells were measured from PBL of nine patients allergic to pollen (2), mold (2), honey (1), mites in house dust (1), mice (1), and one patient with an acute allergic dermatitis. The results are summarized in **Table 1**, and indicate that a high surface expression of P2X4 is a good marker for Siglec-8^+ cells in human PBL. Taken together, our data show that the frequencies of PBL large granular cells with a strong surface expression of P2X4 correlate with the frequencies of Siglec-8^{high} cells in allergic patients.

Analysis of the gallbladder surgical specimen from a patient with chronic calculous cholecystitis (**Figure 8** and **Figure S5**) shows specific co-staining of P2X4 and Siglec-8,

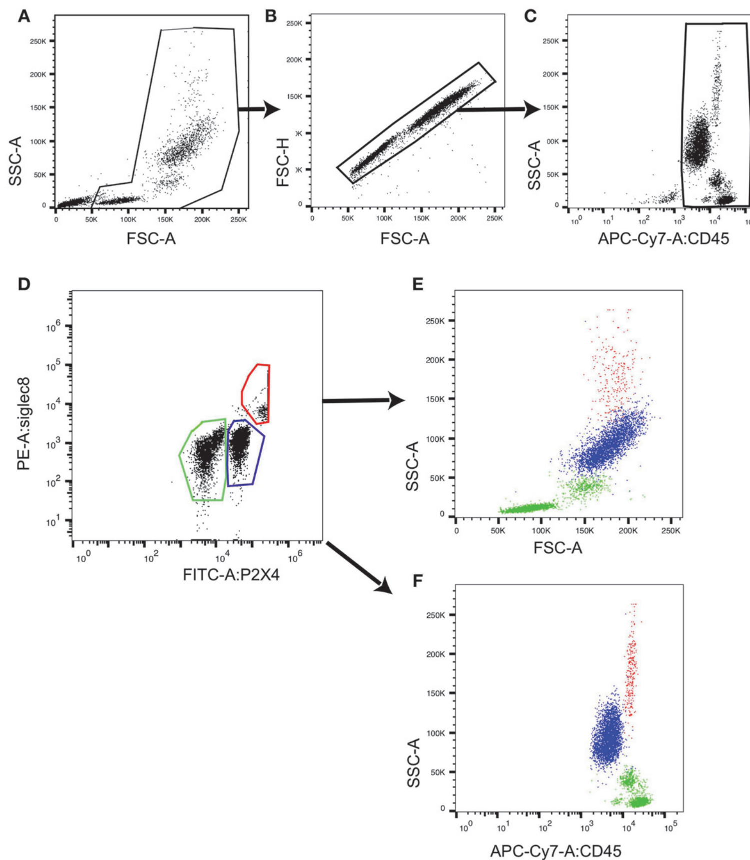

FIGURE 7 | Eosinophils from human PBL express highest levels of P2X4. $CD45^+$ peripheral blood leukocytes defined by the gating strategy presented in **(A–C)** were stained with fluorochrome-coupled anti-P2X4 mAb27 and with anti-Siglec8 mAb, and analyzed by flow cytometry. A representative dot plot shows P2X4 vs. SIGLEC8 expression **(D)** in $CD45^+$ leukocytes. Three gates outlined in red (gate 1), blue (gate 2), and green (gate 3) define $P2X4^{high}SIGLEC8^{high}$, $P2X4^{med}SIGLEC8^{low}$, and $P2X4^{low}SIGLEC8^{low}$ subsets, respectively. **(E,F)** Represent forward (FSC) (respectively, CD45 expression) vs. side scatter (SSC) for cells from each gate, showing that $P2X4^{high}SIGLEC8^{high}$ are large, highly granular cells.

indicating that eosinophils in tissues express those markers at high level. Another section of the same sample was stained with hematoxylin/eosin to visualize the density of eosinophils in the tissue. These results indicate that the anti-hP2X4 mAb27 can be used to visualize eosinophils in inflammatory tissues.

P2X4 Is Expressed by a Higher Fraction of Leukocytes in Males Compared to Females

P2X4 functions in brain microglial cells have been shown to be sex-dependent. Upregulation of P2X4 expression by these cells is required for pain hypersensitivity in male mice, but not in females where lymphocytes may be implicated (37, 38). We therefore compared P2X4 expression on PBL from men and women, as well as from male and female mice using our mAb27.

Men had a higher fraction of P2X4 positive cells compared to women, when all blood $CD45^+$ leucocytes were considered (**Figure 9A**). Similar experiments were then performed with mouse PBL, to test whether this expression pattern across leukocytes was conserved between human and mouse. The results showed a similar distribution as in humans, with lower P2X4 expression in females (**Figure 9B**). It is also interesting to note that the biggest percentages of $Siglec8^{high}P2X4^{high}$ cells identifying eosinophils were observed in two male patients (**Table 1**).

Overall, these results show that human and mouse leukocyte subsets express significant levels of P2X4, suggesting that this purinergic receptor can play a role in their activation. The percentage of P2X4 positive cells appears to be consistently higher in men and male mice.

TABLE 1 | Human PBL eosinophils express high P2X4 levels at the cell surface[a].

Sex and age	Known allergy	% of DP Siglec8^{high} P2X4$^{high\ b}$	Total % of P2X4$^{high\ b}$	Total % of Siglec8$^{high\ b}$
F, 21	Pollen, cats, mites*	1.08	1.08	1.12
F, 50	Honey, propolis**	0.9	0.9	1
M, 48	Mold**	3.94	3.95	4.2
F, 54	Nickel*	1.9	1.95	1.89
F, 51	Pollen, citrus**	5.6	5.7	5.7
F, 56	Atopic dermatitis**	2.23	2.23	2.25
F, 35	Mice*	0.78	0.93	0.96
M, 49	Mites and molds**	4.62	4.64	5.1
M, 44	Not specified*	1.08	1.7	1.09

*Currently stable; **Currently reactive.

[a] Anti P2X4 mAb27 was used for these experiments.

[b] Percentages of P2X4^{high}SIGLEC8^{high}DP, P2X4^{high}, and SIGLEC8^{high} within the CD45^{+} leukocyte population as defined in **Figures 6A–C**.

DISCUSSION

In this work, we report the production and validation of several mAbs against human P2X4. We characterized mAb27(IgG2b) and the mAb29(IgM), and showed that they cross-react against the murine ortholog of this receptor. We used mAb27 to assess the expression of P2X4 on leukocytes. We demonstrated that high expression level of P2X4 is an excellent surface marker for human eosinophils (Siglec-8^{high} cells), in PBL of healthy individuals and allergic patients. We also observed that the expression levels on leukocytes were higher in males compared to females, in mouse and human.

Purinergic P2X receptors are membrane channels that bind extracellular ATP and mediate most of its functions (39). Their roles are partly redundant but they do not have similar expression patterns across tissues and cell types (2, 40). It is therefore important to generate specific reagents such as mAbs to determine their expression range and functional capacity. Antibodies raised against synthetic peptides usually work in Western blot but fail to bind the native protein. In contrast, our mAbs were produced after immunization with the hP2X4 extracellular domain and screened using eukaryotic cells expressing hP2X4. Multiple assays including flow cytometry of transfected cells, immunoprecipitations, and immunochemistry show that these mAbs are specific for hP2X4, and recognize this receptor in native conformation. They did not work in Western blot assay, which is consistent with the presence of disulphide bonds (S-S) and N-linked glycosyl chains in the extracellular domain of P2X4 (41). Intracellular patterns of IHC with our mAbs were very similar to those obtained with commercial polyclonal Abs. The specificity of mAb27 for mouse P2X4 is clearly established by its capacity to label peritoneal cells from WT mice, but not those from P2X4 KO animals. Importantly, we also observed that our anti-hP2X4 mAbs did not cross-react with HEK cells expressing human P2X7 (**Figure S6**)—the member of P2X family that is the most similar to P2X4—further establishing their specificity to the P2X4 receptor. In addition, we found that preincubation of cells expressing hP2X4-mcherry or hP2X7 with mAb27 or mAb29 inhibited the binding of mAb27-FITC to cells expressing hP2X4-mcherry only. Thus, mAb27 binds specifically to P2X4, and binds the same or a closely located epitope as mAb29. Human and mouse (or rat) P2X4 amino-acid sequences are about 87% similar to each other, thus it was interesting to test whether our mAbs cross-react with murine P2X4 specifically. We indeed showed that the mAbs can recognize the mouse receptor in flow cytometry, immunoprecipitation, and IHC experiments. This highlights the potential use of our mAbs in murine species.

Interestingly, three monoclonal antibodies against the ectodomain of the rat P2X4 receptor have been raised previously by two other groups (42–44). These mAbs reacted with the rat P2X4 receptor in its native conformation and could immunoprecipitate it. However, only the mAb raised by Bo et al. was tested on the human P2X4, and it did not recognize it (42). Thus, our mAbs specific for the human P2X4 and cross-reacting with the murine ortholog represent novel tools allowing studies on the presence and function of this receptor in several tissues and PBL, particularly eosinophils.

Using our anti-P2X4 mAb27, we showed that this purinergic receptor is expressed on the surface of leukocytes (defined as CD45^{+} cells), at variable levels across cell types. We found that the overall P2X4 expression by PBL and spleen leukocytes was significantly higher on males, compared to females. Importantly, this difference stands for both mice and humans, indicating that sexual dimorphism could be a fundamental feature of the conserved, hence essential, functions of the receptor. This was observed consistently in the context of a significant difference in the overall proportion of P2X4 expressing leukocytes between human (60–80%) and mouse (20–40%), reflecting the low frequency of neutrophils in mouse blood. Interestingly, differences in the P2X4 functions between sexes have been documented, regarding neuropathic pain. While the P2X4 dependent microglia-neuron signaling is required for chronic pain hypersensitivity in males (39, 45) microglia do not contribute to pain hypersensitivity in female mice. Allodynia is in fact abolished in Rag 1 KO female mice, indicating that it requires the presence of adaptive immune cells (38). In females, peripheral nerve injury (PNI) induces an upregulation of P2X4 receptor in spinal dorsal horn, but at a much lower level when compared to males (46). It will be interesting to explore further whether a higher expression of P2X4 by myeloid cells from the blood or spleen of males may also participate to pain signaling. Taken together, all these observations raise the question of a possible sexual dimorphism of the functions mediated by P2X4. Interestingly, a recent study showed that deletion of P2X4 receptor was neuroprotective, and enhanced recovery from ischemic stroke in female mice only (47). Whether or not the sexual dimorphism of P2X4 expression on leukocytes is important for human pathologies remains to be clarified.

