Journal of Immunological Methods 526 (2024) 113626

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Methods for studying P2X4 receptor ion channels in immune cells

Ronald Sluyter [a,*], Tahnee B.-D. McEwan [a], Reece A. Sophocleous [a], Leanne Stokes [b]

[a] Molecular Horizons and School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia
[b] School of Pharmacy, University of East Anglia, Norwich, Norfolk NR4 7TJ, UK

ARTICLE INFO

Keywords:
Adenosine 5′-triphosphate
Calcium
Fluorescence technique
Leukocyte
Ligand-gated ion channel
Patch clamping
Purinergic receptor

ABSTRACT

The P2X4 receptor is a trimeric ligand-gated ion channel activated by adenosine 5′-triphosphate (ATP). P2X4 is present in immune cells with emerging roles in inflammation and immunity, and related disorders. This review aims to provide an overview of the methods commonly used to study P2X4 in immune cells, focusing on those methods used to assess *P2RX4* gene expression, the presence of the P2X4 protein, and P2X4 ion channel activity in these cells from humans, dogs, mice and rats. *P2RX4* gene expression in immune cells is commonly assessed using semi-quantitative and quantitative reverse-transcriptase-PCR. The presence of P2X4 protein in immune cells is mainly assessed using anti-P2X4 polyclonal antibodies with immunoblotting or immunochemistry, but the use of these antibodies, as well as monoclonal antibodies and nanobodies to detect P2X4 with flow cytometry is increasing. Notably, use of an anti-P2X4 monoclonal antibody and flow cytometry has revealed that P2X4 is present on immune cells with a rank order of expression in eosinophils, then neutrophils and monocytes, then basophils and B cells, and finally T cells. P2X4 ion channel activity has been assessed mainly by Ca^{2+} flux assays using the cell permeable Ca^{2+}-sensitive dyes Fura-2 and Fluo-4 with fluorescence microscopy, spectrophotometry, or flow cytometry. However, other methods including electrophysiology, and fluorescence assays measuring Na^{+} flux (using sodium green tetra-acetate) and dye uptake (using YO-PRO-1^{2+}) have been applied. Collectively, these methods have demonstrated the presence of functional P2X4 in monocytes and macrophages, microglia, eosinophils, mast cells and $CD4^{+}$ T cells, with other evidence suggestive of functional P2X4 in dendritic cells, neutrophils, B cells and $CD8^{+}$ T cells.

1. Introduction

Ion channels play essential roles in immune cells, inflammation and immunity (Feske et al., 2015). Among these is the P2X4 receptor, which belongs to the P2X family of purinergic receptors. P2X receptors are trimeric ligand-gated ion channels activated by adenosine 5′-triphosphate (ATP) and other nucleotides (Illes et al., 2021). Activation of P2X receptors causes the rapid flux of Ca^{2+}, Na^{+} and K^{+} across the plasma membrane, with P2X4 having the greatest permeability to Ca^{2+} among all P2X receptors (Stokes et al., 2017). Repeated or prolonged P2X4 activation can also lead to the uptake of organic cations, including fluorescent dyes such as YO-PRO-1^{2+} (Stokes et al., 2017), although this phenomenon is not widely reported. P2X4 is located on the cell-surface membrane and in intracellular compartments, being most abundant in lysosomes, with cell activation altering the distribution of this receptor depending on the cell type or activation stimulus (Kanellopoulos et al., 2021). P2X4 is most reported as a homomeric receptor, but heteromeric receptors containing P2X4 subunits with P2X7 or other P2X subunits have been described (Kanellopoulos et al., 2021; Stokes et al., 2017) and are not discussed further in this article.

P2X4 is present in various cell types including immune cells and has well-established roles in the nervous system (Sophocleous et al., 2022), including pain (Sophocleous and Sluyter, 2023), and in the cardiovascular system (Bragança and Correia-de-Sá, 2020). P2X4 also functions in inflammation and immunity, with emerging roles in sepsis (Antonioli et al., 2019), allergy (Matsuoka et al., 2022), inflammatory bowel disorders (Sluyter, 2023), and other inflammatory and immune disorders including those relating to the central nervous system such as multiple sclerosis (Domercq and Matute, 2019) and amyotrophic lateral sclerosis (Volonté et al., 2023).

This review aims to provide an overview of the techniques commonly used to study P2X4 in immune cells, focusing on those methods used to assess *P2RX4* gene expression, the P2X4 protein, and P2X4 ion channel activity in these cells. It is beyond the scope of this article to discuss the many techniques used to study downstream effects of P2X4 activation in isolated immune cells (or those in animal models), which are various

* Corresponding author at: School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia.
E-mail address: rsluyter@uow.edu.au (R. Sluyter).

https://doi.org/10.1016/j.jim.2024.113626
Received 26 October 2023; Received in revised form 28 January 2024; Accepted 1 February 2024
Available online 3 February 2024

and many, and most of which are commonly used by immunologists and others to study immune cell functions such as phagocytosis, cytokine and prostaglandin release, and cell proliferation and migration.

2. Methods for studying *P2RX4* gene expression and the P2X4 protein in immune cells

Knowledge about the expression and distribution of P2X4 in immune cells has been advanced by use of the traditional techniques reverse-transcriptase (RT)-PCR, including quantitative RT-PCR (qPCR), immunoblotting, immunochemistry combined with either light or fluorescent microscopy, and flow cytometry. Examples of the methods used and the expression and presence of P2X4 in immune cells are summarised in Table 1. While RT-PCR and qPCR serve as convenient and highly accessible techniques for most laboratories, these alone do not provide evidence as to whether *P2RX4* or *P2rx4* mRNA from humans and other animals, respectively, is translated to P2X4 protein. Moreover, such techniques require the isolation of specific immune subsets from primary tissues to accurately ascertain whether mRNA coding for P2X4 protein is expressed in such cells. Nevertheless, methods, such as fluorescence activated cell sorting or immunomagnetic cell separation technologies, can be used for isolating immune cell populations and subsets from tissues, including blood, for the subsequent study of mRNA. Further, RT-PCR and qPCR remain useful screening tools, especially for cell lines which can serve as models of immune cells, noting that such lines are typically derived from malignant cells and thus do not fully recapitulate the primary immune cell of interest.

