

# Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses

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**Abstract** Extracellular ATP and related nucleotides promote a wide range of pathophysiological responses via activation of cell surface purinergic P2 receptors. Almost every cell type expresses P2 receptors and/or exhibit regulated release of ATP. In this review, we focus on the purinergic receptor distribution in inflammatory cells and their implication in diverse immune responses by providing an overview of the current knowledge in the literature related to purinergic signaling in neutrophils, macrophages, dendritic cells, lymphocytes, eosinophils, and mast cells. The pathophysiological role of purinergic signaling in these cells include among others calcium mobilization, actin polymerization, chemotaxis, release of mediators, cell maturation, cytotoxicity, and cell death. We finally discuss the therapeutic potential of P2 receptor subtype selective drugs in inflammatory conditions.

**Keywords** ATP · Extracellular nucleotides · P2 receptors · Inflammatory cells · Regulation of immune responses

## Abbreviations

cAMP	Cyclic adenosine monophosphate
DCs	Dendritic cells
ECP	Eosinophil cationic protein
ERK	Extracellular signal-regulated kinases
fMLP	fMet-Leu-Phe receptor
HLMC	Human lung mast cell
HMC	Human mast cell
IP <sub>3</sub>	Inositol 1,4,5-triphosphate

LAD 2	Leukocyte adhesion deficiency
LCL	Lymphoblastoid cell lines
MIP-2	Macrophage inflammatory protein-2
MoDCs	Monocyte-derived dendritic cells
MSU	Monosodium urate
E-NTPDase1	Ecto-nucleoside triphosphate diphosphohydrolase 1
PLC	Phospholipase C

## Introduction

Inflammation encompasses a complex homeostatic mechanism that enables the body to detect and fight foreign nonself antigens and restore tissue integrity. While mediators of inflammation originating from outside are often responsible for initiating the immune response, some constitutive intracellular molecules from the host—that are implicated in housekeeping functions during normal physiology—can be released into the extracellular space and regulate the function of immune cells, and consequently equally participate and/or modulate inflammatory responses under pathological conditions [1, 2]. Evidence has accumulated showing that ATP and related purine and pyrimidine nucleotides fulfill these requirements [3–5] further evidenced by recent studies using bioluminescence imaging techniques that visualized ATP release in vivo in response to contact allergen [6], irradiation, allograft rejection [7], and intraperitoneal LPS administration [8].

Indeed, while ATP is found inside almost every living cell—predominantly known for its central role in the cellular energy metabolism-, it is also widely distributed outside the cell where it appears to influence a diverse scale of biological processes such as the generation of chemotactic signals and/or the activation of different immune cells, causing inflammatory cells to migrate, proliferate, differentiate, or release diverse inflammatory mediators [9, 10]. Under

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inflammatory conditions, ATP is often regarded to be released passively following cellular stress or cell death. However, nucleotides can also be released into the extracellular space via nonlytic mechanisms through regulated transport as a neurotransmitter from neurons or from immune cells during inflammation and even under normal physiological conditions; this process is regulated via an autocrine feedback mechanism [11, 12].

Extracellular ATP and the related purine and pyrimidine nucleotides exert their functions via signaling through membrane-bound purinergic P2 receptors. These receptors are widely expressed throughout the body on various immune and nonimmune cells. The P2 receptors are subdivided into two families: the G protein-coupled P2Y receptors and the ligand-gated P2X ion channels. The International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification recognizes eight distinct P2Y receptor subtypes: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> [13] and seven P2X subunits that may form six homomeric (P2X1–P2X5 and P2X7), and at least six heteromeric P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, and P2X4/6, receptors [14].

Either constitutively present or induced under pathological conditions, P2 receptors are differentially expressed on a large scale of cells including immune cells (Table 1) [13, 15–17]. Although P2 receptors are clearly implicated in diverse inflammatory reactions [18], the mechanisms involved are complex and not fully elucidated. Their expression profiles and biological activities are diverse as evidenced by results in the literature regarding eosinophils [10, 19], neutrophils [20, 21], mast cells [22, 23], monocytes/macrophages [24], lymphocytes [25, 26], and others [4]. Identifying the precise receptors and pathways involved is a first step in the challenge which could lead to the discovery of a new therapeutic class of drugs that suppress inflammation.

This review summarizes the history and the current knowledge regarding the role of purinergic signaling in different inflammatory cells and focuses on the purinergic receptor distribution in immune cells and their implication in diverse immune responses.

## Neutrophils

Neutrophils (Fig. 1) are essential effector cells of the innate immune system, forming the first line of defense responsible for the initial clearance of invading microorganisms. They are highly motile phagocytic cells that rapidly migrate from the blood circulation [27]. Their recruitment and activation into peripheral tissues is crucial for an efficient host defense but needs to be tightly regulated in order to avoid host damage. The latter is under control of a diverse set of neutrophil chemoattractants such as chemokines,

eicosanoids, cytokines and other peptides [28], but also extracellular ATP and its derivatives can play a role herein. In this chapter, the current knowledge regarding the effects of extracellular nucleotides in neutrophil function and its influence on the immune response will be presented.

Ward et al. were the first to describe the role of purines in neutrophil functionality, showing the release of ATP/ADP from platelets to induce an enhanced superoxide anion production in stimulated rat and human neutrophils [29–31]. The involvement of a purinergic pathway in the platelet–neutrophil interaction was corroborated by others who reported its implication in the respiratory burst in human neutrophils isolated from peripheral blood of healthy donors [32, 33]. Suh et al. characterized the P2X7 receptor subtype to be involved in the purinergic mechanism of enhanced respiratory burst, consequently suggesting its presence on human neutrophils [34]. In contrast, others refuted the presence of P2X7 receptors in human neutrophils by means of a large scale of techniques [35, 36]. While on mRNA level, human neutrophils were previously reported to express P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>14</sub> (at that time called GPR105) [37], and P2X7 [38, 39], only the P2Y<sub>2</sub> and P2Y<sub>14</sub> but not P2Y<sub>1</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> receptors were initially reported to be functionally active [40, 41]. However, later on also the P2Y<sub>6</sub> and P2Y<sub>11</sub> were assigned to a specific task in neutrophil functionality [35, 42] (see below). Besides the ambiguous data on P2X7 and a single paper claiming the involvement of P2X1 in neutrophil chemotaxis [43], more information on the expression of other P2X receptor subtypes in neutrophils is lacking. Hence further investigation regarding the functional expression of the different P2X receptor subunits in this cell type is required.

Functionally, Ward et al. [44] observed an increase in intracellular calcium levels in rat neutrophils during stimulation by ATP, which was furthermore corroborated by others [29, 45–48]. The purinergic pathway that induces the elevation of calcium levels in neutrophils involves the activation of phospholipase C (PLC) which generates inositol 1,4,5-triphosphate (IP<sub>3</sub>) that subsequently induces calcium mobilization [45, 46, 49, 50]. The P2Y<sub>2</sub> receptor was identified to be the receptor subtype implicated in these responses [40, 50, 51]. Although not mediated via an increased Fcγ receptor-triggered Ca<sup>2+</sup> response but via tyrosine phosphorylation, ATP released from platelets and exogenously applied ATP showed to potentiate actin polymerization with associated increased IgG-mediated phagocytosis and to enhance respiratory burst in neutrophils [33]. P2U—now being characterized to functionally correspond to the P2Y<sub>2</sub> receptor based on the agonist profile reported in the referred study—was again suggested as the P2 receptor responsible for this actin polymerization process in neutrophils [52]. As actin polymerization is also involved in cell motility such as chemotaxis and phagocytosis, exogenous ATP, and adenosine have indeed been shown to induce