Flow cytometry experiments using our anti-P2X4 mAb27 revealed that this purinergic receptor is highly expressed by large, granular cells with high levels of the Siglec-8 surface marker. Additionally, we verified by RT-PCR that sorted CD45^{+} P2X4^{high} cells from human PBL expressed Siglec-8 mRNA (**Figure S7**). This sialic acid-binding lectin is strongly expressed

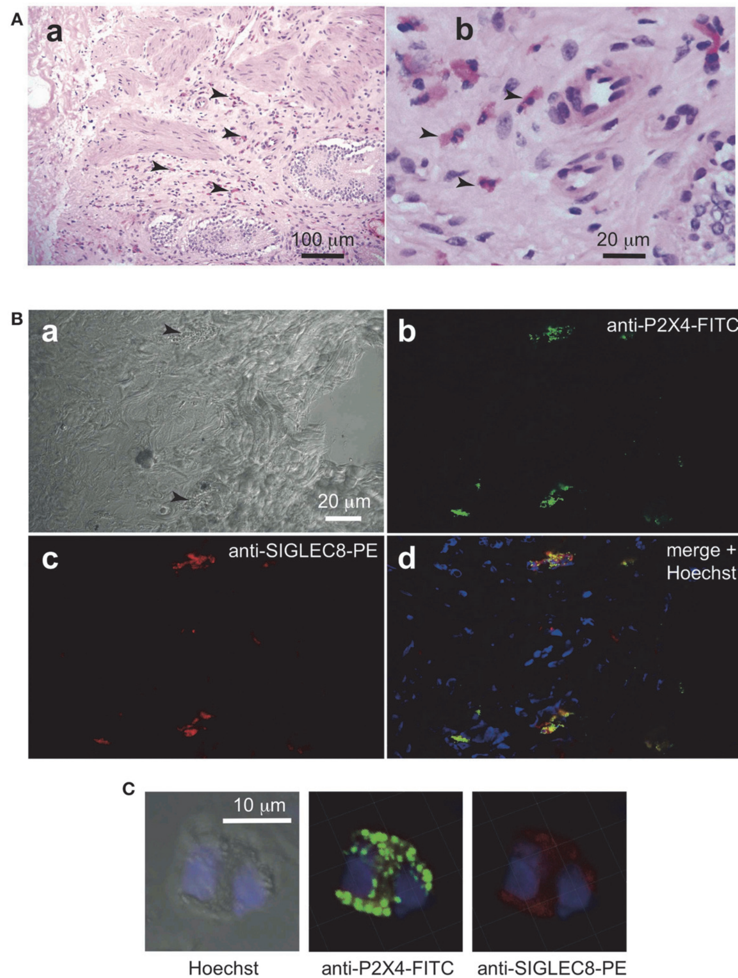

FIGURE 8 | Anti-hP2X4 staining identifies eosinophils in gall bladder sections. Cryosections (5 μm) of freshly isolated gall bladder sample from a patient with cholecystitis diagnosis were stained by hematoxylin-eosin **(A)**. Eosinophils are indicated by arrows. In **(B)**, two granulocytes are indicated by black arrows in the bright field image (a). Sections were stained with anti-hP2X4-FITC mAb27 (b), anti Siglec-8-PE (c). Merged images with Hoechst staining is shown in (d). Images acquired by confocal microscopy indicate that Siglec8-positive eosinophils express high levels of P2X4. **(C)** Illustrates the respective intracellular distributions of Siglec-8 and P2X4. Isotypic control is shown in **Figure S5**.

by eosinophils (48), by mast cells and to a lesser extent by basophils (49, 50). In human PBL, the P2X4^{high} cells do not correspond to mast cells that are not present in blood (51–53), which strongly suggests that they are eosinophils. In addition, P2X4^{high} cells in gallbladder sections were clearly eosinophils and not mast cells. Like mast cells, basophils are very rare among human PBL (54). In allergic patients, a basophil population can be detected as CD123^{+} cells, but they express only low levels of P2X4, supporting that P2X4^{high} cells are eosinophils (**Figure S4**). Other granulocytes (essentially neutrophils), monocytes/macrophages (CD45^{+}, CD14^{+}) and B cells (CD45^{+}, CD20^{+}) express intermediate levels of P2X4, while T lymphocytes are negative. These results confirmed previous observations reporting that P2X4 was expressed by myeloid cells, in particular by microglial cells in the brain. We also observed a strong surface expression of P2X4 on eosinophils in PBL of both healthy donors and patients with allergic pathologies. In these individuals, eosinophils are 2–3 times more frequent in PBL than in healthy people (51). Hence, the strong P2X4 expression by eosinophils is also seen on circulating and activated cells during

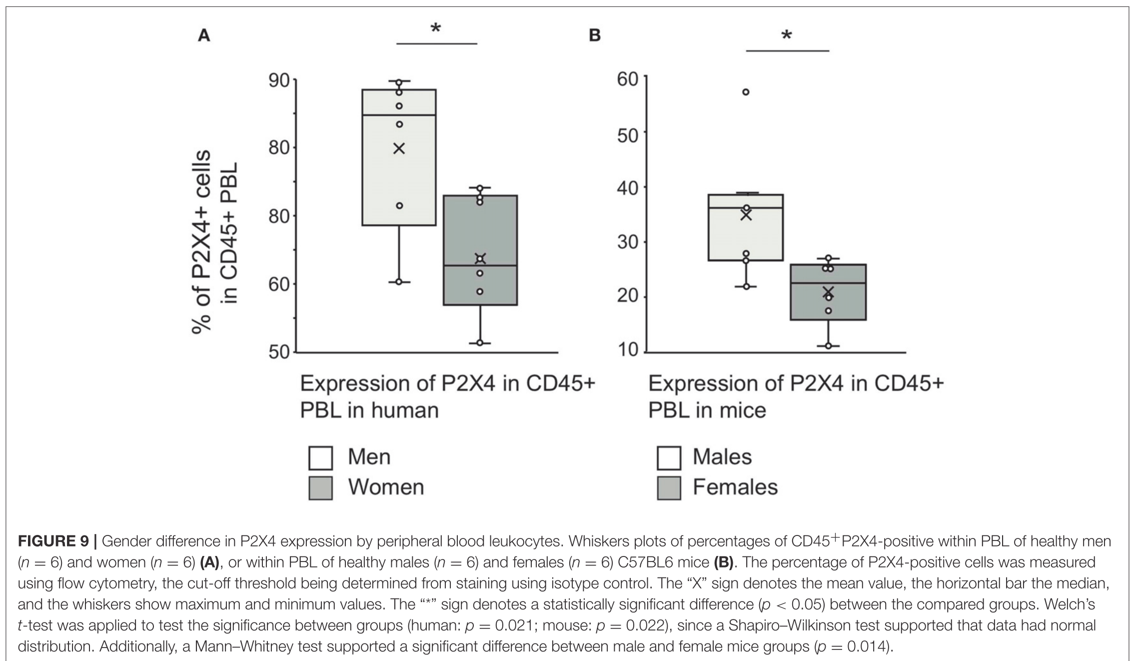

FIGURE 9 | Gender difference in P2X4 expression by peripheral blood leukocytes. Whiskers plots of percentages of $CD45^{+}$P2X4-positive within PBL of healthy men ($n = 6$) and women ($n = 6$) **(A)**, or within PBL of healthy males ($n = 6$) and females ($n = 6$) C57BL6 mice **(B)**. The percentage of P2X4-positive cells was measured using flow cytometry, the cut-off threshold being determined from staining using isotype control. The "X" sign denotes the mean value, the horizontal bar the median, and the whiskers show maximum and minimum values. The "*" sign denotes a statistically significant difference ($p < 0.05$) between the compared groups. Welch's t-test was applied to test the significance between groups (human: $p = 0.021$; mouse: $p = 0.022$), since a Shapiro–Wilkinson test supported that data had normal distribution. Additionally, a Mann–Whitney test supported a significant difference between male and female mice groups ($p = 0.014$).

allergy. Since P2X4 detects extracellular ATP at micromolar concentrations, these observations suggest that eosinophils may use this receptor for ATP-induced activation.

Eosinophils are terminally differentiated cytotoxic effector cells that control immune homeostasis and exert immunomodulatory functions. They provide host protection against parasitic, fungal, bacterial, and viral infections (55, 56) but also contribute to tissue damage during infections, asthma and autoimmune diseases. The differentiation of eosinophils from progenitor cells is controlled by inflammatory stimuli such as IL3, IL5, and CCL11/eotaxin; mature eosinophils are released to the blood (26) and infiltrate thymus, spleen and lymph nodes, as well as Peyer's patches and bone marrow where they promote plasma cell survival (57). Our results suggest that extracellular ATP, even at low concentration, may play a role in eosinophil activation, maybe at the degranulation step through which these cells release active compounds.