To date, the presence of P2X4 protein in immune cells has largely depended on the use of anti-P2X4 polyclonal antibodies by immunoblotting or immunochemistry, although in some instances such antibodies have been used for flow cytometry (Table 1). Of these, rabbit polyclonal antibodies (polyAb) to the intracellular (Cat. No. APR-002) or extracellular (Cat. No. APR-024) epitopes of P2X4 from Alomone Labs (Jerusalem, Israel) appear to be the most widely employed anti-P2X4 polyAb (Table 1). These, like most anti-P2X4 polyAb, afford the advantage of binding P2X4 from various species. However, these polyAb should be used with caution, with several immunoblotting studies failing to report the molecular weight of the band detected or reporting a band larger than that predicted by the *P2RX4* or *P2rx4* gene sequence (44 kDa) or as a result of glycosylation (58 kDa) (Hu et al., 2002). Thus, where possible the specificity of any anti-P2X4 polyAb used should be validated by comparing the binding of antibodies in P2X4 null and transfected cell lines, or in cells or tissues from wild-type and *P2rx4* knockout mice for example.

As recently reviewed (Kanellopoulos et al., 2021), several monoclonal antibodies (mAb) and nanobodies (Nb) to P2X4 are available, with a number used to study the distribution of P2X4 in immune cells. The first of these, an anti-P2X4 mAb, demonstrated the presence of P2X4 in the thymus and spleen (and many other tissues) of rats by immunoblotting (Bo et al., 2003). In relation to isolated immune cells, an anti-mouse P2X4 mAb (Nodu 246) has revealed the presence of P2X4 (predominately intracellular) in mouse bone marrow-derived macrophages (BMDMs), microglia and peritoneal mast cells by immunochemistry and/or flow cytometry (Bergmann et al., 2019). This same study also reported similar findings with mouse BMDMs and peritoneal mast cells using an anti-mouse P2X4 Nb (Nb 325) or an anti-mouse/human/rat P2X4 Nb (Nb 271). Furthermore, an anti-mouse/human/rat P2X4 mAb (mAb27) has been used to demonstrate the presence of P2X4 on various human immune cell subsets by flow cytometry with a rank order of expression on eosinophils (high P2X4), neutrophils and monocytes (intermediate P2X4), basophils and B cells (low P2X4), and T cells (near absent P2X4) (Paalme et al., 2019). This study also reported that P2X4 was present at greater amounts on peripheral blood leukocytes from male humans and mice compared to peripheral blood leukocytes from female humans and mice. While this difference has not been extensively explored, sex differences with P2X4 have been observed in relation to mouse models of neuropathic pain (Taves et al., 2016) and stroke (Verma et al., 2017). Collectively, this suggests that studies of P2X4 in immune cells and inflammation and immunity should consider sex differences as a potential variable. However, it should be noted that a recent study has indicated that microglial P2X4 are essential for nociception and neuropathic pain regardless of sex (Gilabert et al., 2023). Finally, it is also worth noting that P2X4 can be found at greater amounts in intracellular compartments (e.g. lysosomes) than at the cell-surface (Qureshi et al., 2007; Stokes and Surprenant, 2009) and when testing for expression, this should be considered in the experimental design.

3. Pharmacological reagents for studying P2X4 ion channel activity in immune cells

The identification of P2X receptors in cells has extensively relied on pharmacological reagents, with each P2X receptor historically recognised by a distinct agonist profile, with the design and discovery of P2X subtype-specific antagonists (Müller and Namasivayam, 2021) and less specific positive modulators (Stokes et al., 2020) greatly advancing this process. However, such advances are best validated by the exploration of such pharmacological reagents against recombinant receptors heterologously expressed in P2X receptor null cells for the species being investigated due to species-specific differences in the pharmacological profiles of each P2X receptor subtype (Sluyter et al., 2023).

Heterologously expressed human P2X4 is activated by ATP with a half-maximal effective concentration (EC_{50}) of 0.48 μM (Bianchi et al., 1999). Human P2X4 can be activated partly by 2′(3′)-O-(4-benzoylbenzoyl)-ATP (BzATP) (EC_{50} of 0.49 μM; maximum effect (E_{max}) of 80% of ATP), 2-methylthio-ATP (2MeSATP) (EC_{50} of 2.19 μM; E_{max} of 80% of ATP), α,β-methylene ATP (α,β-meATP) (EC_{50} of 8.32 μM; E_{max} of 50% of ATP) and adenosine-5′-O-(3-thio) triphosphate (ATPγS) (EC_{50} of 10.96 μM; E_{max} of 61% of ATP) (Bianchi et al., 1999). Heterologously expressed P2X4 is not activated by adenosine 5′-diphosphate (ADP), with prior reports of receptor activation by this nucleotide subsequently attributed to the presence of contaminating ATP in commercial ADP preparations (Mahaut-Smith et al., 2000; Micklewright et al., 2018; Sophocleous et al., 2020a).

ATP at a concentration of 100 μM, which fully activates P2X4 (see references in Table 2), is commonly used to identify P2X4 ion channel activity in immune cells (Tables 3–5). This concentration helps distinguish P2X4 from P2X7, which are also present in immune cells but typically activated by ATP at concentrations >100 μM (Sluyter, 2017). The presence of P2X4 has occasionally been shown by revealing that the concentration of ATP required to activate a response is less than that required for BzATP (Nguyen et al., 2020), with BzATP being a more potent activator of P2X7 than ATP (Sluyter, 2017). However, given that multiple P2X receptors are likely to be present in immune cells (Alberto et al., 2022) and most are responsive to nucleotides that also activate P2X4, agonist responses alone are insufficient to establish the presence of functional P2X4 in immune cells.