**Table 1** Expression profiles and functional responses of the purinergic receptor subtypes in different immune cells

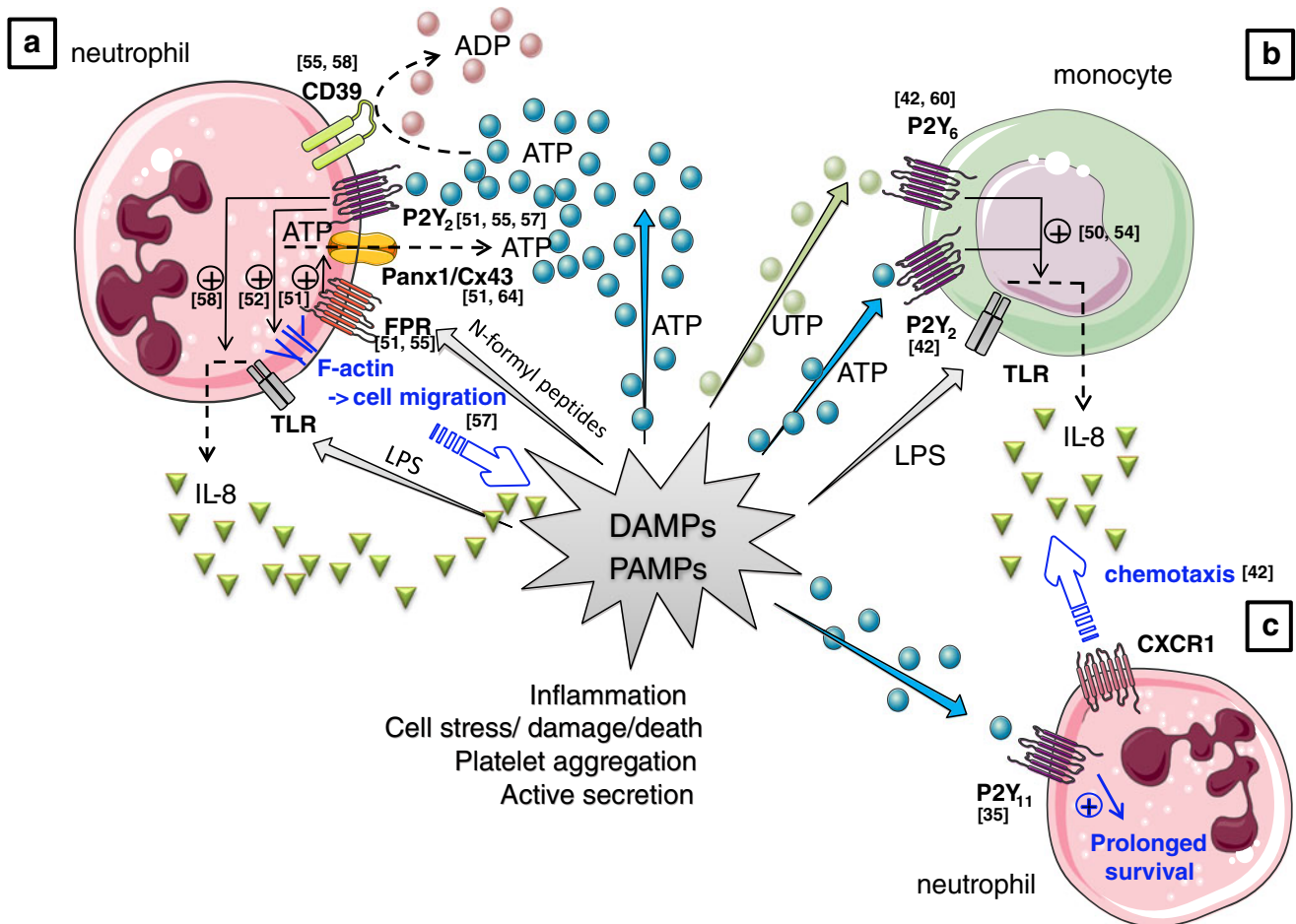
Inflammatory cell type	Functional response to purines	P2 receptor subtype (expression profile and/or involvement in functional response)	References
Neutrophils	Undefined roles	P2Y <sub>1</sub> , P2Y <sub>4</sub> , P2Y <sub>11</sub> , P2Y <sub>14</sub> , and P2X7 <sup>a</sup>	[34, 36, 38–40]
	Calcium mobilization	P2Y <sub>2</sub>	[40, 50, 51]
	Actin polymerization	P2Y <sub>2</sub>	[52]
	Primary granule release	P2Y <sub>2</sub>	[40]
	Chemotaxis	P2Y <sub>2</sub> , P2Y <sub>6</sub> , and P2X1	[43, 55, 57, 58]
	Reduced cAMP accumulation	P2Y <sub>14</sub>	[41]
	Delay in constitutive neutrophil apoptosis	P2Y <sub>11</sub>	[35]
Macrophages	Undefined roles	P2Y <sub>4</sub> , P2Y <sub>6</sub> , P2Y <sub>11</sub> , P2Y <sub>12</sub> , P2Y <sub>13</sub> , P2Y <sub>14</sub> , and P2X1–P2X6	[75–78]
	Intracellular calcium increase	P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>4</sub> , P2Y <sub>11</sub> , P2X4, and P2X7	[73–78]
	IL-1 $\beta$ /IL-18 maturation/release via the NLRP3 inflammasome, caspase-1 and cytosolic K <sup>+</sup> depletion	P2X7	[24, 78, 80, 82–88, 90–96, 108]
	Release of cathepins, PGE <sub>2</sub> , MMP-9 phosphatidilserine (caspase independent)	P2X7	[8, 111–114]
	Promoting chemotaxis/phagocytosis <sup>b</sup>	P2Y <sub>2</sub> , P2Y <sub>12</sub> , P2X1, and P2X3	[115, 117–119]
	Regulation of autophagy	P2X4 and P2X7	[98, 100, 108, 109]
Dendritic cells	Multinucleated giant cells formation	P2X7	[73, 83, 120, 121]
	Undefined roles	P2X1, P2X4, P2X5, P2X7, P2Y <sub>1</sub> , P2Y <sub>4</sub> , P2Y <sub>6</sub> , and P2Y <sub>11</sub>	[123–125, 127]
	Regulation in cytokine release	P2Y <sub>11</sub>	[128–131]
	DC maturation	P2Y <sub>11</sub> , P2Y <sub>12</sub> , and P2Y <sub>14</sub>	[123, 126, 128, 129, 131, 134]
	Apoptosis DC migration	P2X7 P2Y <sub>2</sub> and P2Y <sub>11</sub>	[136, 137] [125, 141]
Lymphocytes B and T cells	Undefined roles	P2X2, P2X3, P2X5, P2X6, and all P2Y	[21, 150–153]
	T cell activation (p38 MAPK activation and IL-2 gene transcription )	P2X1, P2X4, and P2X7	[166, 168]
	T cell activation (CD62L shedding)	P2X7	[25, 178, 179]
	cAMP accumulation	P2Y <sub>14</sub>	[154]
	Inhibition of immunosuppressive potential of Tregs	P2X7	[169]
Natural killer cells	Regulation of NK cytotoxicity and chemotaxis	P2Y <sub>11</sub>	[183]
Eosinophils	Undefined roles	P2Y <sub>1</sub> , P2Y <sub>4</sub> , P2Y <sub>6</sub> , P2Y <sub>11</sub> , P2Y <sub>14</sub> , P2X1, P2X4, and P2X7	[10, 19, 191, 192]
	Chemotaxis	P2Y <sub>2</sub>	[141, 193]
	Release of chemokines and cytokines	P2Y <sub>2</sub> , P2X1, P2X7, and P2Y <sub>6</sub>	[196, 197]
Mast cells	Undefined roles	P2X1, P2X4, P2X6, P2X7, P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>11</sub> , P2Y <sub>12</sub> , and P2Y <sub>13</sub>	[23, 200, 213, 214]
	Degranulation	P2Y <sub>13</sub> and P2Y <sub>14</sub>	[215, 216, 221]

<sup>a</sup> Ambiguous data reporting the presence [34, 38] or absence [36] of P2X7 on neutrophils

<sup>b</sup> Ambiguous data reporting the ATP to be a long-range chemoattractant without phagocytic activity [115] vs. ATP as an autocrine/paracrine signal limited to influence chemotaxis but accompanied with phagocytosis by locally inducing lamellipodial membrane extensions [117, 118]

chemotaxis of neutrophils [52–54]. Chen et al. reported a specific role for purinergic P2Y<sub>2</sub> receptor and the A3 adenosine receptor subtype respectively in gradient sensing and controlling migration speed of neutrophils in an autocrine response to ATP release during stimulation of human

neutrophils with the chemoattractant *N*-formyl-Met-Leu-Phe (FMLP) [55]. The same researchers translated their findings into an in vivo model showing A3 and P2Y<sub>2</sub> receptors to control the recruitment of neutrophils to the lung in a mouse model of sepsis [35, 56]. The involvement