Although eosinophil count in blood and biopsy samples may not correlate with disease severity, there is a clear evidence of eosinophil activation during multiple pathologies. Eosinophils are implicated in allergic diseases as well as in asthma and in antiparasitic immune responses (58). The P2X4 expression by eosinophils should be considered in conjunction with recent reports showing that this purinergic receptor enhances allergic responses (20, 21). However, the immune functions of these cells remain poorly understood, partly because of the lack of specific surface markers and tumor cell lines. As a new surface marker of human eosinophils, P2X4 appears as a useful target to get insight into their biology. Importantly, Siglec-8 cannot be used as a human cell marker, in *in vitro* culture or *in vivo* studies because its engagement by specific antibodies results in apoptosis of eosinophils and inhibition of mediator release from mast cells (50), highlighting the importance of new markers for this cell type.

CONCLUSION

In this work, we have produced and validated anti-P2X4 mAbs. Using the IgG2b mAb27, we show that expression level of P2X4 by myeloid cells is higher in males compared to females, with potentially important consequences for several pathologies. We also report that among human PBL, eosinophils are by far the cell type expressing the highest level of P2X4 on the cell surface, suggesting that ATP-dependent activation could be important in the eosinophil biology in healthy and pathological contexts.

ETHICS STATEMENT

Animal handling and maintenance were performed according to the interdisciplinary principles and guidelines for the use of animals in research, testing and education (FELASA) prepared by Ad Hoc Committee on Animal Research (The New York Academy of Sciences, New York, NY, USA). The animal experiments described in this study were authorized by the Ethical and Animal Welfare Committee of Estonia (Tartu University, ERC nr 181T-1). Blood samples used in the current study were obtained from healthy donors in accordance with the principles of the Helsinki Declaration of 1975 and subsequent

amendments by the World Medical Assembly. Permission No. 160 was issued to SB on 18.02.2013 by the Ethics Review Committee (ERC) on Human Research of the National Institute for Health Development, Tallinn.

AUTHOR CONTRIBUTIONS

VP, AR, JK, and SR conceived the project. VP, AR, HA, KR, AG, JT, C-SC, MB, JK, and SR designed experiments. VP, AR, KM, HA, KR, BT, AG, C-SC, MB, JK, and SR performed wet-lab experiments. VP, AR, KM, MT, AG, JT, C-SC, PB, MB, JK, and SR performed primary data analysis. HA, KR, MT, BT, AG, JT, AR, JK, TT, and C-SC provided resources. VP, MT, PB, JK, TT, and SR wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02074/full#supplementary-material

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ORIGINAL ARTICLE

Sex-dependent expression levels of VAV1 and P2X7 in PBMC of multiple sclerosis patients

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Abstract

Multiple sclerosis (MS) is an inflammatory autoimmune disorder of the central nervous system and the leading cause of progressive neurological disability in young adults. It decreases the patient's lifespan by about 10 years and affects women more than men. No medication entirely restricts or reverses neurological degradation. However, early diagnosis and treatment increase the possibility of a better outcome. To identify new MS biomarkers, we tested the expression of six potential markers (P2X4, P2X7, CXCR4, RGS1, RGS16 and VAV1) using qPCR in peripheral blood mononuclear cells (PBMC) of MS patients treated with interferon β (IFNβ), with glatiramer acetate (GA) or untreated. We showed that P2X7 and VAV1 are significantly induced in MS patients. In contrast, the expression of P2X4, CXCR4, RGS1 and RGS16 was not significantly modified by MS in PBMC. P2X7 and VAV1 are essentially induced in female patients, suggesting these markers are connected to sex-specific mechanisms. Strikingly, VAV1 expression is higher in healthy women than healthy men and IFNβ treatment of MS reduced VAV1 expression in female MS patients while it up-regulated VAV1 in male MS patients. Our data point to the differential, sex-dependent value of MS markers and treatment effects. Although *rgs16* expression in PBMC was not a valid MS marker in patients, the strong upregulation of P2X4 and P2X7 induced in the spinal cord of WT mice by EAE was abrogated in *rgs16*KO mice suggesting that *rgs16* is required for P2X4 and P2X7 induction by neurological diseases.

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1 | INTRODUCTION

Multiple sclerosis (MS) is a heterogeneous inflammatory autoimmune disorder of the central nervous system (CNS) and the leading cause of progressive neurological disability in young adults.[1] The disease generally manifests in patients aged 20-50 and affects women more often than men. The central mechanism of MS is the migration of autoreactive cells across the blood–brain barrier (BBB) into the brain. These cells target and attack the protective myelin sheaths that surround axons and enable neurons to transmit signals. Progression of the disease involves the loss of oligodendrocytes, which are responsible for generating and maintaining the myelin sheath.[2] Symptoms of MS depend on the location and severity of brain lesions. The most common form of the disease is characterized by relapses that are followed by remissions. During relapses, symptoms manifest or get worse. In the periods of remission, patients recover wholly or partially.[3] No genetic or environmental factors have been identified as being solely responsible for MS but genetic predisposition, viral infections and several environmental risk factors are all believed to play a role in the aetiology of the disease.[4]

There is currently no cure for MS. However, there are several treatments that reduce the recurrence and severity of relapses, shorten the relapse periods, stimulate tissue repair, ease the symptoms and prevent disability resulting from MS progression.[5] Still, no medication entirely restricts or reverses neurological degradation.[6] A promising approach in MS treatment is immune system reconstitution after immune ablation, followed by autologous haematopoietic stem cell transplantation.[7] Early diagnosis and treatment increase the possibility of a better long-term prognosis for MS patients.[6] Hence, there is a strong interest in novel biomarkers that could be used for the early detection of MS. Genes showing MS-specific expression patterns would be of particular interest. Such biomarkers may be helpful not only for diagnosing MS but also for monitoring the progression of the disease and evaluating the impact of treatments.[8]

The most common experimental model for studying MS is experimental autoimmune encephalomyelitis (EAE), a condition that can be artificially induced in mice. This disease model mimics the immuno- and neuropathological mechanisms of MS, including brain inflammation, demyelination, gliosis and axonal loss.[9]

In the present study, we assessed the expression of six genes for their potential as biomarkers for MS. For this we measured their expression in peripheral blood mononuclear cells (PBMC) of healthy individuals and patients with MS receiving different disease-modifying drugs. The choice of PBMC to test MS markers was based on the availability of such samples, that are collected from each patient in the frame of treatment protocols. Also, the markers tested in this study are expressed and potentially induced in immune cells. PBMC have been used to test MS biomarkers in multiple studies.[10–12]

Since MS is more frequently diagnosed in women, we also tested for any sex-specific differences, to see whether the potential biomarkers would be equally useful in both biological sexes.

The genes we chose for testing were as follows.

Two purinergic receptors – **P2X4** and **P2X7** – were selected because both are up-regulated in the nervous system during MS.[13,14] Postmortem studies have shown that the P2X7 receptor is more highly expressed in the microglia of MS patients than in non-MS controls.[15] Functional and non-functional genetic variants in the sequences of both P2X4 and P2X7 have also been identified as possible factors that modulate MS predisposition.[16]

CXCR4 is a G protein-coupled receptor (GPCR) that is involved in T cell migration[17] and has previously been reported to have lower expression in MS patients compared to healthy controls.[18]

The proteins **RGS1** and **RGS16** (Regulators of G protein signalling 1 and 16) belong to a family that downmodulates biochemical pathways downstream of GPCRs. RGS activity dissociates the receptor-coupled heterotrimeric G protein, thereby blocking signalling through the receptor and inhibiting any downstream biochemical pathways. Different RGS proteins are known to modulate different receptors, although sometimes their specificities can overlap. RGS1 has been previously associated with MS,[19,20] although its expression and role in PBMC of MS patients remain controversial.[21–24] RGS16 was selected as a potential candidate since it restricts proinflammatory responses[25] and inhibits T-cell migration by regulating the G-protein-coupled receptors and CXCR4 signalling.[17] Both RGS1[26] and RGS16 are induced by type I IFN which might be relevant during some MS treatments. Furthermore, our preliminary analyses have suggested that the expression of purinergic receptors is differentially affected in MS models using RGS16-deficient mice.

Finally, **VAV1** has been previously shown to be involved in EAE[27] and also, in a seminal paper, in multiple sclerosis.[28] This guanine nucleotide exchange factor (GEF) functions in transducing T cell antigen receptor (TCR) signals. After phosphorylation, VAV1 activates the small GTPases. VAV1 also acts as an adaptor for proteins which are involved in TCR, BCR and other signalling pathways.[29] Importantly, a knock-in mouse with a $VAV1^{R63W}$ mutation showed decreased effector T-cell functions and a less severe EAE due to impaired VAV adaptor functions.[27]

In the second part of this study, we investigated P2X4 and P2X7 gene expression in the spinal cords and P2X4 protein expression on the surface of blood cells of

wild-type (WT) and RGS16 deficient (RGS16KO) mice in the EAE model to determine whether G protein regulation and purinergic signalling could be directly connected in the context of MS.

By comparing the expression of the six biomarkers in healthy individuals and patients with MS receiving different treatments, our data reveal contrasting effects of two of the platform therapies, Interferon-β (IFNβ) and glatiramer acetate (GA). Moreover, we find differences in how men and women respond to treatment. EAE in RGS16 deficient mice also demonstrates the role of this RGS in the P2X4 and P2X7 induction by the disease.

2 | MATERIALS AND METHODS

2.1 | Blood sampling

Samples of venous blood were collected from MS patients (n = 87) and healthy controls (n = 40). These specimens were provided by West Tallinn Central Hospital and East Tallinn Central Hospital during the time period of 2013-2018. All the procedures and analyses of this study were carried out according to the principles of the Helsinki Declaration of 1975 and following amendments by the World Medical Assembly. Approval No 160 was granted on 18 February 2013 to Sirje Rüütel Boudinot from the Ethics Review Committee on Human Research of the National Institute for Health Development, Tallinn. All blood donation was voluntary, and patients gave their signed agreement to be part of the scientific study.