P2X4 ion channel activity can be positively modulated by ivermectin, with an EC_{50} in the range of 0.11 to 0.26 μM (Khakh et al., 1999b; Priel and Silberberg, 2004; Sophocleous et al., 2020a). As such, this compound has been widely used, most commonly at 3 μM, to identify P2X4 in immune cells (Tables 3–5). One caveat in the interpretation of studies using ivermectin to establish the presence of P2X4 in immune cells, is that this compound can potentiate human P2X7 activity, although not mouse (Nörenberg et al., 2012) or rat (Khakh et al., 1999b) P2X7 activity. Thus, ivermectin alone may also be insufficient to establish the presence of functional P2X4, at least in human immune cells.

The establishment of P2X4 ion channel activity in immune cells has been greatly advanced by the design and discovery of specific P2X4 antagonists (Table 2). Of these, 5-BDBD has been most used, commonly at a concentration of 10 μM (Tables 3–5). This compound was originally

Table 1
P2RX4 or *P2rx4* mRNA and P2X4 protein in immune cells.

Cell type	Species	mRNA	Protein (IB)	Protein (IC)	Protein (FC)	Reference
$CD34^+$ stem and progenitor cells	Human	qPCR				Wang et al. (2004)
Leukocytes	Human				Mouse mAb (mAb27, in-house)	Paalme et al. (2019)
Leukocytes (mononuclear)	Human		Rabbit polyAb (Alomone, APR-002)			Liu et al. (2016)
Leukocytes	Mouse				Mouse mAb (mAb27)	Paalme et al. (2019)
Leukocytes (peritoneal cavity)	Mouse				Mouse mAb (mAb27)	Paalme et al. (2019)
Spleen cells (whole tissue lysates)	Mouse				Mouse mAb (2G11, in-house)	Bo et al. (2003)
Thymus cells (whole tissue lysates)	Mouse				Mouse mAb (2G11)	Bo et al. (2003)
Monocytes (HL-60)	Human	RT-PCR				Berchtold et al. (1999); Yoon et al. (2006)
Monocytes (THP-1)	Human		Rabbit polyAb (APR-002)			Stokes and Surprenant (2009)
Monocytes (THP-1)	Human	qPCR		Rabbit polyAb (Alomone)		Layhadi and Fountain (2017)
Monocytes	Human	qPCR				Wang et al. (2004)
Monocytes	Human		Rabbit polyAb (Alomone)			Grahnert et al. (2009)
Monocytes	Human			Rabbit polyAb (APR-002 and APR-024)		Liu et al. (2016)
Monocytes	Human				Mouse mAb (mAb27)	Paalme et al. (2019)
Macrophages (THP-1)	Human	qPCR		Rabbit polyAb (Alomone)		Layhadi and Fountain (2017)
Macrophages (lung)	Human	RT-PCR				Myrtek et al. (2008)
Macrophages (lung)	Human		Rabbit polyAb (APR-002)		Rabbit polyAb (APR-002)	Stokes and Surprenant (2009)
Macrophages (monocyte-derived, GM-CSF)	Human	RT-PCR				Berchtold et al. (1999); Vargas-Martínez et al. (2020)
Macrophages (monocyte-derived, GM-CSF)	Human	qPCR		Rabbit polyAb (Alomone)		Layhadi et al. (2018)
Macrophages (monocyte-derived, M-CSF)	Human	RT-PCR				Hanley et al. (2004)
Macrophages (monocyte-derived, M-CSF)	Human	qPCR		Rabbit polyAb (Alomone)		Layhadi and Fountain (2019)
Macrophages (DH82)	Dog	RT-PCR		Goat polyAb (SAB2500734, Sigma)		Sophocleous et al. (2020b)
Macrophages (J774)	Mouse	RT-PCR	Rabbit polyAb (Roche)	Rabbit polyAb (Roche)		Coutinho-Silva et al. (2005)
Macrophages (J774)	Mouse	RT-PCR				Ito and Matsuoka (2008)
Macrophages (J774)	Mouse		Rabbit polyAb (APR-002)			Dhuna et al. (2019); Stokes and Surprenant (2009)
Macrophages (RAW264.7)	Mouse	qPCR	Rabbit polyAb (Alomone)			Kawano et al. (2012)
Macrophages (RAW264.7)	Mouse	qPCR	Rabbit polyAb (Alomone, APR-024)			Zumerle et al. (2019)
Macrophages (bone marrow-derived, M-CSF)	Mouse	qPCR				Zumerle et al. (2019)
Macrophages (bone marrow-derived, LCCM)	Mouse			Rat mAb (Nodu 246, in-house)	Rat mAb (Nodu 246) and llama Nb (Nb 271 in-house)	Bergmann et al. (2019)
Macrophages (lung)	Mouse	RT-PCR				Kessler et al. (2011)
Macrophages (paw)	Mouse			Rabbit Ab (in-house)		Ulmann et al. (2010)
Macrophages (peritoneal)	Mouse		Rabbit polyAb (APR-002)			Stokes and Surprenant (2009)
Macrophages (peritoneal)	Mouse		Rabbit polyAb (APR-002)	Rabbit polyAb (APR-002)		Qureshi et al. (2007)
Macrophages (peritoneal)	Mouse			Rabbit Ab (in-house)		Ulmann et al. (2010)
Macrophages (peritoneal)	Mouse	RT-PCR	Rabbit Ab (Abcam)	Rabbit Ab (Biorbyt)	Rabbit Ab (Biorbyt)	Aminin et al. (2016)
Macrophages (splenic)	Mouse	RT-PCR		Rabbit polyAb (Roche)		Coutinho-Silva et al. (2005)
Macrophages (subcapsular sinus)	Mouse		Rabbit polyAb (APR-024)		Rabbit polyAb (APR-024)	Zumerle et al. (2019)
Macrophages (NR8383)	Rat	RT-PCR		Rabbit polyAb (Alomone)		Bowler et al. (2003)
Macrophages (NR8383)	Rat	RT-PCR	Rabbit polyAb (APR-002)	Rabbit polyAb (APR-002)	Rabbit polyAb (APR-002)	Stokes and Surprenant (2009)
Microglia (BV-2)	Mouse				Mouse mAb (mAb27)	Paalme et al. (2019)
Microglia (BV-2 and *P2rx7* KO BV-2)	Mouse		Rabbit polyAb (APR-002)	Rabbit polyAb (Alomone)		Dhuna et al. (2019)
Microglia (C8-B4)	Mouse	qPCR	Rabbit polyAb (Alomone)	Rabbit polyAb (Alomone)		Toulme and Khakh (2012)
Microglia (cortex)	Mouse	RT-PCR, qPCR				Light et al. (2006)
Microglia (cortex)	Mouse	qPCR	Rabbit polyAb (APR-002)	Rabbit polyAb (APR-002)		Nguyen et al. (2020)
Microglia (unspecified)	Mouse			Mouse mAb (Nodu 246)		Bergmann et al. (2019)