**Fig. 1** Purinergic mechanisms in neutrophil biology. **a** Activation of the formyl-peptide receptor (*FPR*) by *N*-formyl peptides produced by the degradation of either bacterial or host cells (which constitutes together with many other molecules, including ATP, the group of PAMPs and DAMPs) stimulates localized ATP release from neutrophils [51, 55] which occurs through pannexin-1 (*panx1*) and/or connexin 43 (*Cx43*) hemichannels [51, 64] resulting in the activation of nearby  $P2Y_2$  receptors on the neutrophils [51, 55, 57]. This autocrine  $P2Y_2$  receptor activation will subsequently amplify gradient sensing of chemotactic signals (e.g., *N*-formyl peptides [51, 55] and interleukin (IL)-8 [57] by stimulating F-actin to the leading edge [51, 52, 55].  $P2Y_2$  receptor ligation is also implicated in the potentiation of IL-8 production by human neutrophils in response to the bacterial

endotoxin LPS [58], a mechanism under tight control by the ectonucleotidase “Ecto-nucleoside triphosphate diphosphohydrolase 1” (E-NTPDase1 or *CD39*) expressed on the surface of neutrophils [55, 58] by breaking down ATP to ADP. **b** Next to neutrophilic  $P2Y_2$  receptors, extracellular nucleotides (UTP and ATP) were also reported to regulate neutrophil migration by controlling TLR-induced IL-8 release from monocytes via  $P2Y_2$  and/or  $P2Y_6$  receptors expressed on the latter cell types [42, 60]. **c** ATP, via  $P2Y_{11}$  might also cause a long-lasting delay in constitutive neutrophil apoptosis [35]. DAMP damage-associated molecular patterns, LPS lipopolysaccharide, PAMP pathogen-associated molecular patterns, TLR toll-like receptor. Values between brackets in the figure represent reference numbers

of the neutrophilic  $P2Y_2$  receptor in neutrophil migration was corroborated by several other research papers by the group of Sévigny who described that  $P2Y_2$  receptor activation on neutrophils is required for IL-8 (released from monocytes/macrophages and/or epithelial cells at sites of inflammation) to induce most efficient neutrophil chemotaxis [57], and that  $P2Y_2$  receptors on neutrophils are implicated in the potentiation of IL-8 production by human neutrophils themselves, a mechanism under tight control by the ectonucleotidase “Ecto-nucleoside triphosphate diphosphohydrolase 1” (E-NTPDase1 or *CD39*) expressed on the surface of neutrophils [58]. E-NTPDase1 also

regulates the migration speed of neutrophils but not their ability to detect the orientation of the gradient field generated by fMLP that induces an autocrine ATP release via stimulation of the formyl peptide receptor [59]. Besides neutrophilic  $P2Y_2$  receptors, extracellular nucleotides were also reported to regulate neutrophil migration by controlling TLR-induced IL-8 release from monocytes via  $P2Y_2$  and/or  $P2Y_6$  receptors expressed on the latter cell types [42, 60] or potentiate LPS-induced transendothelial migration of neutrophils via  $P2Y_2$  receptors on endothelial cells via an IL-8 independent way [61]. Similarly, macrophages stimulated with extracellular ATP produce macrophage inflammatory

protein-2 (MIP-2) which is an important factor for neutrophil migration [62]. Vaughan et al. reported that exposure of neutrophils to physiologically relevant concentrations of ATP causes a long-lasting delay in constitutive neutrophil apoptosis mediated via P2Y<sub>11</sub> receptors [35].

While purinergic activation of neutrophils thus seems to be clearly a regulatory component implicated in the immune response mediated by neutrophils [63], the activation of neutrophils can conversely also lead to the release of ATP, which was reported to occur via connexin (Cx43) and pannexin-1 (panx-1) hemichannels [51, 64]. Stimulation of the formyl peptide receptor, which colocalizes with P2Y<sub>2</sub> nucleotide receptors on the cell surface, forms a purinergic signaling system that facilitates neutrophil activation and leads to ATP release through panx1 hemichannels [51]. While UDP-glucose, an agonist at P2Y<sub>14</sub> receptors, is also reported to be functionally active in neutrophils—as evidenced by inducing an inhibition of forskolin-stimulated cAMP accumulation and an increase in ERK1/2 phosphorylation—, it does not enhance neutrophil degranulation triggered by fMLP [41].

In conclusion, with the exception of a single study suggesting P2X1 to be implicated in chemotaxis of neutrophils [43], a fast majority of publications describes the P2Y<sub>2</sub> receptor to be the main P2 receptor subtype implicated the functional regulation of neutrophils regarding calcium mobilization, actin polymerization, cell motility, and an autocrine feedback mechanism releasing ATP through hemichannels. Neutrophil apoptosis on the other hand rather seems to be mediated via P2Y<sub>11</sub> receptors [35] (Table 1).

## Macrophages

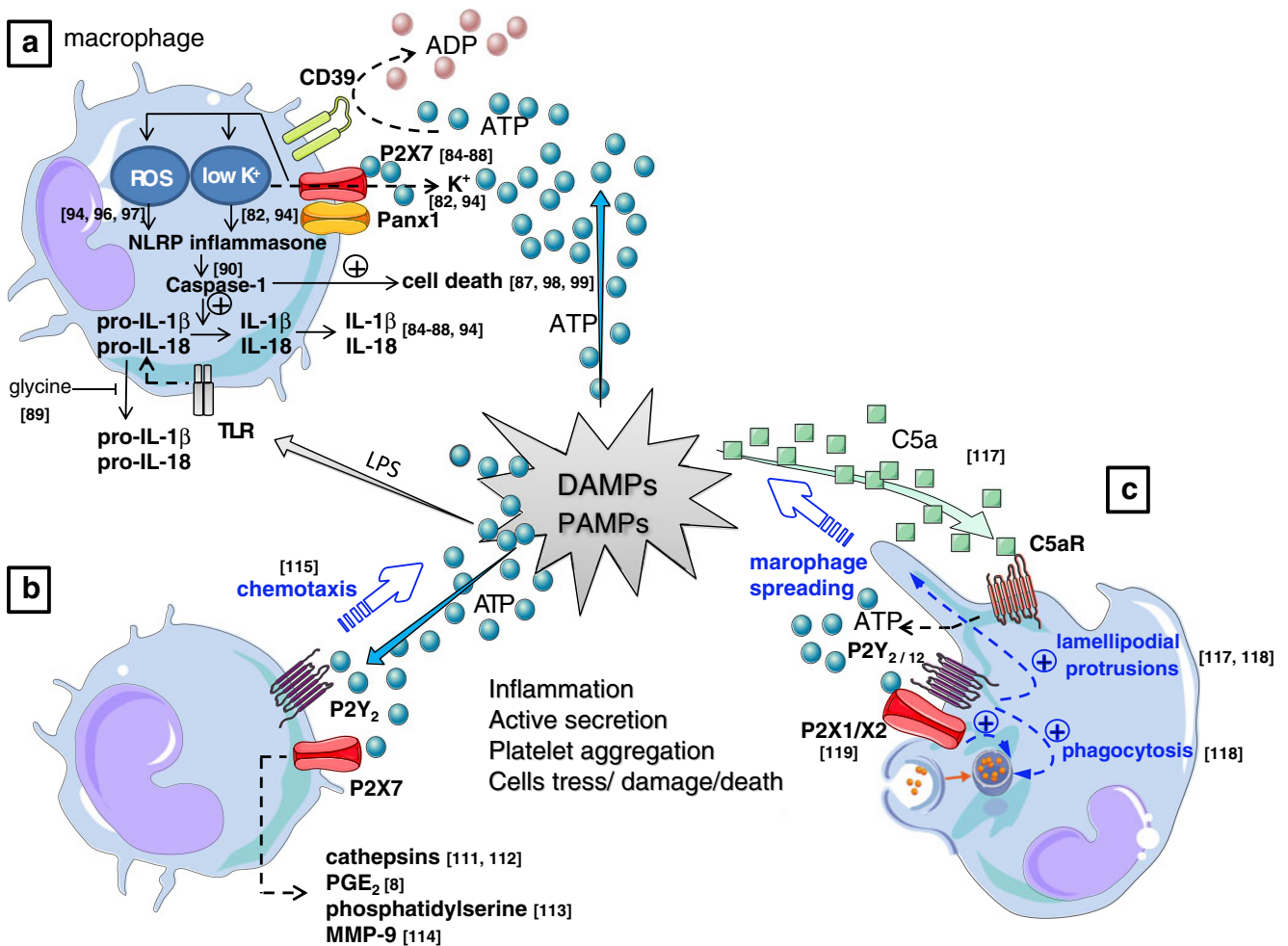
Macrophages (Fig. 2) are phagocytes [65] that are strategically located throughout the body tissues where they ingest and process foreign material and dead cells. Macrophages are implicated in antigen presentation and immunomodulation by production of various cytokines, playing an important role in the initiation, maintenance, and resolution of inflammation. Macrophages are known to be able to rapidly change their function in response to local microenvironmental signals [66]; such a signal derived from dying, necrotic, and apoptotic cells is ATP.