Blood samples (usually 10-20 mL) were obtained from patients of different biological sex (26 males and 61 females) and with a broad age range (from 20 to 73 years old). The median age of patients was 40 years (41 for males and 39 for females). After patients were informed of the potential side effects of GA or IFN-based treatment, they were invited to indicate their choice, which was validated by medical doctors. For analysis, all patients were divided into three groups according to their current treatment that had been prescribed by their doctor: patients treated with IFNβ-based drugs (Avonex, Betaferon, Rebif, Extavia; n = 40), patients treated with glatiramer acetate (Copaxone; n = 27) and patients nontreated at the moment they donated blood (n = 20). It should be noted that this procedure might add factors of variations.

The healthy control group had a similar male-to-female ratio compared to MS patients (14 males and 26 females), and their age varied from 20 to 64 years with the median age being 34 years (34 years for males and 35 for females). The sexes and ages of all participants in different treatment groups are shown in Table S1.

An identification number was added to all blood specimens at the point of collection. The anonymity of donors was kept for sample processors and analysts, only their biological sex, birth year, age and treatment option were provided by the medical practitioner who collected the samples. Their disease duration remained unknown to the researchers.

2.2 | Processing of blood

Peripheral blood mononuclear cells were extracted from whole blood using Ficoll-Paque TM PREMIUM reagent (GE Healthcare UK Limited, United Kingdom) according to the manufacturer's suggested protocol. PBMC were stored in cell lysis buffer RLT (QIAGEN, Germany) at a temperature of −20°C until further handling. RNA was extracted from PBMC using RNeasy Plus Mini Kit (QIAGEN, Germany) according to the manufacturer's suggested protocol. RNA concentration and absorbance ratios were measured using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan).

2.3 | Mice and EAE induction

All procedures on animals were performed with permission and according to the rules and guidelines of the Ethical and Animal Welfare Committee of Estonia (the University of Tartu, ERC nr 27 and 28). The mice EAE induction experiments took place in the Tallinn University of Technology Vivarium in the years 2015 to 2018. The mice used in these experiments were 2 months to a year old when euthanized. Thirty-two wild-type C57BL/6 (15 male and 17 female) and 15 RGS16 knockout mice (RGS16KO, 7 male and 8 female) were used in the mouse spinal cord study. For blood analysis, 20 C57BL/6 mice (10 males, 10 females) and 14 RGS16 knockout mice (7 males, 7 females) were used.

RGS16KO mice that had been generated using the classical Cre-Lox recombination system on C57BL/6 mice,[30] were received as a generous gift from Professor Kirk Druey, NIAID, Bethesda, USA. The RGS16KO mice offspring born in the Tallinn University of Technology Vivarium were genotyped before using in the experiments as described in the protocol by Lopez.[31] The sex of the mice was determined visually.

Experimental autoimmune encephalomyelitis was induced on day 0 by subcutaneous injections of myelin oligodendrocyte glycoprotein (MOG) peptide fragment 35-55 (Storkbio Ltd.; containing amino acids 35-55 of MOG) into the left and right hip (40 μL per side) and both hind paws (10 μL per paw). Each mouse received in total 12.5 μL (50 μg) of MOG35-55 in 37.5 μL phosphate-buffered saline (PBS) and 50 μL of complete Freund's adjuvant (Sigma-Aldrich) containing 28 μg/mL

desiccated *Mycobacterium tuberculosis (M. tb)* powder H37 RA (Difco Laboratories Inc). At 1 hour and 48 hours after MOG injections, 250 ng of Pertussis toxin (PTX; Invitrogen) in 200 μL of PBS was injected intraperitoneally. The clinical score of EAE was visually determined every day from day 7 on a scale from 0-5 based on Hooke Laboratories protocol.[32] The mice of EAE experiments were euthanized by cervical dislocation on day 19 or 20 of the experiment. To collect and analyse blood samples, 5 minutes before euthanizing, each mouse was given an intraperitoneal injection of 100 μL of 5 times diluted heparin (LEO Pharma, 5000 I.U./mL) solution in PBS to keep the blood from coagulating after death. Blood was collected from the eye socket or if necessary, from the heart into separately marked tubes. The spinal cords of mice were frozen immediately after hydraulic extrusion.

Mortar and pestle were used to homogenize spinal cords in liquid nitrogen. The cells were lysed in TRIzol (Thermo Fisher Scientific, USA) and mRNA was extracted using TRIzol according to the manufacturer's suggested protocol. DNase treatment was performed using DNase Treatment Kit (Ambion, USA). RNA concentration and absorbance ratios were measured using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan).

2.4 | cDNA synthesis and qPCR

Complementary DNA (cDNA) was synthesized with RevertAid Reverse Transcriptase (Thermo Scientific, USA), RiboLock RNase Inhibitor (Thermo Scientific, USA) and oligodT (18) primer (Oligomer, Finland) according to the manufacturer's suggested protocol. In most cases, 500 ng of total RNA was used as the template. All cell, RNA and cDNA samples not used immediately were stored at temperatures −20 or −80°C.

Relative expression of individual genes was quantified until cycle 35 by qPCR on a ROCHE LightCycler 480 II thermal cycler. All reactions were conducted in a volume of 10 μL, containing 0.2 μL 10 mM gene-specific F + R primers, cDNA that was obtained from 5 ng of RNA and 2 μL of 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX; Solis Biodyne, Estonia). Primer sequences were selected from Harvard Medical School's primer bank and their specificity was checked with MFEprimer 2.0, primers themselves were synthesized by TAG Copenhagen A/S (Denmark). For human samples, 6 pairs of primers were used, each targeting a different biomarker candidate (Table 1, primers hP2X4, hP2X7, hRGS1, hRGS16, hVAV1 and hCXCR4). For mouse samples, only the purinergic receptors were analysed (Table 1, primers mP2X4 and mP2X7). We also targeted specific endogenous reference genes: the gene for ribosomal protein lateral stalk subunit P0 in humans (primer hRPLP0) and the genes for phosphoglycerate kinase 1 and succinate dehydrogenase complex subunit A in mice (primers mPGK1 and mSDHA; Table 1).

All cDNA and primer mixes were prepared and pipetted to the 384-well (Bioplastics B70515L, natural) or 96-well (Bioplastics B17489L, white) qPCR plates in a total volume of 10 μL for every reaction according to the standard protocol. The plates were sealed with cover sheets (Bioplastics Opti-Seal cat. No. 157300) and centrifuged for 30 seconds at 300 rpm in a microplate centrifuge.

A reference sample (interplate control, IPC) was included on every plate to allow combining the results across different qPCR runs. For plates containing human samples, cDNA from control patient P1 was used as IPC. For plates containing mouse samples, a mixture of mouse spine cDNAs was used as IPC. For every cDNA and primer combination, three reactions on the same plate were measured. When the results were not sufficiently close to each other, another test with these materials was performed.

TABLE 1 Primers used in real-time quantitative PCR.

Primer	Forward sequence	Reverse sequence
hRPLP0	5′-CCT CAT ATC CGG GGG AAT GTG-3′	5′-GCA GCA GCT GGC ACC TTA TTG-3′
hP2X4	5′-CTC ATC CTG GCC TAC GTC AT-3′	5′-CCC TTG ACC TTG GTC GTA AC-3′
hP2X7	5′-TAT GAG ACG AAC AAA GTC ACT CG-3′	5′-GCA AAG CAA ACG TAG GAA AAG AT-3′
hRGS1	5′-TGC TGC TGA AGT AAT GCA ATG-3′	5′-AGT CTT CAC AAG CCA GCC AG-3′
hRGS16	5′-AGT CTG CAG GTT CAT CCT CG-3′	5′-GAT CCG ATC AGC TAC CAA GC-3′
hVAV1	5′-CAA CCT GCG TGA GGT CAA C-3′	5′-ACC TTG CCA AAA TCC TGC ACA-3′
hCXCR4	5′-ACT ACA CCG AGG AAA TGG GCT-3′	5′-CCC ACA ATG CCA GTT AAG AAG A-3′
mPGK1	5′-ATG DCG CTT DCC AAC AAG CTG-3′	5′-GCT CCA TTG TCC AAG CAG AAT-3′
mSDHA	5′-GGA ACA CTC CAA AAA CAG ACC T-3′	5′-CCA CCA CTG GGT ATT GAG TAG AA-3′
mP2X4	5′-CCC TTT GCC TGC CCA GAT AT-3′	5′-CCC TTT GCC TGC CCA GAT AT-3′
mP2X7	5′-TGG CAC CGT CAA GTG GGT CTT GCA C-3′	5′-CCT TTG ACC TTG GTG TGC ACG GAG CTG-3′

2.5 | Cell staining and flow cytometer analysis

50 µL of blood from every mouse was used for staining. 5ul of P2X4-FITC antibody (a monoclonal antibody made in our laboratory[33] conjugated with fluorescein isothiocyanate) was added to the blood, suspended and then incubated for 15 minutes in dark at RT. After that, blood cells were treated for 10 minutes with 1 mL of Red Blood Cell Lysing Buffer (RBC) and suspended in the PBS to be analysed with the FACS device FACSCalibur™ as soon as possible to get reliable results. The cell intake from stained samples was 30 000 cells per acquisition. All results were saved for further analysis with CellQuestTM data analysis software.

2.6 | Data analysis

For analysis of gene expression, the ΔΔct method was used to obtain relative mRNA expression compared to the endogenous control gene(s) and IPC. These calculations were performed and charts were created in MS Excel. The data gathered from FACS analysis was also processed in MS Excel.