(*continued on next page*)

Table 1 (*continued*)

Cell type	Species	mRNA	Protein (IB)	Protein (IC)	Protein (FC)	Reference
Microglia (cortex)	Rat	qPCR	Rabbit polyAb (Alomone)			Nasu-Tada et al. (2006); Ohsawa et al. (2007)
Microglia (cortex)	Rat		Rabbit polyAb (APR-002)	Rabbit polyAb (APR-002)		Qureshi et al. (2007)
DCs (monocyte-derived)	Human	RT-PCR				Berchtold et al. (1999); Ferrari et al. (2000b)
DCs (bone marrow-derived)	Mouse	qPCR	Rabbit polyAb (Alomone)			Sakaki et al. (2013)
Granulocytes	Human				Mouse mAb (mAb27)	Paalme et al. (2019)
Eosinophils	Human	RT-PCR				Ferrari et al. (2000a)
Eosinophils	Human			Mouse mAb (mAb27)	Mouse mAb (mAb27)	Paalme et al. (2019)
Mast cells (LAD2 and lung)	Human	RT-PCR				Wareham et al. (2009)
Mast cells (bone marrow-derived)	Mouse	qPCR	Rabbit polyAb (Alomone)			Yoshida et al. (2020); Yoshida et al. (2022)
Mast cells (connective tissue)	Mouse	qPCR				Yoshida et al. (2020)
Mast cells (peritoneal)	Mouse	qPCR				Yoshida et al. (2022)
Mast cells (peritoneal)	Mouse				Rat mAb (Nodu 246) and llama Nb (Nb 271 or Nb 325 in-house)	Bergmann et al. (2019)
B cells (RPMI 8226)	Human	RT-PCR				Farrell et al. (2010)
B cells (CLL)	Human			Rabbit polyAb (in-house)		Sluyter et al. (2001)
B cells	Human	qPCR	Rabbit polyAb (Alomone)			Lee et al. (2006)
B cells	Human				Mouse mAb (mAb27)	Paalme et al. (2019)
$CD4^+$ T cells (Jurkat and blood)	Human	RT-PCR				Berchtold et al. (1999)
$CD4^+$ T cells (Jurkat and blood)	Human	qPCR	Rabbit polyAb (Alomone)	Rabbit polyAb (Alomone)		Woehrle et al. (2010)
$CD4^+$ T cells (Jurkat, Tn, Tcm and Teff)	Human	RT-PCR, qPCR				Wang et al. (2014)
T cells	Human				Mouse mAb (mAb27)	Paalme et al. (2019)
$CD4^+$ T cells and Tregs	Human	qPCR				Trabanelli et al. (2012)
$CD4^+$ T cells (adipose tissue and blood)	Human				Mouse Ab (Abcam)	Ruiz-Rodríguez et al. (2019)
γδ T cells	Human	qPCR				Manohar et al. (2012)

Abbreviations: Ab, antibody (where antibody type was not disclosed); CLL, $CD19^+$ chronic lymphocytic leukaemia cells; DCs, dendritic cells; FC, flow cytometry; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; IB, immunoblotting; IC, immunochemistry; KO, knockout; LCCM, L929 cell-conditioned medium; mAb, monoclonal antibody; Nb, nanobody; polyAb, polyclonal antibody; qPCR, quantitative RT-PCR; RT-PCR, reverse transcriptase-PCR; Tcm, central memory T cells; Teff, effector T cells; THP-1 macrophages, phorbol 12-myristate 13-acetate-differentiated THP-1 cells; Tn, naïve T cells.

Companies: Abcam, Abcam Cambridge, UK; Alomone, Alomone Labs, Jerusalem, Israel; Biorbyt Ltd., Cambridge, UK; Roche, Roche Biosciences, Palo Alto, CA; Sigma, Sigma Chemical Co., St Louis, MO. Where known, catalogue number provided in parenthesis, with company name not repeated. Where the catalogue number or other company identifier was not disclosed in the original publication, only the company is listed.

Table 2
Specific P2X4 antagonists.