The influence of exogenous nucleotides on macrophages was identified by Cohn and Parks which paved the way for further research into the involvement of extracellular purine and pyrimidine nucleotides during inflammation [67]. The authors showed adenine nucleotides to be implicated in the pinocytic vesicle formation in mouse macrophage cultures. Later Silverstein and colleagues revealed ATP to induce a mechanism which facilitates calcium fluxes in mouse macrophages [68–71]. Since calcium is mobilized during stimulation with ATP, assessment of calcium levels is used in the

functional characterization of the receptors involved in purinergic responses. The P2Z receptor—later on being characterized as the P2X7 receptor [72]—was the first P2 receptor subtype identified to augment intracellular calcium levels in murine and human macrophages [73, 74].

The expression and functional characterization of other P2 receptor subtypes in macrophages was assessed in different cell lines: all murine and rat macrophage cell lines were shown to express multiple P2Y and P2X subtypes. However, only P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> receptors were demonstrated to be functionally implicated in evoking ATP-mediated increases of cytosolic calcium levels [75–77]. In humans, alveolar macrophages were reported to express all P2 receptor subtypes except P2Y<sub>12</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, and P2X<sub>6</sub> [78], whereas only, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>11</sub>, and P2X<sub>7</sub> exhibit functional responses in inducing an intracellular calcium elevation.

Besides phagocytosis and antigen presentation, macrophages are implicated in inflammatory reactions via the secretion of a diverse set of mediators [79]. A role for extracellular ATP in modulating the mediators' expression and/or release was first reported by Tonneti et al. who observed increased production of nitric oxide, IL-1 $\beta$ , and TNF- $\alpha$  by ATP in lipopolysaccharide-treated mouse macrophages [80, 81]. IL-1 $\beta$  maturation and release in response to ATP in murine macrophages was confirmed by Perregaux and Gabel who reported a concomitant decrease in intracellular K<sup>+</sup> as a requisite [82]. In contrast to murine macrophages, in human macrophages the expression of TNF- $\alpha$  was reported to be downregulated, whereas IL-1 $\beta$  secretion was also here upregulated by extracellular nucleotides [78]. In rat alveolar macrophages, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 cytokine release was also reported to be facilitated by P2X7 receptor activation, while TNF- $\alpha$  release was not affected [83]. The P2Z/P2X7 was indeed earlier identified to be involved in the increase in IL-1 $\beta$  production in human macrophages [24, 84–87]. Later on, secretion of IL-1 $\beta$  and IL-18 in macrophages was found to be mediated by caspase-1, in association with the involvement of the NLRP3 inflammasome [88]. Priming of macrophages with PAMPs such as LPS is sufficient to stimulate synthesis of pro-IL-1 $\beta$ , but secretion of the active IL-1 $\beta$  cytokine requires a secondary signal that is provided by ATP through P2X7 ligation, yielding the cleavage of pro-IL-1 $\beta$  into active IL-1 $\beta$  by the cysteine proteases caspase-1. In macrophages, both pro-IL-1 $\beta$  and processed IL-1 $\beta$  are reported to be released via two distinct pathways respectively; the former pathway being caspase-1 and panx-1 independent but blocked by glycine [89] while the latter does depend on these structures, with both pathways being independent of cell death processes [88]. Caspase-1 itself is expressed as an inactive procaspase-1 that needs cleavage and activation in association with protein complexes called inflammasomes; the specific



**Fig. 2** **a** Challenging macrophages with PAMPs such as LPS stimulates synthesis of pro-IL-1 $\beta$  and pro-IL-18. Secretion of the active cytokines requires a secondary signal that is provided by ATP through P2X7 ligation [84–88], yielding the conversion of pro-IL-1 $\beta$  into active IL-1 $\beta$  by NLRP3 inflammasome activated caspase-1 enzymatic cleavage [90]. The activation process of caspase-1 is initiated via P2X7-mediated cytosolic K<sup>+</sup> outflow [82, 94], or alternatively via the activation of ROS [94, 96, 97]. Also the pro-IL-1 $\beta$  and pro-IL-18 can be released via a distinct caspase-1 independent pathway which is blocked by glycine [89]. Prolonged stimulation with ATP also leads to caspase-1 dependent, but IL-1 $\beta$  and IL-18 cytokine processing independent cell death in macrophages [87, 98, 99]. **b** P2X7 receptor activation has also been shown to signal caspase-1 and IL-1 $\beta$ /IL-18 independent release of cathepsins [111, 112], PGE<sub>2</sub> [8], phosphatidylserine [113], and MMP-9 [114], all of which are implicated in cellular processes that play a defined role in inflammation. ATP is also reported

to act as a long-range “find me” signal (chemoattractant) to recruit monocytes and macrophages [115]. **c** The role of ATP as “real” direct chemoattractant for macrophages is however controversial; the purinergic system can also act as an indirect chemoattractant that navigates macrophages in a gradient of the “real” chemoattractant C5a via auto-crine release of ATP induced by the C5a receptor (C5aR), yielding an amplification in the gradient sensing via an orientated generation of lamellipodial membrane protrusions involving P2Y<sub>2</sub> and P2Y<sub>12</sub> receptors to furthermore induce an indirect effect of chemotaxis and the promotion of phagocytosis [117, 118]. Enhanced phagocytic effect in macrophages by purinergic stimuli can also involve the ligation of P2X1 or P2X3 receptors [119]. DAMP damage-associated molecular patterns, LPS lipopolysaccharide, MMP-9 matrix metalloproteinase-9, PAMP pathogen-associated molecular patterns, TLR toll-like receptor, PGE<sub>2</sub> prostaglandin E<sub>2</sub>. Values between brackets in the figure represent reference numbers

inflammasome that is activated by P2X7 activation contains the adaptor molecule NLRP3 [90]. The NLRP3 inflammasome can be activated via P2X7-ligation by DAMPs such as ATP [91], but also biglycan [92] and serum amyloid A [93] are reported to elicit signaling through P2X7. P2X7-ligation can lead to pore formation and cytosolic K<sup>+</sup> outflow which results in activation of caspase-1 and the subsequent secretion of active IL-1 $\beta$  and IL-18 in macrophages that had been

primed with LPS [94] confirming the early observations by Perregaux and Gabel as mentioned above [82]. However, unlike caspase-1 activated by TLR agonists (extracellular pathogenic products) and ATP, the activation of the macrophage inflammasome by intracellular bacteria is reported to proceed normally in the absence of P2X7 receptor-mediated cytoplasmic K<sup>+</sup> perturbations [95]. ATP also shows to process and secrete IL-1 $\beta$  and IL-18 via the activation of

reactive oxygen species (ROS) in rat alveolar macrophages through P2X7 [96]. ATP-mediated ROS production can activate ERK1/2 and subsequently caspase-1, which is required for IL-1 $\beta$  and IL-18 processing [94, 96, 97]

While prolonged stimulation with ATP also leads to caspase-1-dependent cell death in macrophages [98], this process seems to be independent from the concomitant occurring IL-1 $\beta$  and IL-18 cytokine processing and release [87, 99]. Biswas et al. reported that ATP-treated human monocytes/macrophages undergo rapid cell autophagy which involves the activation of P2X7 receptors and is associated with augmented mycobactericidal activity of macrophages [100]. Some intracellular pathogens modulate the function of the macrophage phagosome in order to escape the host defense responses [101]. However the host macrophages have developed a purinergic-mediated mechanism to counteract the escape efforts of these pathogens such as *Mycobacterium* [102], *Vibrio cholerae* [103], *Pseudomonas aeruginosa* [104], and *Chlamydia trachomatis* [105]; P2X7 receptor ligation can stimulate the inflammasome associated with secretion of cytokines but it can also lead to the direct killing of intracellular pathogens in infected macrophages, involving macrophage cell death and potentially related to macrophage autophagy [106, 107]. The co-expression of P2X4 receptors with P2X7 receptors was subsequently found to suppress P2X7-mediated autophagy and to facilitate the release of pro-inflammatory mediators in mouse macrophage RAW264.7 cells, consequently enhancing inflammation [108]. This association of P2X4 with P2X7 was also described in relation to macrophage cell death [109] but for which the underlying molecular mechanism is not yet unveiled. The effects by P2X7 receptor activation can also be tempered by E-NTPDase1 which degrades ATP at the cell surface of macrophages, potentially contributing to the fact that P2X7 is activated by higher concentrations of ATP compared with other P2 receptors [110].