RGS16KO mice blood data gathered from FACS had an approximately normal distribution (controlled with Shapiro–Wilk Normality Test) and the *t*-test was used to evaluate the statistical significance of these results. The rest of the data obtained in this study cannot be considered normally distributed, therefore the statistical significance of the results was estimated by performing the non-parametric Mann–Whitney *U*-test. If the *P*-value obtained for the *t*-test or Mann–Whitney *U* test is smaller than .05 then the difference between groups can be considered statistically significant. Additional significance thresholds were defined at values .01, .001 and .0001. Spearman correlation coefficients were calculated for correlation analysis of our six biomarker candidate genes in PBMC. Their statistical significance was evaluated with *P*-values. All the statistical evaluations were performed with either R (version 3.5.2) or the statistics software GraphPad Prism 8.0.1.

3 | RESULTS

3.1 | P2X7 is up-regulated in PBMC of MS patients while P2X4 is induced by IFNβ treatment

To analyse the P2X4 receptor expression in MS patients, we first merged the data obtained for all treated and non-treated MS patients (all MS patients, n = 87) and compared them with the healthy control group (n = 40). In this merged data set, P2X4 receptor expression was significantly increased in MS patients compared to healthy controls ($P = .0165$; Figure 1A). Furthermore, the seven samples with the highest P2X4 expression are all from patients with MS (Figure 1A,B). There is less statistical power in comparing each of the treatment groups separately to the control group and a significant difference was observed only between healthy controls and patients treated with interferon-β (IFNβ) and IFNβ-derived drugs ($P = .0359$; Figure 1B). Hence, these results do not provide significant evidence of P2X4 induction by MS. In fact, *IFNβ* treatment rather than MS could explain why many patients of the entire dataset had P2X4 upregulated. However, a trend towards higher expression was visible nonetheless in other MS patients compared to healthy controls.

Performing the same comparisons for P2X7 revealed a similar trend for all MS patients (treated or untreated) with a higher expression compared to healthy controls ($P = .01$; Figure 1C). Again, all individuals exhibiting the highest expression are in the MS group, rather than in the healthy control group (Figure 1C). However, in contrast to P2X4, P2X7 was significantly more expressed in both non-treated ($P = .002$) and GA-treated MS patients ($P = .019$) compared to the healthy control group (Figure 1D). No significant P2X7 up-regulation was observed after treatment with IFNβ-based drugs, compared to healthy controls, suggesting that this treatment may inhibit MS-induced P2X7 induction.

3.2 | IFNβ treatment, but not MS, induces significant up-regulation of GPCR pathway components CXCR4, RGS1 and RGS16

The combined data from all patients with MS suggests a significant up-regulation of all tested GPCR pathway components (data not shown). However, further analysis of the data from different treatment groups reveals additional patterns. In particular, we find that for all three markers (CXCR4, RGS1 and RGS16) the observed increases in mRNA expression are largely driven by the largest subset of the data, the 40 patients treated with different IFNβ-based drugs. In fact, the expression levels of those genes in untreated patients are close to their expression in healthy controls. GA does seem to induce a slight increase in expression, but it is not of statistical significance for any of the three genes. In contrast to GA, IFNβ treatment leads to a significant up-regulation for the expression of all these genes, compared to healthy controls ($P < .0001$ for

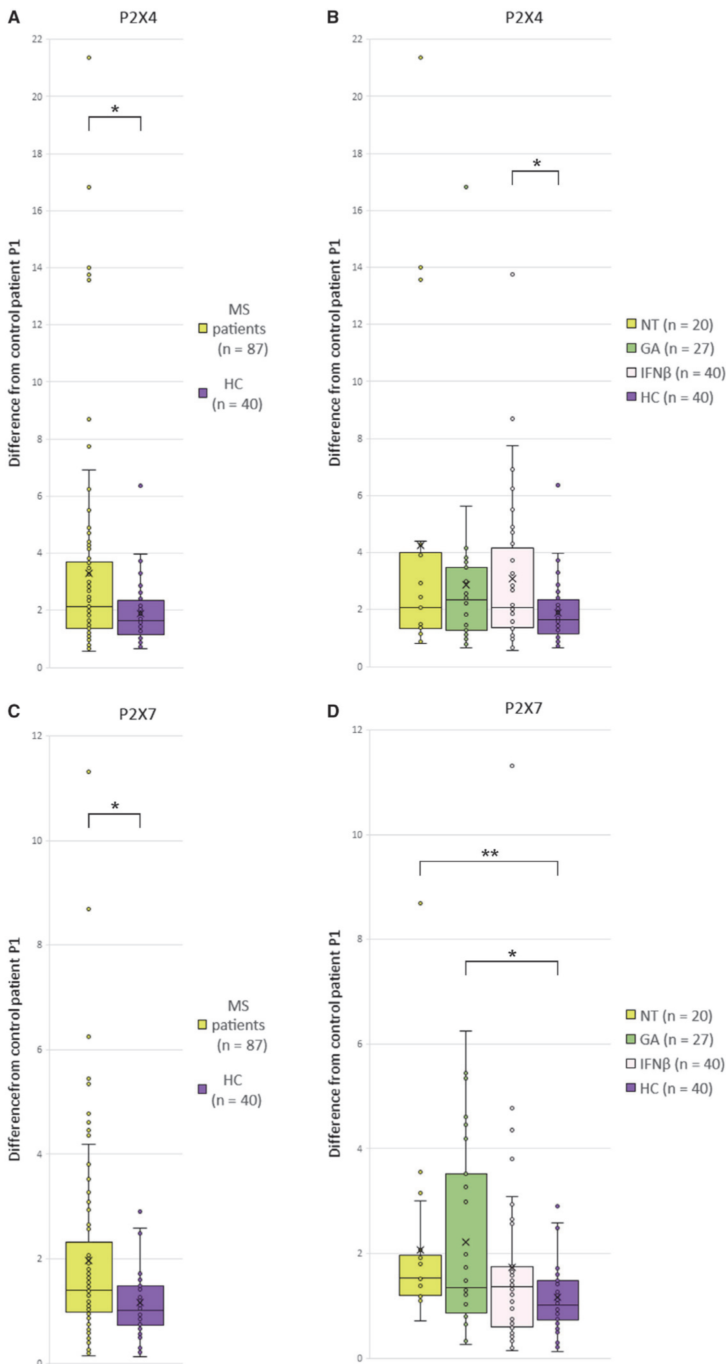

FIGURE 1 Relative P2X4 and P2X7 gene expression in human PBMC. A and C: yellow – MS patients (treated and not treated), violet – healthy control group. B and D: yellow – non-treated MS patients, green – GA-treated MS patients, pink – IFNβ-treated MS patients, violet – healthy control group. (A) P2X4 expression is increased in MS patients compared to the healthy control group ($P = .0165$). (B) P2X4 expression is increased among IFNβ-treated patients compared to the healthy control group ($P = .0359$). (C) P2X7 expression is increased in MS patients compared to the healthy control group ($P = .01$). (D) P2X7 expression is higher in non-treated MS patients ($P = .002$) and GA-treated patients ($P = .019$) compared to the healthy control group. *Y*-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. *P-value < .05, **P-value < .01.

all), non-treated patients ($P = .019$, $P = .0004$, and $P \leq .0001$ accordingly) and GA-treated patients ($P = .0078$, $P = .0006$, and $P = .0003$ accordingly, Figure 2A-C).

Hence, our results do not support the hypothesis of MS itself substantially affecting the expression of CXCR4, RGS1 and RGS16.

3.3 | VAV1 expression is higher in GA-treated MS patients

The combined data from all patients suggests a significant increase in the expression of VAV1 ($P = .0291$, Figure 3A). It is important to note that there is no significant difference between healthy controls and patients treated with IFNβ (Figure 3D), although a trend of increased expression can be seen for both untreated patients and those treated with GA. GA-treated patients are the only ones for which the difference is statistically significant ($P = .0018$/healthy control group). Interestingly, IFNβ-treated patients exhibit significantly lower VAV1 expression when compared to GA-treated patients ($P = .01$) indicating that these two treatments do not affect similarly VAV1 expression level (Figure 3B).

3.4 | Striking differences in biomarker expression patterns in men and women

Multiple sclerosis is more frequent in women than men. Moreover, it is believed that the pathogenesis is often faster in men than in women, suggesting that the mechanisms might be partly different. We, therefore, compared the expression patterns of our six selected biomarker candidates between women and men.

3.4.1 | P2X4 is significantly up-regulated only in male MS patients treated by IFNβ

P2X4 expression was significantly higher only in IFNβ-treated MS patients. Strikingly, this occurred only in men ($P = .05$ compared to NT male MS patients and $P = .0253$ compared to the male healthy control group; Figure 4B). There was no such rise in female MS patients. The median value of P2X4 expression in IFNβ-treated female patients was even a little lower than in female NT or GA MS patients (Figure 4C). Still, we see a tendency of slight up-regulation of P2X4 in all female patient groups compared to the healthy control group (although this result is not supported by the statistical analysis; Figure 4C).