	IC_{50} (μM) or known inhibition if IC_{50} not reported				
Antagonist	Human	Dog	Mouse	Rat	Reference
5-BDBD	0.35–5.8	5.8	2.0	0.75–3.5	Abdelrahman et al. (2017); Balázs et al. (2013); Coddou et al. (2019); Sophocleous et al. (2020a)
BAY-1797	0.11–0.24		0.11	0.23	Shen et al. (2023); Werner et al. (2019)
BX430	0.46–1.9	7.8	>10	66	Ase et al. (2015); Pasqualetto et al. (2023); Shen et al. (2023); Sophocleous et al. (2020a)
MRS4719	0.50		Yes		Toti et al. (2022)
NC-2600	Yes		Yes		D'Antongiovanni et al. (2022)
NP-1815-PX	0.26		Yes	Yes	D'Antongiovanni et al. (2022); Matsumura et al. (2016)
PSB-12054	0.19		1.8	2.1	Hernandez-Olmos et al. (2012)
PSB-12062	0.93–1.8		1.8	0.93	Hernandez-Olmos et al. (2012)

Abbreviation: IC_{50}, half-maximal inhibitory concentration.

but incompletely described in a patent (Fisher et al., 2005), with the first published study using recombinant human P2X4 reporting a half-maximal inhibitory concentration (IC_{50}) of 1.0–2.0 μM to this receptor (Balázs et al., 2013), with somewhat similar EC_{50} values against recombinant dog, mouse and rat P2X4 subsequently reported by others (Abdelrahman et al., 2017; Coddou et al., 2019; Sophocleous et al., 2020a) (Table 2). Despite its popularity, some (Ase et al., 2015; Sophocleous et al., 2020a) but not others (Abdelrahman et al., 2017) report that 5-BDBD only partly impairs recombinant human P2X4 responses. As such, the specific P2X4 antagonists including BX430 (Ase et al., 2015), MRS4719 (Toti et al., 2022) and PSB-12062 (Hernandez-Olmos et al., 2012) have been used occasionally to study P2X4 in immune cells (Tables 3–5). The specific P2X4 antagonists BAY-1797 (IC_{50} of 0.21 μM) (Werner et al., 2019), NC-2600 (D'Antongiovanni et al., 2022), NP-1815-PX (Matsumura et al., 2016) and PSB-12054 (IC_{50} of 0.19 μM) (Hernandez-Olmos et al., 2012) afford alternative options. Each of the above antagonists, except NC-2600, are commercially available from at least one company. These companies, listed in alphabetical order, include Biorbyt Ltd. (Cambridge, UK), MedChemExpress (Monmouth Junction, NJ), Santa Cruz Biotechnology Inc. (Dallas, TX), Sellick Chemicals (Houston, TX), Sigma Chemical Co. (St Louis, MO) and/or Tocris Bioscience (Bristol, UK).

While historically of value to help establish the presence of P2X4 ion channel activity in immune cells, the future use of the non-selective P2X4 antagonists, which can inhibit other P2X receptors, such as

Table 3
Functional P2X4 in monocytes and macrophages.

Cell type	Species	Method	Method Details	ATP (μM)	Evidence for P2X4	Reference
Monocytes (THP-1)	Human	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	100	3 μM IVM	Li and Fountain (2012)
Monocytes (THP-1)	Human	Ca^{2+} flux	Fluo-4, Berthold Technologies Mithras LB 940 luminometer	100	100 μM 5-BDBD	Fagerberg et al. (2016)
Monocytes	Human	Ca^{2+} flux	Fura-2, Zeiss Axiovert 135 microscope	100	3 μM IVM	Grahnert et al. (2009)
Macrophages (THP-1)	Human	Ca^{2+} flux	Fura-2, Nikon TE2000-U microscope or Perkin Elmer Victor3 1420 plate reader	50	5 μM BX430	Ase et al. (2015)
Macrophages (THP-1)	Human	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	100	10 μM 5-BDBD, 10 μM PSB-12062, *P2RX4* shRNA, 3 μM IVM	Layhadi and Fountain (2017)
Macrophages (lung)	Human	Electrophysiology	HEKA EPC9 amplifier	100	3 μM IVM	Stokes and Surprenant (2009)
Macrophages (lung)	Human	Ca^{2+} flux	Fura-2, Zeiss Attofluor microscope	100		Myrtek et al. (2008)
Macrophages (monocyte-derived, M-CSF)	Human	Electrophysiology	Axon Instruments Axopatch 200B amplifier	100–3000		Hanley et al. (2004)
Macrophages (monocyte-derived, M-CSF)	Human	Electrophysiology	HEKA EPC10 amplifier	100	3 μM IVM	Stokes et al. (2011)
Macrophages (monocyte-derived, M-CSF)	Human	Ca^{2+} or Na^{+} flux	Fluo-3 or sodium green tetra-acetate, Nikon Diaphot 300 microscope	300		Hanley et al. (2004)
Macrophages (monocyte-derived, M-CSF)	Human	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	100	10 μM 5-BDBD, 10 μM PSB-12062, 3 μM IVM	Layhadi and Fountain (2019)
Macrophages (monocyte-derived, GM-CSF)	Human	Electrophysiology	Axon Instruments Axopatch 200B amplifier	10	3 μM IVM	Vargas-Martínez et al. (2020)
Macrophages (monocyte-derived, GM-CSF)	Human	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	100	5 μM BX430, 10 μM PSB-12062, 3 μM IVM	Layhadi et al. (2018)
Macrophages (monocyte-derived, GM-CSF)	Human	Ca^{2+} flux	Calbryte 590 AM, Zeiss LSM880 microscope	50	0.65 μM MRS4719	Toti et al. (2022)
Macrophages (DH82)	Dog	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	10	30 μM 5-BDBD, 100 μM paroxetine, 50 μM TNP-ATP, 3 μM IVM	Sophocleous et al. (2020b)
Macrophages (J774)	Mouse	Electrophysiology	Axon Instruments Axopatch 200B amplifier	10–100		Coutinho-Silva et al. (2005)
Macrophages (J774)	Mouse	Electrophysiology	HEKA EPC9 amplifier	100	3 μM IVM	Stokes and Surprenant (2009)
Macrophages (J774)	Mouse	Electrophysiology	Fura-2, Hitachi F-2500 spectrometer	3–30	5.7 μM IVM	Ito and Matsuoka (2008)
Macrophages (J774)	Mouse	Ca^{2+} flux	Fura-2, Photon Technology photometer	100		Coutinho-Silva et al. (2005)
Macrophages (RAW264.7)	Mouse	Ca^{2+} flux	Fluo-4, Hitachi F-2500 spectrometer	ND	*P2rx4* shRNA	Kawano et al. (2012)
Macrophages (RAW264.7)	Mouse	Ca^{2+} flux and waves	Fluo-4, Zeiss LSM700 microscope	Endogenous (photoactivated caged-IP_3)	100 μM 5-BDBD, 5 U/mL apyrase, *P2rx4* siRNA	Zumerle et al. (2019)
Macrophages (bone marrow, M-CSF)	Mouse	Ca^{2+} flux and waves	Fluo-4, Zeiss LSM700 microscope	Endogenous (photoactivated caged-IP_3)	5 U/mL apyrase	Zumerle et al. (2019)
Macrophages (lung)	Mouse	Electrophysiology	HEKA EPC9 amplifier	100	3 μM IVM	Kessler et al. (2011)
Macrophages (peritoneal)	Mouse	Electrophysiology	Axon Instruments Axopatch 200A amplifier	30	3 μM IVM	Qureshi et al. (2007)
Macrophages (peritoneal)	Mouse	Electrophysiology	HEKA EPC9 amplifier	100	3 μM IVM	Stokes and Surprenant (2009)
Macrophages (peritoneal)	Mouse	Ca^{2+} flux	Fura-2, Photon Technology photometer	1–100		Coutinho-Silva et al. (2005)
Macrophages (peritoneal)	Mouse	Ca^{2+} flux	Fura-2, Molecular Devices Metafluor Imaging system	20 (3 applications)	3 μM IVM, *P2rx4* KO	Ulmann et al. (2010)
Macrophages (peritoneal)	Mouse	Ca^{2+} flux	Fura-2, Zeiss Axiovert 200 or Observer ZI microscope	30	10 μM phenolphthalein, 10 μg/mL anti-P2X4 polyAb (Biorbyt, ord100036), 2 U/mL apyrase, *P2rx4* siRNA	Aminin et al. (2016)
Macrophages (peritoneal;	Mouse	Ca^{2+} flux	Fura-2, SLM Aminco Bowman spectrofluorometer	10	3 μM IVM	Seil et al. (2010a); Seil et al. (2010b)