Besides the caspase-1 dependent processes described above, P2X7 receptor activation has also been shown to signal caspase-1 and IL-1 $\beta$ /IL-18 independent release of cathepsins [111, 112], prostaglandin (PG)E<sub>2</sub> [8], phosphatidylserine [113], and matrix metalloproteinase 9 [114], all of which are implicated in cellular processes that play a defined role in inflammation.

Extracellular purines and pyrimidines might also be implicated in controlling the movement of macrophages; Elliott et al. reported that ATP released from apoptotic cells acts as a long-range “find me” signal (chemoattractant) to recruit motile monocytes and macrophages. The authors showed that the increased recruitment of monocytes/macrophages to apoptotic cell supernatants in a transwell migration assay and in an in vivo murine subcutaneous air-pouche model was reduced by apyrase and under P2Y<sub>2</sub><sup>-/-</sup> conditions [115]. The identification of the P2Y<sub>2</sub> receptor in purinergic-mediated chemotaxis of macrophages is however

not in agreement with the initial observation by McCloskey et al. [116] who observed that ADP was a chemoattractant for the murine J774 macrophage cell line because this agonist is not active on the P2Y<sub>2</sub> receptor. However, Elliot et al. could not exclude the possibility that other chemotactic factors work alone or together with nucleotides in mediating the observed chemoattractant effect. Moreover, the role of nucleotides in chemotaxis remains equivocal as evidenced by several recent papers that do not consider ATP any longer as a “real” direct chemoattractant for macrophages. One report describes ATP as an indirect chemoattractant that navigates macrophages in a gradient of the chemoattractant C5a via autocrine release of ATP, generating amplification in gradient sensing via a “purinergic feedback loop” [117]. The same paper also reports the stimulation of macrophages with ATP to generate lamellipodial membrane protrusions that induce an indirect effect of chemotaxis [117]. The latter two mechanisms were found to involve P2Y<sub>2</sub> and P2Y<sub>12</sub> receptors [117]. The same authors confirmed in another recent paper that ATP does not recruit macrophages but locally induces lamellipodial membrane extensions and that ATP can promote chemotaxis and phagocytosis via autocrine/paracrine signaling involving P2Y<sub>2</sub> and P2Y<sub>12</sub> receptors but that it is itself not a chemoattractant as was evidenced from a microscope-based real-time chemotaxis assay that allows quantification of migration velocity and chemotaxis [118]. The increase in phagocytotic effect of macrophages by P2Y<sub>2</sub> and/or P2Y<sub>12</sub> ligation also contrasts to the findings of Elliott et al. who characterized ATP as a long-range chemoattractant -as discussed above- but without any effect on phagocytic activity [115]. Marques-da-Silva et al. on the other hand confirmed an enhanced phagocytic effect in macrophages by purinergic stimuli but proposed the engagement of P2X1 or P2X3 receptors based on the agonists profile [119]

Macrophages can furthermore undergo fusion with other macrophages to form multinucleated giant cells (MGC), a common feature of granulomas that develop during various inflammatory reactions. The involvement of purinergic receptors in MGC formation was first reported by the group of Di Virgilio who showed that high levels of P2X7 expression leads to spontaneous macrophage fusion in vitro [73, 120], being confirmed by Lemaire and Leduc [83]. Both groups later on attributed this effect to the C terminal part of the P2X7 receptor [121].

In conclusion, the implication of purinergic P2 receptors in inflammatory responses is evident in macrophages, being dominated by the P2X7 receptor subtype. Recent evidence suggests the possible regulatory function for P2X4 in P2X7-mediated responses. Further research is needed in order to assess whether other purinergic P2 receptors might contribute to the regulation of macrophage function (Table 1).

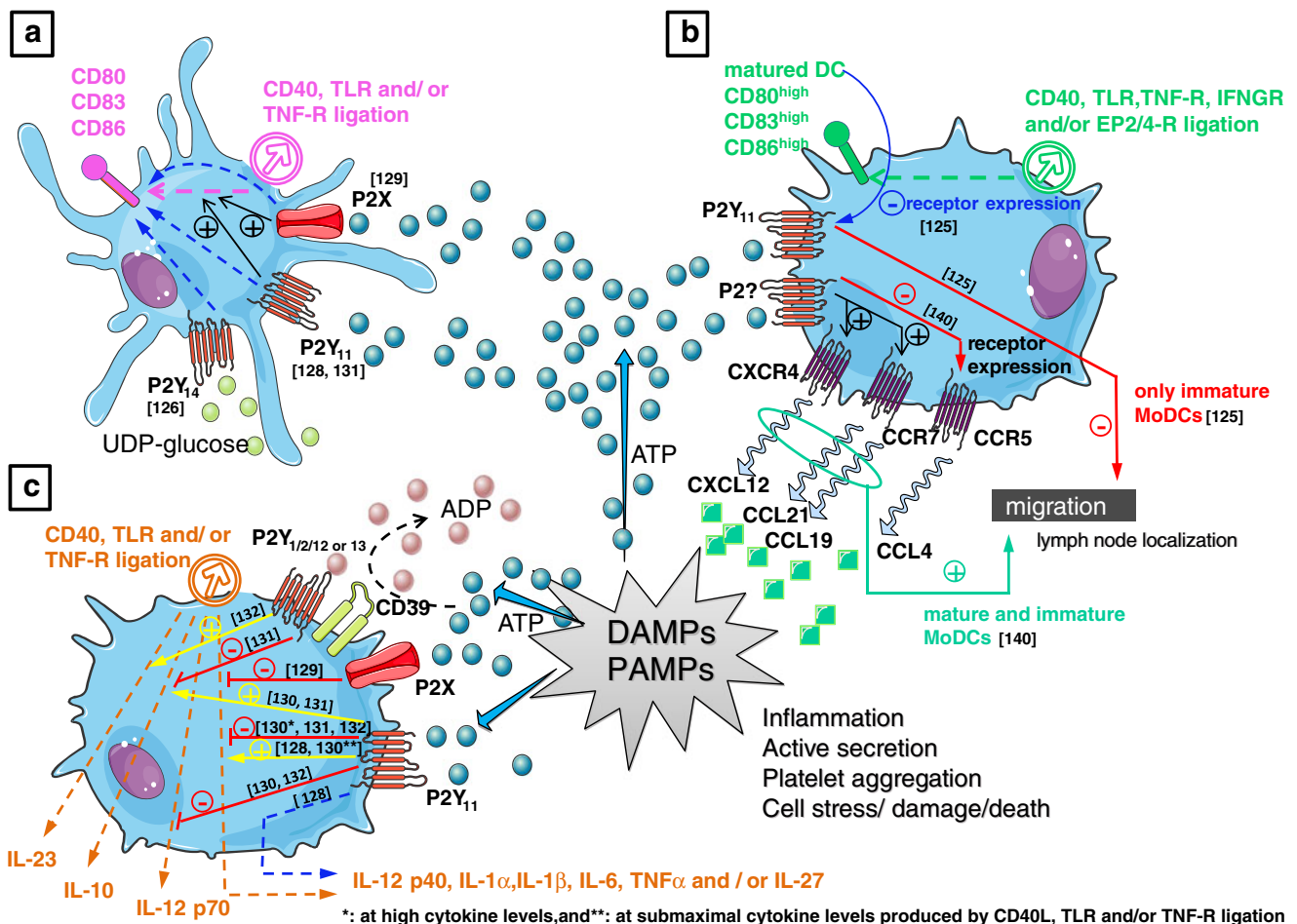
## Dendritic cells

Dendritic cells (DCs) (Fig. 3) are the most potent antigen presenting cells of the immune system. In the absence of infection, DCs participate in central and peripheral T cell tolerance. During an infection, a dramatic increase in the movement of DCs from the tissue via afferent lymphatics into lymph nodes occurs where they trigger a specific T cell response [122].