3.4.2 | P2X7 expression was increased mainly in MS female patients

Our results show that only non-treated MS female patients had increased P2X7 expression compared to the

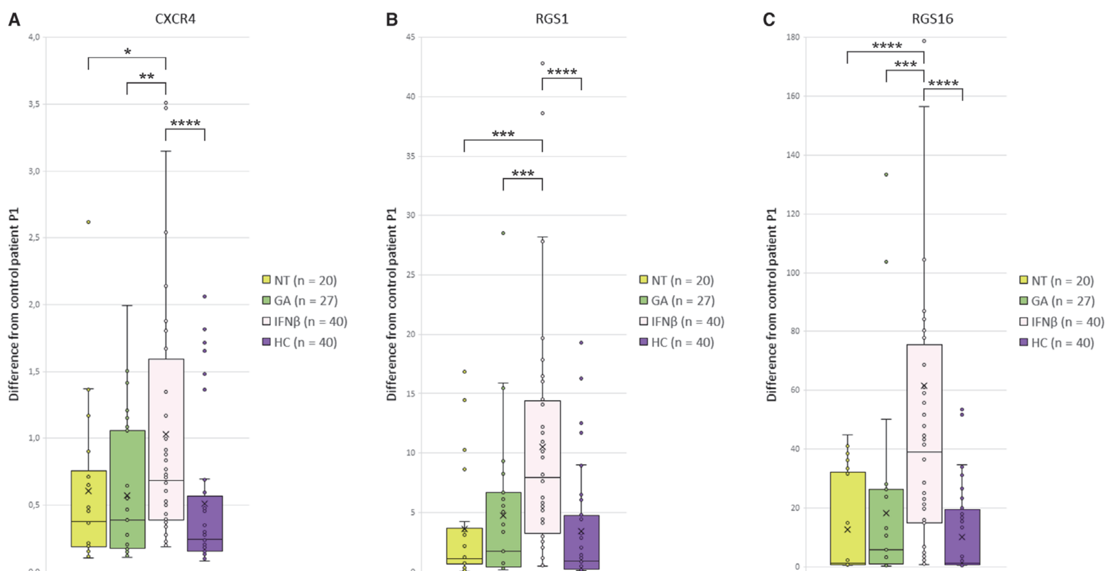

FIGURE 2 Relative CXCR4, RGS1 and RGS16 gene expression in human PBMC. A-C: yellow – non-treated MS patients, green – GA-treated MS patients, pink – IFNβ-treated MS patients, violet – healthy control group. (A-C) IFNβ-treated MS patients have higher CXCR4, RGS1 and RGS16 levels compared to non-treated MS patients ($P = .019$, $P = .0004$, and $P \le .0001$ accordingly), GA-treated MS patients ($P = .0078$, $P = .0006$, and $P = .0003$ accordingly), and healthy control group (all $P < .0001$). *Y*-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. *P-value < .05, **P-value < .01, ***P-value < .001, ****P-value < .0001.

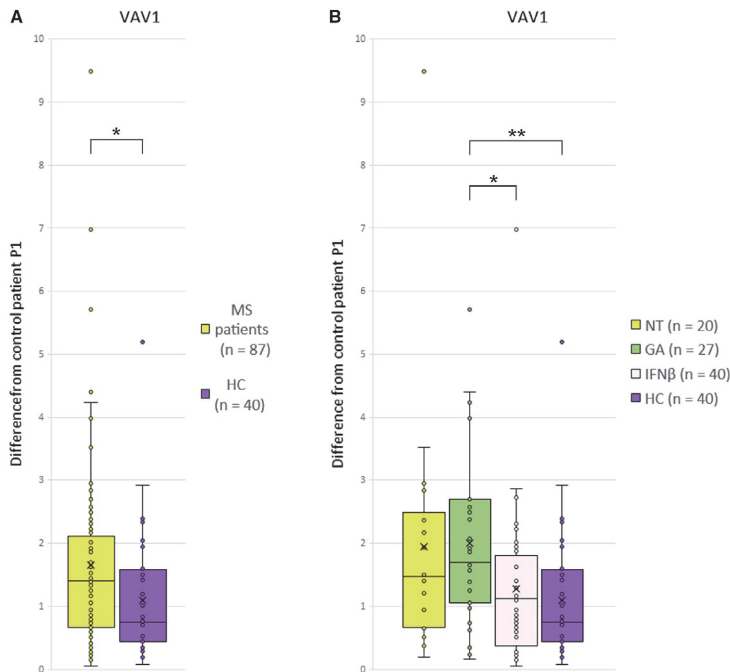

FIGURE 3 Relative VAV1 gene expression in human PBMC. A: yellow – MS patients (treated and not treated), violet – healthy control group. B: yellow – non-treated MS patients, green – GA-treated MS patients, pink – IFNβ-treated MS patients, violet – healthy control group. (A) VAV1 expression level is elevated in PBMC of MS patients compared to the healthy control group ($P = .0291$). (B) GA-treated MS patients have increased VAV1 expression compared to IFNβ-treated MS patients ($P = .01$) and the healthy control group ($P = .0018$). *Y*-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. **P*-value <.05, ***P*-value <.01, ****P*-value <.001, *****P*-value <.0001.

healthy control group ($P = .0018$/healthy control women; Figure 4D). Among men, the P2X7 expression levels were similar in all the observed groups – non-treated, IFNβ-treated, GA-treated MS patients and the healthy control group (Figure 4E). Furthermore, P2X7 was also expressed at higher levels in GA-treated female patients than in the healthy control group ($P = .0175$; Figure 4F), indicating that this treatment did not inhibit P2X7 induction in women in contrast to the trend observed for IFNβ treatment.

3.4.3 | VAV1 up-regulation in non-treated MS female patients was inhibited by IFNβ, while this treatment raised VAV1 levels in men

VAV1 expression levels were higher in female MS patients compared to male patients. In particular, VAV1 expression levels were much higher in non-treated women with MS compared to non-treated men with MS ($P = .0077$), or healthy controls ($P = .0105$/healthy women, and $P = .0042$/healthy men; Figure 5A).

Furthermore, the impact of MS treatments on VAV1 expression was strikingly different in men and women (Figure 5B,C). Among men, VAV1 was expressed in PBMC of non-treated MS patients at similar levels as in healthy controls, but IFNβ treatment increased the expression significantly compared to non-treated male MS patients ($P = .0365$) and the healthy men control group ($P = .0478$, Figure 5B). Non-treated female MS patients had significantly higher VAV1 expressions compared to IFNβ-treated female MS patients ($P = .003$; Figure 5C) but not GA-treated female MS patients. The VAV1 expression levels of IFNβ-treated female MS patients were in fact remarkably similar to the healthy control group. Taken together, these observations show the opposite effects of the IFNβ treatment on VAV1 expression in women and men.

These contrasted responses between men and women were also supported by pairwise correlation analysis of P2X7, P2X4 and VAV1 expression levels in PBMC (Table S2). A positive correlation of VAV1 expression with P2X, only in women MS patients, was very clear. Also, the significant correlation of P2X4 with P2X7 and with VAV1,

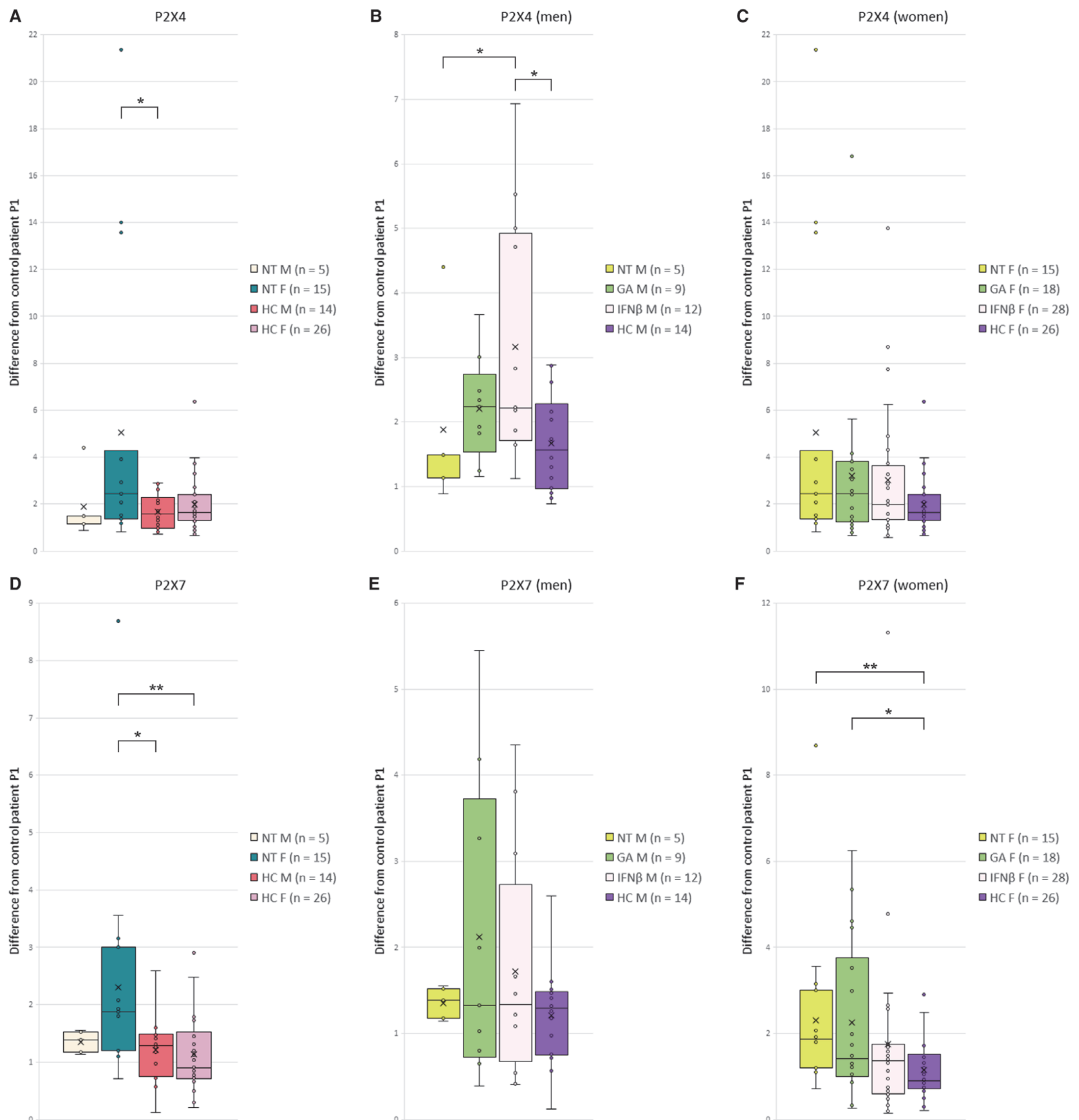

FIGURE 4 Relative sex-dependent P2X4 and P2X7 gene expression in human PBMC. A and D: beige – non-treated MS male patients, marine blue – non-treated MS female patients, red – healthy control group men, pink – healthy control group women. B and E: men. C and F: women. B, C, E and F: yellow – non-treated MS patients, green – GA-treated MS patients, pink – IFNβ-treated MS patients, violet – healthy control group. (A) Comparison of P2X4 expression by sex between NT MS patients and the healthy control group. (B, C) IFNβ treatment increases P2X4 expression among men compared to non-treated patients ($P = .05$) and the healthy men control group ($P = .0253$) but not among women. (D-F) P2X7 expression is elevated in non-treated female MS patients compared to healthy men ($P = .0446$) and women ($P = .00177$) but is not elevated in non-treated male MS patients. IFNβ inhibits the P2X7 induction in women, however, GA treatment does not. GA-treated women have higher P2X7 expression compared to healthy women ($P = .0175$). *Y*-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. *P-value < .05, **P-value < .01.