(*continued on next page*)

Table 3 (*continued*)

Cell type	Species	Method	Method Details	ATP (μM)	Evidence for P2X4	Reference
wildtype and *P2rx7* KO)						
Macrophages (peritoneal; wildtype and *P2rx7* KO)	Mouse	Dye uptake	YO-PRO-1^{2+}, Photon Technology spectrofluorometer	100	3 μM IVM	Seil et al. (2010a)
Macrophages (subcapsular sinus)	Mouse	Ca^{2+} flux and waves	Fluo-4, Zeiss LSM700 microscope	Endogenous (photoactivated caged-IP_3)		Zumerle et al. (2019)
Macrophages (NR8383)	Rat	Electrophysiology	HEKA EPC9 amplifier	10–1000	3 μM IVM	Bowler et al. (2003)
Macrophages (NR8383)	Rat	Electrophysiology	HEKA EPC9 amplifier	100	3 μM IVM	Stokes and Surprenant (2009)

Abbreviations: ATP, adenosine 5′-triphosphate; IP_3, inositol-1,4,5-trisphosphate; IVM, ivermectin; KO, knockout; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; ND, not disclosed; polyAb, polyclonal antibody; shRNA, short hairpin interfering RNA; siRNA, small interfering RNA; THP-1 macrophages, phorbol 12-myristate 13-acetate-differentiated THP-1 cells; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) ATP.
Company: Biorbyt, Biorbyt Ltd., Cambridge, UK.

Table 4
Functional P2X4 in microglia.

Cell type	Species	Method	Method Details	ATP (μM)	Evidence for P2X4	Reference
Microglia (BV-2)	Mouse	Electrophysiology	Axon Instruments Axopatch 200B amplifier	100	3 μM IVM	Bernier et al. (2008)
Microglia (BV-2)	Mouse	Ca^{2+} flux	Fura-2, Nikon TE2000-U microscope	100	3 μM IVM	Bernier et al. (2008)
Microglia (BV-2)	Mouse	Ca^{2+} flux	Fluo-4, Nikon A1 microscope	≤100		Gilbert et al. (2016)
Microglia (BV-2 and *P2rx7* KO BV-2)	Mouse	Ca^{2+} flux	Fura-2, Molecular Devices FlexStation 3	25	10 μM 5-BDBD, 1 μM PSB-12062, 3 μM IVM	Dhuna et al. (2019)
Microglia (C8-B4)	Mouse	Electrophysiology	Axon Instruments Axopatch 200B or 700A amplifier	100		Toulme and Khakh (2012)
Microglia (C8-B4)	Mouse	Ca^{2+} flux	Fura-2, Olympus IX71 microscope	100		Toulme and Khakh (2012)
Microglia (cortex)	Mouse	Electrophysiology	HEKA EPC-10 amplifier	100 (BzATP, 1000)	3 μM IVM (ATP)	Nguyen et al. (2020)
Microglia (cortex)	Mouse	Ca^{2+} flux	Fura-2, Nikon TE2000-U microscope	100	3 μM IVM, *P2rx4* KO	Bernier et al. (2013)
Microglia (cortex)	Mouse	Ca^{2+} flux	Fluo-3 or Fura-4, Nikon DiaPhot 300 microscope	≤100		Light et al. (2006)
Microglia (cortex)	Mouse	Ca^{2+} flux	Fluo-4, BZ-X780 microscope	100 (BzATP, 1000)	3 μM IVM (ATP)	Nguyen et al. (2020)
Microglia (cortex)	Mouse	Dye uptake	YO-PRO-1^{2+}, Nikon TE2000-U microscope	100		Bernier et al. (2013)
Microglia (cortex)	Rat	Ca^{2+} flux	Fura-2, non-specified microscope	50	100 μM TNP-ATP	Nasu-Tada et al. (2006)
Microglia (cortex)	Rat	Ca^{2+} flux	Fura-2, Nikon TMD-300 microscope	50	*P2rx4* shRNA	Ohsawa et al. (2007)