Human dendritic cells were found to express mRNA for P2X1, P2X4, P2X5, and P2X7 as well as for P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, and P2Y<sub>14</sub> receptors [123–126]. The

expression of P2X7 on protein level in DCs during immune responses was suggested by Buell et al. who observed immunopositive staining within human tonsils [127].

Functional responses induced by purinergic stimuli in DCs involve the release of IL-1 $\beta$  and TNF- $\alpha$  in LPS-matured DCs [124]. While Wilkin et al. [128] also reported ATP to synergize with TNF- $\alpha$  and LPS in increasing IL-12 secretion, la Sala et al. [129] reported ATP to inhibit LPS—and soluble CD40 ligand (sCD40L)—induced expression of IL12, but also of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. When discriminating between IL-12p40 and IL-12p70, the stable ATP analogue ATP $\gamma$ S potentiated the IL-12p40 release from



**Fig. 3** Purinergic responses in monocyte-derived dendritic cells (*MoDCs*). **a** *MoDC* maturation: chronic stimulation of DCs with non-cytotoxic doses of ATP increases membrane expression of CD80, CD83, and CD86. Moreover, ATP also enhanced soluble CD40 ligand (*sCD40L*), LPS, and/or TNF- $\alpha$ -induced maturation of *moDCs* via P2X receptor activation [129]. These processes might also involve P2Y<sub>11</sub> [128] and/or P2Y<sub>14</sub> [126] receptor activation. **b** *MoDC* migration: ATP provides a signal for enhanced lymph node localization of DCs; ATP increased migration of immature and mature DCs to CXCL12, CCL19, and CCL21, whereas responses to CCL4 were reduced [140]. In contrast, exposure of *MoDCs* to gradients of ATP inhibited their migratory capacity via P2Y<sub>11</sub> receptors [125]. During maturation (by *sCD40L*, LPS, TNF- $\alpha$ , INF $\gamma$ , and/or PGE<sub>2</sub>), *MoDCs* downregulated

P2Y<sub>11</sub> receptor expression which yield mature DCs that are less sensitive to ATP-mediated inhibition of migration [125]. **c** *MoDC* cytokine release—equivocal data in the literature report either a potentiating or inhibitory effect on *sCD40L* LPS and/or TNF- $\alpha$ -induced expression of inflammatory cytokines. *CCL19* chemokine (C–C motif) ligand 19, *CCR7* C–C chemokine receptor type 7, *CXCL12* chemokine (C–X–C motif) ligand 12, *CXCR4* C–X–C chemokine receptor type 4, *DAMP* damage-associated molecular patterns, *INF $\gamma$*  interferon gamma, *IFNGR* INF $\gamma$  receptor, *LPS* lipopolysaccharide, *PAMP* pathogen-associated molecular patterns, *PGE<sub>2</sub>* prostaglandin E<sub>2</sub>, *EP2/4-R* PGE<sub>2</sub> receptor 2 and/or 4, *TLR* toll-like receptor, *TNF- $\alpha$*  tumor necrosis factor alpha, *TNFR* TNF receptor. Values between brackets in figure represent reference numbers



human monocyte-derived DCs (MoDCs) induced by TNF- $\alpha$ , lipopolysaccharides (LPS), or by sCD40L [130]. The combinations ATP $\gamma$ S-TNF- $\alpha$  and ATP $\gamma$ S-sCD40L were however unable to induce detectable bioactive IL-12p70 production and at concentrations of LPS that induced a significant stimulation of IL-12p40 and IL-12p70, the effect of ATP $\gamma$ S was inhibitory. The authors also showed that ATP $\gamma$ S synergized with LPS and sCD40L, but not TNF- $\alpha$ , to stimulate IL-10 production [130]. Except for the increase in IL-12p40 levels by ATP $\gamma$ S, above data were corroborated by another study reporting that ATP $\gamma$ S inhibited the levels of IL-12p40, IL-12p70, and TNF $\alpha$  produced during concomitant LPS challenge, whereas the IL-10 secretion was observed to be increased [131]. Conversely, IL10 production in these DCs could be reduced by ADP $\beta$ S stimulation, which equally reduced IL-12 levels [131]. The inhibition by ATP of IL-12p40 and IL-12p70-production by DCs was also observed by Schnur et al. who moreover found that ATP also inhibited IL-27 expression but enhanced IL-23 expression in *E. Coli* induced MoDCs [132].

The involvement of the purinergic system in DC functionality was equally confirmed by assessing the maturation status based on the levels of DC surface markers. The implication of P2 receptors in DC maturation was already suggested by Schnur et al. in showing that activation and maturation of DCs was blocked by suramin [133]. Later studies showed that in combination with TNF- $\alpha$ , ATP increased the expression of the DC surface markers CD80, CD83, and CD86 confirming a maturation-promoting effect [123]. Similarly, la Sala et al. reported that chronic stimulation of DCs with low, noncytotoxic doses of ATP increased membrane expression of CD54, CD80, CD83, and CD86, slightly reduced the endocytic activity of DCs, and augmented their capacity to promote proliferation of allogeneic naive T lymphocytes. Moreover, ATP also enhanced LPS- and sCD40L-induced CD54, CD83, and CD86 expression [129]. While Wilkin et al. assigned these processes to cAMP accumulation via P2Y<sub>11</sub> receptor activation in human DCs based on the rank order of potency of various ATP analogues [128], a study on mice DCs reported that also ADP—via P2Y<sub>12</sub>—can promote antigen endocytosis by DCs and enhance T cell response [134]. Moreover, also the P2Y<sub>14</sub> receptor was proposed to be implicated in DC activation/maturation as evidenced by the induction of Ca<sup>2+</sup> fluxes and an increase in expression of the costimulatory molecule CD86 during stimulation with UDP glucose [126].

Other functional responses induced by purinergic stimuli in human DCs involve the reorientation of dendrites and changes in cell shape via functionally coupled P2Y receptors but not P2X receptors, being deduced from the pharmacological selectivity of the applied agonists [135]. However in this study, prolonged exposure to ATP was found to be toxic to dendritic cells that swelled, lost typical

dendrites, and eventually died, which was highly suggestive for the expression of the cytotoxic receptor P2X7, as confirmed by ability of dendritic cells to become permeate to dyes such as Lucifer yellow or ethidium bromide [124]. ATP via P2X7 indeed also mediates apoptosis of dendritic cells as was confirmed in murine DCs [136, 137]. Epidermal Langerhans cells have previously been shown to express membrane ATPase which appears to protect them against the cytotoxic effects of extracellular ATP [138]. Similarly, human peripheral blood-derived DCs express the ecto-apyrase CD39 and the ecto-5P-nucleotidase CD73 as demonstrated by RT-PCR which rapidly hydrolyze extracellular ATP [123].

The reorientation of dendrites and changes of cell shape by P2Y receptor activation as observed by Liu et al. [135] suggests a possible role for ATP in mediating DC chemotaxis. The chemotactic effect of ATP was confirmed by Idzko et al. in immature but not mature DCs via P2Y receptors while P2X receptor activation only showed minor responses [139]. ATP indeed provides a signal for enhanced lymph node localization of DCs but it may, at the same time, diminish the capacity of DCs to amplify type 1 immune responses as was reported by la Sala et al. [140]. In contrast, exposure of MoDCs and CD1a<sup>+</sup> dermal DCs to gradients of ATP inhibited their migratory capacity in a dose-dependent manner. However, during maturation, MoDCs downregulated P2Y<sub>11</sub> receptor expression which yielded mature DCs that are less sensitive to ATP-mediated inhibition of migration [125]. These data indicate that the physiologic consequences of exposure to ATP on DC migration depend on ATP concentration, DC type, and DC maturational stage. The authors speculated that the formation of ATP gradients at sites of inflammation may transiently inhibit the migration of local DCs, thus prolonging the time of antigen encounter [125]. Also, the P2Y<sub>2</sub> receptor appears to be involved in ATP-triggered migration of DCs as was reported for myeloid DCs in an experimental model of asthmatic airway inflammation [141]. The importance of ATP in dendritic cell on airway inflammation was earlier reported to be implicated in the pathogenesis of human asthma and experimental asthmatic airway inflammation in mice [142]. The authors showed that all the cardinal features of asthma, including eosinophilic airway inflammation, Th2 cytokine production and bronchial hyper-reactivity, were abrogated when lung ATP levels were locally neutralized using apyrase or when mice were treated with broad-spectrum P2-receptor antagonists. In addition to these effects of ATP in established inflammation, Th2 sensitization to inhaled antigen was enhanced by endogenous or exogenous ATP. The adjuvant effects of ATP were due to the recruitment and activation of lung myeloid dendritic cells that induced Th2 responses in mediastinal lymph nodes [142].