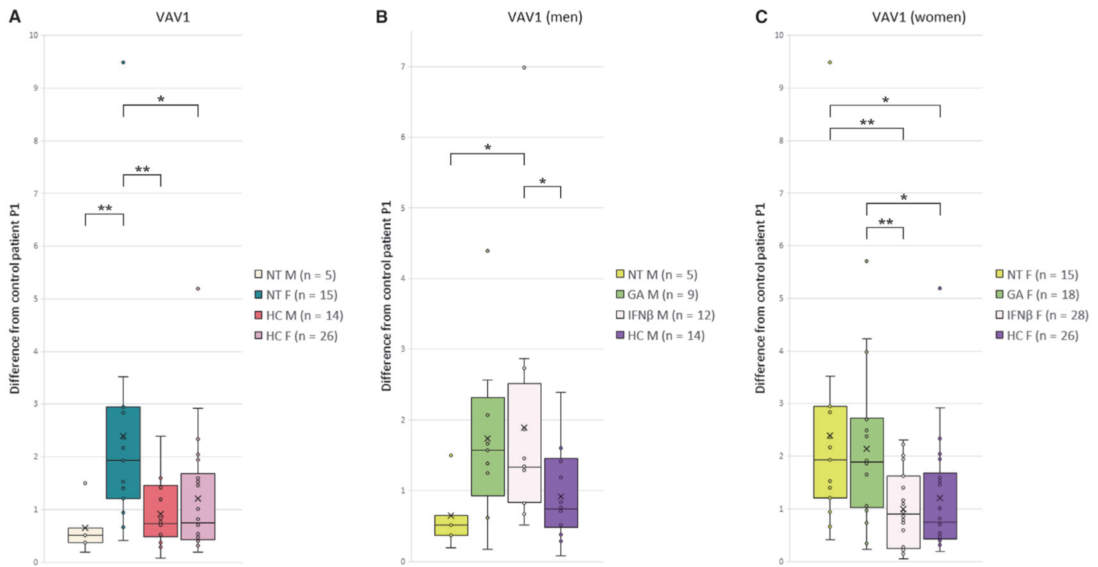

FIGURE 5 Relative sex-dependent VAV1 gene expression in human PBMC. A: beige – non-treated male MS patients, marine blue – non-treated female MS patients, red – healthy men control group, pink – healthy women control group. B: men. C: women. B and C: yellow – non-treated MS patients, green – GA-treated MS patients, pink – IFNβ-treated MS patients, violet – healthy control group. (A) Non-treated MS female patients have raised VAV1 expression compared to non-treated MS male patients ($P = .0077$), healthy men ($P = .0042$), and healthy women ($P = .0105$). (B) IFNβ treatment increases VAV1 expression in MS male patients compared to non-treated MS male patients ($P = .0365$) and healthy men ($P = .0478$). (C) Non-treated ($P = .0105$) and GA-treated ($P = .0126$) female MS patients have higher VAV1 expression compared to healthy women. IFNβ treatment lowers VAV1 expression in MS female patients compared to non-treated ($P = .003$) and GA-treated ($P = .0028$) female MS patients. *Y*-axis indicates fold-change compared to control patient P1. Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. *P-value < .05, **P-value < .01.

suggests that P2X4 follows a relatively similar expression pattern as P2X7 and VAV1, the markers identified in women in this study.

3.5 | RGS16 is required for the P2X4 and P2X7 up-regulation in the mouse spinal cord by EAE

Our data of differential up-regulation patterns of P2X4 and P2X7 in female and male MS patients were reminiscent of some of our previous observations in *rgs16* deficient (*rgs16* KO) mice. To investigate the possible connection between our biomarker candidates, we designed an experiment in which the expression of P2X4 and P2X7 was directly measured from the spinal cord tissue of RGS16KO and control (wild-type, WT) mice, both in healthy individuals and in those in whom EAE (the mouse model of MS) had been induced.

In the spinal cord of WT mice, the expression of both P2X4 and P2X7 is significantly increased during EAE (both $P < .0001$). In RGS16KO mice this increase is not present (Figure 6A,B). This striking observation demonstrates that in a mouse model of MS, P2X4 and P2X7 are induced in an RGS16-dependent manner, possibly mediated by its role in inhibiting the migration of T-lymphocytes to the brain.[34]

During the characterization of anti-P2X4 monoclonal antibodies by flow cytometry,[33] we noticed that the regulation of P2X4 expression on the surface of mouse blood cells by *rgs16* was sex-dependent, pointing to a mechanism that may be relevant also for the implication of these genes in autoimmune neurological disorders. The level of P2X4 expression was similar in females for WT and RGS16KO mice. In contrast, it was much lower in RGS16KO males compared to WT ($P = .0068$, Figure 6C), indicating that the control mechanism of *rgs16* over P2X4 expression is sex-dependent. RGS16 was thus necessary for P2X4 expression on blood cells in male mice but not in female mice.

In conclusion, the induction of the MS marker P2X7 and the other purinergic receptor P2X4 in the spinal cord was *rgs16*-dependent both in males and females. However, P2X4 expression on PBMC at the steady state required *rgs16* only in males, indicating that part of the *rgs16* regulatory pathway of P2X expression is sex-dependent.

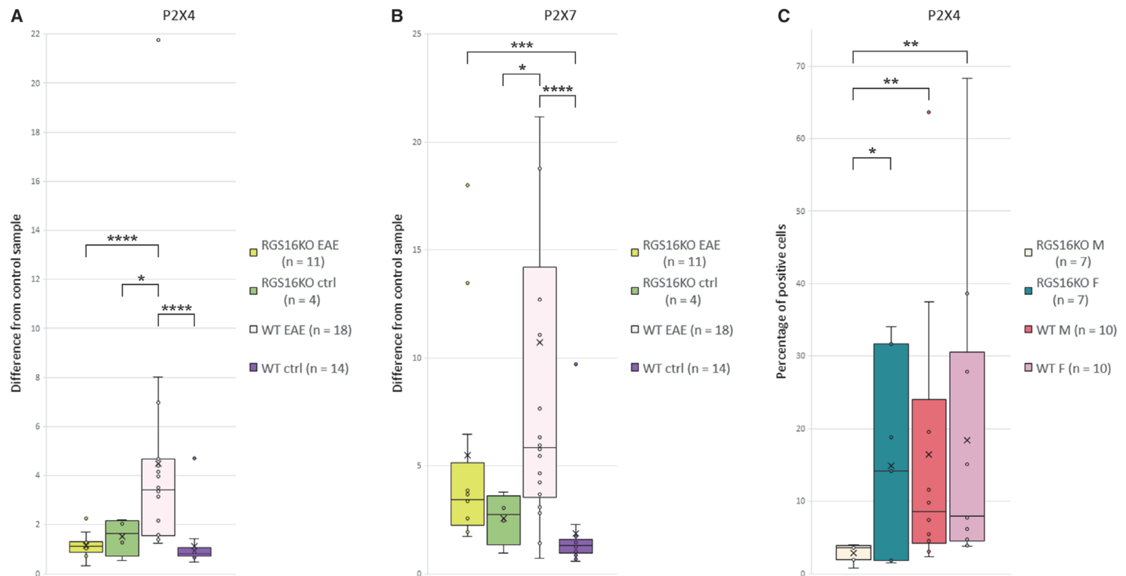

FIGURE 6 Relative P2X4 and P2X7 gene expression in mouse spinal cords and P2X4 protein expression on the blood cell surface. A and B: yellow – RGS16KO EAE mice, green – RGS16KO control mice, pink – WT EAE mice, violet – WT control mice. C: beige – RGS16KO male mice, marine blue –RGS16KO female mice, red – WT male mice, pink – WT female mice. (A) During EAE, P2X4 expression increases in the spinal cords of WT mice compared to WT control mice ($P<.0001$), RGS16KO control mice ($P=.0424$), and RGS16KO EAE mice ($P<.0001$). (B) During EAE, P2X7 expression is amplified in the spinal cords of WT mice compared to the control mice ($P<.0001$). (C) Fewer cells of RGS16KO male mice have P2X4 expressed on their surface compared to RGS16KO female mice ($P=.0439$), WT male mice ($P=.0068$), and WT female mice ($P=.002$). *Y*-axis indicates fold-change compared to the control sample (A and B) or the percentage of cells expressing P2X4 protein on their surface (C). Dots – individual values, horizontal line inside the box – median value, x – mean value, the upper line of the box – upper quartile, the lower line of the box – lower quartile, whiskers – greatest or lowest value excluding outliers. **P*-value < .05, ***P*-value < .01, ****P*-value < .001, *****P*-value < .0001.