Abbreviations: ATP, adenosine 5′-triphosphate; BzATP, 2′(3′)-O-(4-benzoylbenzoyl) ATP; IVM, ivermectin; KO, knockout; TNP-ATP, TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) ATP; shRNA, short hairpin interfering RNA.

2,4,6-trinitrophenol-ATP (Haines et al., 1999), Brilliant Blue G (Jiang et al., 2000), phenolphthalein (King et al., 2005), and paroxetine (Dao-Ung et al., 2015) should be avoided. Finally, anti-P2X4 antibodies (Williams et al., 2019) or fragments thereof (Westlund et al., 2021) with blocking activity provide emerging alternatives to establish P2X4 ion channel activity in immune cells.

4. Methods for studying P2X4 ion channel activity in immune cells

A review of the literature reveals that P2X4 ion channel activity has been studied mostly in macrophages (Table 3) and microglia (Table 4), possibly due to the broader role of purinergic signalling in the central nervous system and due to these cells readily adhering to solid surfaces aiding their study. P2X4 ion channel activity however has been extended to monocytes (Table 3) and various other immune cells including dendritic cells, eosinophils, mast cells, and $CD4^+$ T cells (Table 5). Curiously, P2X4 ion channel activity in dendritic cells, neutrophils and B cells has not been widely investigated nor well-established using ivermectin and P2X4 antagonists (Table 5). Despite numerous reports of functional P2X4 in $CD4^+$ T cells (Table 5), to the best of our knowledge, P2X4 ion channel activity has not been directly investigated in $CD8^+$ T cells. One study however has reported that P2X4 mediates migration of effector memory $CD8^+$ T cells isolated from kidney transplant recipients (Doan Ngoc et al., 2022).

P2X4 ion channel activity in immune cells has been assessed mainly by Ca^{2+} flux assays using the cell permeable Ca^{2+}-sensitive dyes, commonly Fura-2 (Fura-2/AM) or Fluo-4 (Fluo-4/AM), with fluorescence microscopy, spectrophotometry, fluorimetry and/or flow cytometry (Tables 3–5). Most commonly P2X4 activation is studied following the addition of exogenous ATP. On occasion, however, paracrine P2X4 activation by endogenous ATP, induced by photoactivated caged-inositol-1,4,5-trisphosphate (IP_3) to promote intercellular Ca^{2+} waves, has been employed in mouse RAW264.7 macrophages (Zumerle et al., 2019) and human T cells (Wang et al., 2014). Moreover pioneering studies by Junger and colleagues have studied P2X4-mediated Ca^{2+} fluxes induced by endogenous ATP in human $CD4^+$ T cells following T cell receptor activation with anti-CD3 (Woehrle et al., 2010) or anti-CD3/anti-CD28 (Ledderose et al., 2016; Ledderose et al., 2021) mAb, or in response to the migratory stimulus CXCL12 (stromal cell derived

Table 5
Functional P2X4 in other innate and adaptive immune cells.

Cell type	Species	Method	Method Details	ATP (mM)	Evidence for P2X4	Reference
Leukocytes (mononuclear, adipose and blood)	Human	Ca^{2+} flux	Fura-2, BioTek Synergy HTX spectrophotometer	100	0.5 μM 5-BDBD	Ruiz-Rodríguez et al. (2019)
DCs (monocyte-derived)	Human	Ca^{2+} flux	Fura-2, Perkin Elmer LS-3B fluorometer	100		Berchtold et al. (1999)
DCs (monocyte-derived)	Human	Ca^{2+} flux	Fura-2, LS50 Perkin Elmer fluorometer	ND		Ferrari et al. (2000b)
DCs (bone marrow-derived)	Mouse	Ca^{2+} flux	Fluo-4, Hitachi F-2500 spectrometer	1000	10 μM TNP-ATP, *P2rx4* shRNA	Sakaki et al. (2013)
Eosinophils	Human	Ca^{2+} flux	Fura-2, Zeiss microscope	1–1000		Ferrari et al. (2000a)
Eosinophils	Human	Ca^{2+} flux	Fura-2, Shimadzu RF-54 spectrometer	1–1000	Attributed largely to P2Y	Mohanty et al. (2001)
Granulocytes (gut)	Mouse	Ca^{2+} flux	Fluo-8, non-specified flow cytometer	100 (ATPγS)	*P2rx4* KO	Tani et al. (2021)
Mast cells (LAD2 and lung)	Human	Electrophysiology	HEKA EPC10 amplifier	100	3 μM IVM	Wareham et al. (2009)
Mast cells (bone marrow-derived)	Mouse	Ca^{2+} flux	Fura-2, Hitachi F-2700 spectrometer	100	*P2rx4* KO	Yoshida et al. (2020); Yoshida et al. (2019); Yoshida et al. (2022)
B cells	Human	Ca^{2+} flux	Fura-2, Olympus microscope	1000		Lee et al. (2006)
$CD4^+$ T cells (Jurkat and blood)	Human	Ca^{2+} flux	Fluo-4, BD FACSCalibur flow cytometer or Zeiss LSM 510 META microscope	Endogenous (CD3 activation)	30 μM TNP-ATP, *P2RX4* siRNA	Woehrle et al. (2010)
$CD4^+$ T cells (Jurkat, Tn, Tcm and Teff)	Human	Ca^{2+} flux or waves	Fluo-4 and Fura-Red, Olympus FluoView FV1000 microscope or BD FACSCanto flow cytometer	Endogenous (photoactivated caged-IP_3)	10 μM 5-BDBD	Wang et al. (2014)
$CD4^+$ T cells	Human	Ca^{2+} flux	Fluo-4, Leica DMI6000B microscope	Endogenous (CD3/CD28 activation)	100 μM suramin, 10 μM 5-BDBD	Ledderose et al. (2016); Ledderose et al. (2021)
$CD4^+$ T cells	Human	Ca^{2+} flux or mitochondrial Ca^{2+}	Fluo-4 or Rhod-2, Leica DMI6000B microscope	Endogenous (CXCL12 activation)	10 μM 5-BDBD	Ledderose et al. (2020); Wang et al. (2014)
$CD4^+$ T cells (splenic and lymphoid)	Mouse	Ca^{2+} flux	Fluo-4, Leica IRBE microscope	Endogenous (CD3 activation)	*P2Rx4* KO	Brock et al. (2022)
$CD4^+$ T cells (splenic and lymphoid)	Mouse	Initial local Ca^{2+} microdomains	Fluo-4 and Fura-Red, non-specified microscope	Endogenous (CD3/CD28 activation)	10 μM 5-BDBD, 1 μM PSB-15417, *P2Rx4* KO	Brock et al. (2022)