In conclusion, nucleotides can significantly modify DC functions such as cytokine release, maturation status and

migration characteristics. Although more *in vivo* studies are welcome to confirm the observations made on the models of asthmatic inflammation, targeting P2 receptor function might indeed represent a promising approach to develop new therapeutics for asthma as evidenced by a recent study in a birth cohort at high risk for development of asthma that reported an attenuated P2X7 function to be associated with lower asthma rates [143] (Table 1).

## Lymphocytes

The three major types of lymphocyte (Fig. 4 and Table 1) are T cells, B cells and natural killer (NK) cells. T cells are characterized by the presence of a T cell receptor (TCR) on the cell surface. There are several subsets of T cells (T helper cells (Th cells), cytotoxic T cells (CTLs), memory T cells, regulatory T cells (Tregs), and natural killer T cells (NKT cells)), each with distinct functions; Th cells, CTLs and Treg cells are reported to possess a purinergic component. Th cells (CD4+ T cells) assist other leukocytes in immunologic processes by means of secreting different sets of cytokine subtypes (Th1, Th2, Th17, etc.) Th cells themselves become activated when they are presented with peptide antigens by MHC class II molecules on the surface of antigen presenting cells (APCs), such as macrophages, dendritic cells, and B cells. CTLs (CD8+ T cells) destroy virally infected cells and tumor cells. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of nearly every cell of the body. Treg cells (CD4+ CD25+ T cells) are crucial for the maintenance of immunological tolerance by dampening the T cell-mediated immunity, terminating the immune reaction and suppressing autoreactive T cells that escaped the process of negative selection in the thymus [144]. B cells are characterized by the presence of a B cell receptor on the cell surface which allows B cells to bind to a specific antigen. The principal functions of B cells are to make antibodies against antigens, to perform the role of APCs, and to develop into memory B cells after activation by antigen interaction [145]. Similar to CTL's, NK cells provide responses to virally infected cells and tumor formations but can act much faster as they have the ability to recognize stressed cells in the absence of antibodies or MHC molecules [146].

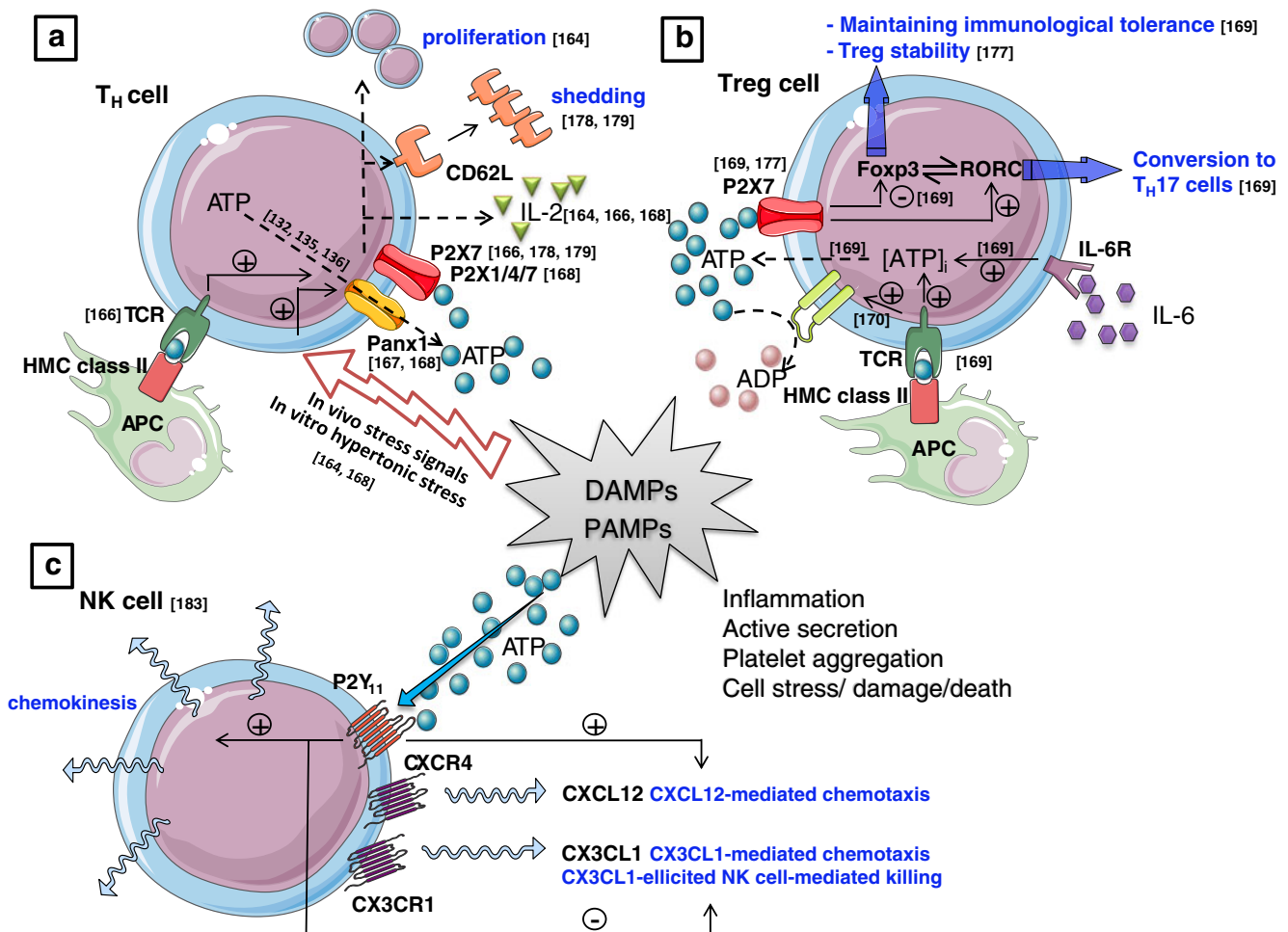
## B and T cells

Based on results generated in the late 1980s and begin 1990s, lymphocytes were initially regarded to exclusively express ionotropic P2X receptors as they react on exogenous ATP via enhanced  $\text{Ca}^{2+}$  influx across the plasma membrane while mobilization of  $\text{Ca}^{2+}$  from intracellular stores (P2Y) could not be observed [147, 148]. Others reported

that P2 receptors are expressed only on B lymphocytes and not on T lymphocytes [149]. However, later studies revealed the presence of both P2X and P2Y receptor subtypes in both T and B lymphocytes [21, 150–153]. By means of confocal immunofluorescence microscopy imaging using a panel of polyclonal antibodies raised against extracellular epitopes of the seven P2X subtypes, Sluyter et al. confirmed the presence of P2X1, P2X2, P2X4, and P2X7 subtypes but not P2X3, P2X5, and P2X6 subtypes on human B lymphocytes [152]. In contrast to the selective expression of P2X receptors in the latter study, Lee et al. [151] demonstrated human B lymphocytes to express mRNA of all P2X and P2Y receptors. All P2Y receptors showed similar rates of expression within 1- or 2-fold of each other. The same is true within the P2X receptor family with the exception of the P2X3 and P2X7 receptors, which were expressed in lower quantities.