4 | DISCUSSION

In this work, we have tested six potential MS markers (P2X4, P2X7, CXCR4, RGS1, RGS16 and VAV1) in PBMC of patients treated with IFNβ, GA or untreated. We showed that P2X7 and VAV1 are significantly induced in MS patients, while it is less clear for P2X4. In contrast, RGS1, RGS16 and CXCR4 are up-regulated by IFNβ but not by MS. IFNβ treatment impairs P2X7 – but not VAV1 (or P2X4) – induction. We also showed that P2X4 is up-regulated in IFNβ-treated male MS patients, while P2X7 and VAV1 are mainly induced in female patients, suggesting that these markers are connected to sex-specific mechanisms. Finally, EAE led to a strong up-regulation of P2X4 and P2X7 in the spinal cord of WT mice, which was abrogated in *rgs16*KO mice suggesting that *rgs16* is required for P2X4 and P2X7 induction and may play a role in the regulation of expression of these activation factors in autoimmune disorders of the central nervous system.

4.1 | Sex-dependent control of purinergic receptors P2X4 and P2X7 expression in PBMC of MS patients

Both P2X4 and P2X7 receptors are ion-gated channels that are activated by ATP. Upon binding of three ATP molecules to the P2X extracellular domain, the receptor is activated and the formation of a nonselective cation channel is induced,[35] which allows the influx of Na^+ and Ca^{++} and efflux of K^+. ATP hydrolysis and a decrease in ATP concentration promote the closure of these pores.[36] Under pathological conditions, ATP release can induce sustained P2X7 receptor activation, with inflammasome assembly and proinflammatory cytokine release. Until now, most studies on purinergic function have focused on P2X7. Less is known about the function of P2X4 under normal conditions and during pathogenesis. However, P2X4 is widely expressed in central and peripheral neurons and microglia, with possible links to MS pathology. P2X4 is also about 1000 times more sensitive to ATP

than the archetypical P2X7 receptor and might potentialize P2X7 activation in the inflammatory context.[37]

Our data reveal that P2X7 induction in PBMC of MS patients is sex-specific, being observed only in women. This up-regulation was maintained in GA-treated women. However, it was not significant in IFNβ-treated female patients, which possibly points to the effects of the treatment.

This contrasting response of P2X7 between male and female MS patients possibly points to distinct inflammatory pathways in PBMC and calls for further studies in the central nervous system. It also provides a marker of the response to IFNβ treatment in women.

In contrast, we did not observe a significant induction of P2X4 in PBMC of MS patients. However, this gene was up-regulated in the optic nerve of Multiple Sclerosis patients as well as in the spinal cord microglia and inflammatory foci in a rat model of EAE.[16] In this work, we also observed the expression of both P2X4 and P2X7 is significantly increased during EAE in the spinal cord of WT mice. This apparent discrepancy is likely explained by the different cell types expressing P2X4 in the nervous system and PBMC.

We also show here that IFNβ treatment up-regulates P2X4 only in male MS patients, again pointing to a sex-dependent regulation of expression, which might cause differential responses of men and women to such treatments. This observation was reminiscent of another sex-specific function of this purinergic receptor, which is necessary for pain hypersensitivity in male mice, while in female mice pain is mediated by adaptive immune cells independently of P2X4.[38] In the same line, the loss of BDNF in microglia has an effect in male mice, but not in female mice.[39] It would be very interesting to study whether chronic IFNβ exposure affects purinergic receptors in mice both in the EAE model and in wild-type animals and if this mechanism is sex-dependent, as it has been reported that IFNβ upregulates P2X4 receptor in microglia.[40] P2X4 expression profile from a mouse fibroblast L929 data set also supports a potential induction of this gene by IFNβ (geo data sets accession number GDS5202).

Taken together, our observations suggest that P2X4 and P2X7 show sex-dependent transcriptional responses in PBMC of MS patients, which are associated with the autoimmune disorder and its treatments. Further studies will be necessary to determine if such sex-dependent regulations also occur in the central nervous system with a potential direct impact on MS pathological mechanisms. Our results also suggest that MS treatments might affect men and women differently through effects on purinergic receptors.

4.2 | VAV1 is another sex-specific MS marker with opposite regulation by IFNβ in men and women

The role of VAV1 in haematopoietic cells has been extensively reviewed by several authors.[41–43] VAV1 activates Rho GTPases such as RAC and CDC42, which in turn stimulate several protein effectors like the MAP-Kinases JNK and p-38 involved in T and B cell proliferation, migration, cytokine production and other effector functions. Mice with VAV1-deficient T cells exhibited severe defects in immune responses.[44,45] Interestingly, VAV1 is also involved in neuroinflammation. *vav1*$^{-/-}$ mice do not develop EAE when injected with MOG, due to a defect of priming and expansion of antigen-specific T cells, while antigen presentation is fully functional in these mice.[43] In humans, the rs2546133-rs2617822 haplotype of the first *VAV1* intron has been associated with a predisposition to MS. The haplotype also segregates with high *VAV1* mRNA expression.[28]

In this study, untreated patients and GA-treated patients showed an increased VAV1 expression compared to healthy controls, confirming a previous report of VAV1 mRNA up-regulation both in PBMC and cerebrospinal fluid in MS.[28] Strikingly, our data show that high levels of VAV1 in MS patients are only observed in women, as for P2X7. We also observed that IFNβ treatment reduced VAV1 expression in female MS patients, while the same treatment up-regulated VAV1 in male MS patients. This contrasted reaction points to the potential sex-specific effects of VAV1 and IFNβ in MS patients. Hence, our data provide a sex-specific biomarker for MS in treated and untreated contexts. Future studies will be needed to identify the mechanisms of VAV1 involvement in MS pathogenesis in men and women, as well as the potential impact of IFNβ-induced VAV1 in male MS patients.

4.3 | RGS pathway components were not up-regulated in PBMC of non-treated patients but were induced by the IFNβ treatment

G protein-coupled receptor signalling plays an important role in several aspects of MS pathogenesis, including lymphocyte migration, cytokine/chemokine production, antigen presentation and T cell differentiation and proliferation.[46] We did not observe significant up-regulation of CXCR4, RGS1 or RGS16 in PBMC of MS patients. Repression of CXCR4 expression has been reported in T cell clones from MS patients treated with GA but not in those derived from patients treated with IFNβ.[47] In

contrast, we did not detect any significant effects of GA on CXCR4 expression in PBMC, compared to healthy controls and nontreated patients. However, CXCR4 was up-regulated by IFNβ treatment.

IFNβ treatment also increased RGS1 and RGS16 expression significantly in PBMC of MS patients. RGS16 up-regulation by type I IFN had been previously reported,[25,48] in line with its implication in antiviral immunity.[25] The clinical implications of RGS induction by IFNβ treatment in MS patient PBMC may be important, with both RGS1 and RGS16 being involved in the control of immune cell migration.[14,27,31,45] In contrast to IFNβ, GA did not affect RGS16 expression levels in PBMC of treated patients, which is consistent with different mechanisms of action of IFNβ and GA on MS.[49]

4.4 | rgs16 was required for p2x4 and p2x7 induction during EAE in mice, and this regulatory pathway comprises sex-dependent components

Our data did not validate RGS1 nor RGS16 up-regulation in PBMC as a relevant MS biomarker in contrast to P2X7 and VAV1. However, our data from EAE in the *rgs16*KO mice established a potentially interesting connection between *rgs16* and purinergic receptors in the context of autoimmune neurological disorders. The implication of the purinergic receptors *p2x4* and *p2x7* during EAE has been well characterized.[50–52] However, the involvement of *rgs16* in *p2x4* and *p2x7* induction is a new finding and suggests that the importance of these three genes, as well as their joined impact on inflammation, should be further characterized in MS. Furthermore, the sex-specific control of *p2x4* by *rgs16* – observed only in male mice – underscores the potential differences in the key pathways controlling neuro-inflammation in males and females.

In conclusion, we have identified sex-dependent expression patterns for the key regulators of inflammation in PBMC of MS patients. We also showed that treatments with GA and IFNβ can modulate the phenotypes in a sex-specific manner, pointing to the importance of this parameter for treatment optimization in clinics. Thus, while VAV1 was induced in PBMC of MS female patients only, IFNβ treatment was reducing its expression in women and increasing it in men. Our data also call for further characterization of the connections between RGS16 and purinergic receptors in the control of neuro-inflammation, again with potential contrast between men and women. Our data are in line with several recent reports that call for sex as a biological variable (SABV) in immunology, pointing to differential outcomes and immune mechanisms between males and females.[53]

AUTHOR CONTRIBUTIONS

AR, O-PS, JS, JK and SRB conceived the project; AR, O-PS, SRB, JS, TT and KG-P were designed experiments and sampling collection. AR, JS, JK and SRB wrote the manuscript. AR, O-PS, SRB, JS and JK edited the manuscript. AR, SRB, KR and TKL performed primary data analysis. All the authors read, corrected and approved the submitted version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest and agree with the submission to the Scandinavian Journal of Immunology.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supervised theses

Helinda-Eliise Saapar, BSc, 2023. “Cellular changes in the lymph node in melanoma metastasis”, (sup) Sirje Rüütel Boudinot, Airi Rump.

Emilia Di Giovanni, MSc, 2022. “Characterization of eosinophils in human melanoma through the detection of P2X4 and Siglec8”, (sup) Serena Meraviglia, Sirje Rüütel Boudinot, Airi Rump.

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Teras, M., Viisileht, E., Pahtma-Hall, M., **Rump, A.**, Paalme, V., Pata, P., Pata, I., Langevin, C., Rüütel Boudinot, Sirje. (2018) Porcine circovirus type 2 ORF3 protein induces apoptosis in melanoma cells. BMC Cancer, 18: 1237.

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Tuuli Katarina Lepasepp, BSc, 2019. “Kalneksiini, P2X4 ja P2X7 tase eksperimentaalse autoimmuunse entsefalomüeliidiga hiirte ajus ja seljaajus võrreldes tervete hiirtega”, (juh) Sirje Rüütel Boudinot, Airi Rump.

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Publikatsioonid

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