Abbreviations: ATP, adenosine 5′-triphosphate; ATPγS, adenosine-5′-O-(3-thio) triphosphate; DCs, dendritic cells; IP_3, inositol-1,4,5-trisphosphate; IVM, ivermectin; KO, knockout; ND, not disclosed; siRNA, small interfering RNA; shRNA, short hairpin interfering RNA; Tcm, central memory T cells; Teff, effector T cells; Tn, naïve T cells; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) ATP.

factor-1α or SDF-1α) (Ledderose et al., 2020; Wang et al., 2014). These same studies have also allowed the study of P2X4 activation in mitochondrial Ca^{2+} responses using Rhod-2 (Rhod-2/AM). More recent advances in technology (Diercks et al., 2019), have allowed for the study of local Ca^{2+} responses in microdomains of mouse T cells using Fluo-4 and widefield fluorescent microscopy and post-processing deconvolution (Brock et al., 2022). Finally, one study has used sodium green tetra-acetate to demonstrate ATP-induced Na^+ fluxes in human macrophages (Hanley et al., 2004) but direct evidence for P2X4 in the process is lacking and to the best of our knowledge this technique has not been used elsewhere to investigate P2X4 in immune cells.

The study of P2X4 ion channel activity in immune cells by measurements of dye uptake appears to be limited to YO-PRO-1^{2+} uptake in mouse macrophages (Seil et al., 2010a) microglia (Bernier et al., 2013). Supporting this activity of P2X4, others have shown ATP-induced YO-PRO-1^{2+} uptake in HEK-293 cells (Dhuna et al., 2019; Virginio et al., 1999) or *Xenopus* oocytes (Khakh et al., 1999a) expressing recombinant human and/or rat P2X4. Whether the paucity of studies using dye uptake measurements reflects the propensity of P2X4 to mediate dye uptake in only some immune cell types or the large (and arguably confounding) signal resulting from P2X7-mediated dye uptake remains to be investigated.

A small number of studies have used electrophysiology, usually whole cell patch clamping, to investigate P2X4 ion channel activity in immune cells (Tables 3–5). This has been applied mainly to adherent cells such as macrophages, microglia and mast cells (and most often in established lines of these cell types) (Tables 3–5). Thus, given the specialised skills, difficulty in patching primary cells and low throughput nature of this technique, electrophysiology approaches are not recommended for the study of P2X4 ion channel activity in immune cells in most instances. Automated patch clamp remains a future possibility, but despite the study of endogenous voltage-gated potassium channel Kv1.3 in primary T cells (Li et al., 2017) this technique has not yet been extended to study other ion channels in immune cells, including P2X4, although this technology has been employed to successfully study HEK-293 cells expressing recombinant human and dog P2X4 (Sophocleous et al., 2020a).

5. Conclusions

A review of the techniques used to study P2X4 in immune cells, reveals that most immune cell types express P2X4, with the majority of P2X4 being found within intracellular compartments and highest amounts of P2X4 expressed on eosinophils relative to other cell types. Furthermore, the relative abundance of P2X4 on immune cells may differ between sexes but the functional significance of this in relation to inflammation and immunity remains to be explored. Of note is the emergence of several mAb and Nb to P2X4, which should advance the field as they become more widely available. In contrast to electrophysiology measurements by patch clamping, use of the cell permeable Ca^{2+}-sensitive dyes Fura-2 and Fluo-4 to detect P2X4-induced Ca^{2+} fluxes by either fluorescence microscopy, spectrophotometry, or flow cytometry appear the methods of choice in most laboratories. The combined use of these methods with specific P2X4 antagonists, many of which are commercially available, afford new possibilities to confirm or establish the presence and activity of P2X4 in immune cells including subsets.

Funding

R.S. and R.A.S. are currently supported by the American Kennel Club

Canine Health Foundation (Grant No. 03181-A). R.S. is also currently supported by the TLR Foundation and the University of Wollongong. L.S. is currently supported by the University of East Anglia.

CRediT authorship contribution statement

Ronald Sluyter: Conceptualization, Writing – original draft, Writing – review & editing. **Tahnee B.-D. McEwan:** Writing – review & editing. **Reece A. Sophocleous:** Writing – review & editing. **Leanne Stokes:** Writing – review & editing.

Declaration of competing interest

The author declares no conflict of interest.

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