The expression of most P2 receptors was suppressed during Epstein–Barr virus-induced B cell transformation into lymphoblastoid cell lines (LCLs); however, the suppression of P2X1, P2X4, P2X5, and P2Y<sub>11</sub> receptors was not as pronounced as for other subtypes, leaving them to be the most abundantly expressed subtypes in LCLs. Only P2X7 receptor was significantly upregulated, suggesting some plasticity in P2-receptor expression in B cells. Western blot analysis confirmed the mRNA data for all P2 receptors [151]. Similarly, Wang et al. [153] showed mRNA from all P2Y receptor subtypes to be expressed in lymphocytes. Among the P2Y receptor subtypes, P2Y<sub>2</sub> and P2Y<sub>12</sub> had highest expression, but did not exceed a 2.5-fold change. The lowest expressed P2Y receptor subtype was P2Y<sub>4</sub>. The P2X4 receptor was found to be the most abundant P2X receptor subtype expressed in lymphocytes compared with the P2X1, P2X4, and P2X7 subtypes that were assessed in that study. In contrast to the study by Lee et al., Wang et al. found similar expression levels between P2X1 and P2X7 [153]. Moore et al. furthermore showed expression of P2Y<sub>14</sub> in peripheral immune cells including lymphocytes [39], which was in a further study reported to be predominantly expressed in T lymphocytes rather than in B lymphocytes [154].

Functionally, the effects of ATP on lymphocytes were investigated extensively. Early observations, from the late 1970s on, already indicated nucleotides to equivocally affect thymocytes/lymphocyte mitogenesis/blastogenesis [147, 155–161]. Barankiewicz et al. suggested that extracellular ATP catabolism may serve as a means of communication between B and T cells in lymphoid organs, with B lymphocytes but not T lymphocytes being the producers of adenosine via ATP breakdown, and T lymphocytes being the recipients of this signal [162]. However, it is now more commonly appreciated that also T lymphocytes can release and hydrolyze extracellular ATP to adenosine, and that ATP is involved in promoting T cell activation while adenosine



**Fig. 4** **a** T helper ( $T_H$ ) cells: hypertonic saline promotes T cell proliferation and enhances IL-2 production of stimulated T cells by inducing the release of cellular ATP and by the autocrine activation of stimulatory P2X receptors [164]. Also, T cell receptor (TCR) stimulation itself triggers the release of ATP from the T cells which - via an autocrine feedback mechanism through P2X7 receptors [166] and/or P2X1 and P2X4 [168]—is required for the effective activation of T cells. This ATP release occurs through pannexin-1 hemichannels [167, 168]. ATP also triggers P2X7 receptor-dependent CD62L shedding in lymphocytes [178, 179] which is associated with T cell activation. **b** T regulatory ( $T_{reg}$ ) cells: CD4<sup>+</sup> helper T cells upon stimulation of the TCR and potentiated by the inflammatory cytokine IL-6, increases the synthesis and release of ATP [169] which subsequently induces an autocrine P2X7-mediated signaling in Tregs [169] that inhibits the suppressive potential and stability of Tregs [177], and promotes their conversion to IL-17-secreting T Th17 effector cells [169]. However,

inhibits T cell functions [163–165]. Indeed, Loomis et al. showed that hypertonic saline promotes T cell proliferation and enhances IL-2 production of stimulated T cells by inducing the release of cellular ATP and by the autocrine activation of stimulatory P2X receptors [164]. The same group also showed that T cells express enzymes that convert ATP to its inhibitory metabolite adenosine which inhibits IL-2 production by activating A2 receptors expressed on the surface of T cells [165]. Later studies demonstrated TCR

stimulation also enhances the expression of CD39 [170] which degrades ATP to ADP and AMP. Consequently, activated Treg cells are able to abrogate the P2X7-mediated suppression of Treg function and stability in an autologous way [169]. **c** Natural killer (NK) cells: NK cells stimulated with ATP increases chemokinesis and chemotaxis to CXCL12 via P2Y<sub>11</sub> receptor activation, while chemotaxis and NK cell-mediated killing in response to CX3CL1 chemokine is inhibited [183]. APC antigen presenting cell, CX3CL1 chemokine (C-X3-C motif) ligand 1, CX3CR1 CX3C chemokine receptor 1, CXCL12 chemokine (C-X-C motif) ligand 12, CXCR4 C-X-C chemokine receptor type 4, DAMP damage-associated molecular patterns, Foxp3 forkhead box P3, HMC class II major histocompatibility complex class II, IL-6R interleukin-6 receptor, PAMP pathogen-associated molecular patterns, RORC retinoic acid receptor-related orphan receptor. Values between brackets in figure represent reference numbers

stimulation itself to trigger the release of ATP from the T cells which—via an autocrine feedback mechanism through P2X7 receptors—is required for the effective activation of T cells as was shown in Jurkat cells and human purified CD4<sup>+</sup> T cells [166]. This ATP release from naive CD4<sup>+</sup> T cells occurs through pannexin-1 hemichannels [167, 168] and the released ATP promotes T cell function as evidenced by p38 MAPK activation and increased IL-2 gene transcription through stimulation of P2X1, P2X4, and P2X7 receptors

[168]. Another recent paper describes that pharmacological antagonism of P2X7 receptors promotes cell-autonomous conversion of naïve CD4<sup>+</sup> T cells into Tregs after TCR stimulation, while the activation of P2X7 by autocrine ATP inhibits the suppressive potential and stability of Tregs [169]. However, TCR stimulation also enhances the expression of CD39 via the induction of the Treg-specific transcription factor Foxp3 [170]. As CD39 is an ectoenzyme that degrades ATP to AMP, activated Treg cells are consequently not only able to abrogate ATP-driven maturation of dendritic cells [170], but also P2X7-mediated suppression of Treg function and stability in an autologous way [169].

The breakdown product of ATP was initially also reported as an inhibitor of lymphocyte mediated cytolysis which was associated with an elevation of cyclic AMP within the cytotoxic T cells [171, 172]. Later on, others also revealed extracellular ATP to be a potent modulator in the cytolytic activity of cytotoxic T cells [173, 174]. Filippini et al. described the ability of both cytotoxic T cells and T helper cells to accumulate ATP in response to TCR crosslinking, suggesting a general role of ATP in the effector functions of T lymphocytes. When secreted by cytotoxic T cells towards the target cell, ATP may contribute to the death of the target cell, while secretion of ATP by T helper cells toward the antigen-presenting cell may modulate the action of lymphokines by involving purinergic receptors or ectoprotein kinases [174]. Similarly, Blanchard et al. provided evidence that ATP is released from cytotoxic T cells during antigen presentation, and that IFN- $\gamma$ -activated macrophages underwent necrosis [173]. Besides the induction of cell death in the cytotoxic T lymphocytes' target cells, also the T cells themselves are reported to undergo ATP-mediated cell death [175]. Unlike cytotoxic T cells and T helper cells, it is the T regulatory cells that are reported to be particularly sensitive to undergo cell death accompanied by CD62L shedding in response to ATP [176, 177]. As it is well documented that ATP triggers P2X7 receptor-dependent CD62L shedding in lymphocytes [25, 178, 179] and because it is also known that the release of CD62L from T cells is associated with T cell activation [180], the involvement of this P2 receptor subtype in T cell responses could be suggested. Indeed, in murine T cells, also Aswad et al. reported that ATP induces the release of CD62L but then associated to the initiation of T cell death [176]. The extent to one or another of both actions depends on the level of P2X7 receptor cell surface expression in the T cell subtype; CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells are particularly sensitive to ATP-induced cell death and this high sensitivity correlates with a high expression level of the P2X7 receptor. T helper cells showed intermediate sensitivity to ATP, whereas cytotoxic T cells are the least sensitive and showed the lowest expression of the P2X7 receptor [177].

Although P2X7 receptors have indeed a strong proapoptotic and/or necrotic activity, it can also support cell growth. Baricordi et al. proposed the operation of an ATP-

based autocrine/paracrine loop that supports lymphoid cell growth in the absence of serum-derived growth factors [181]. Adinolfi et al. described a mechanism that helps to understand the dual effect of P2X7 receptor stimulation on cell growth and cell death; the authors showed that expression and basal activation of the P2X7 receptor modulates mitochondrial ion homeostasis and increases cellular energy stores which correlates with a better survival of P2X7 receptor transfectants in the absence of growth factors. Contrariwise, a strong stimulation of the P2X7 receptor causes a dramatic overflow of basal mitochondrial Ca<sup>2+</sup> levels, a resting mitochondrial potential collapse, and extensive fragmentation of the mitochondrial network, resulting in cell death [182].

The only P2Y receptor for which functional data are available in lymphocytes is P2Y<sub>14</sub>; in T cells UDP, glucose induces significant inhibition of forskolin-stimulated cAMP accumulation via P2Y<sub>14</sub> receptors [154].

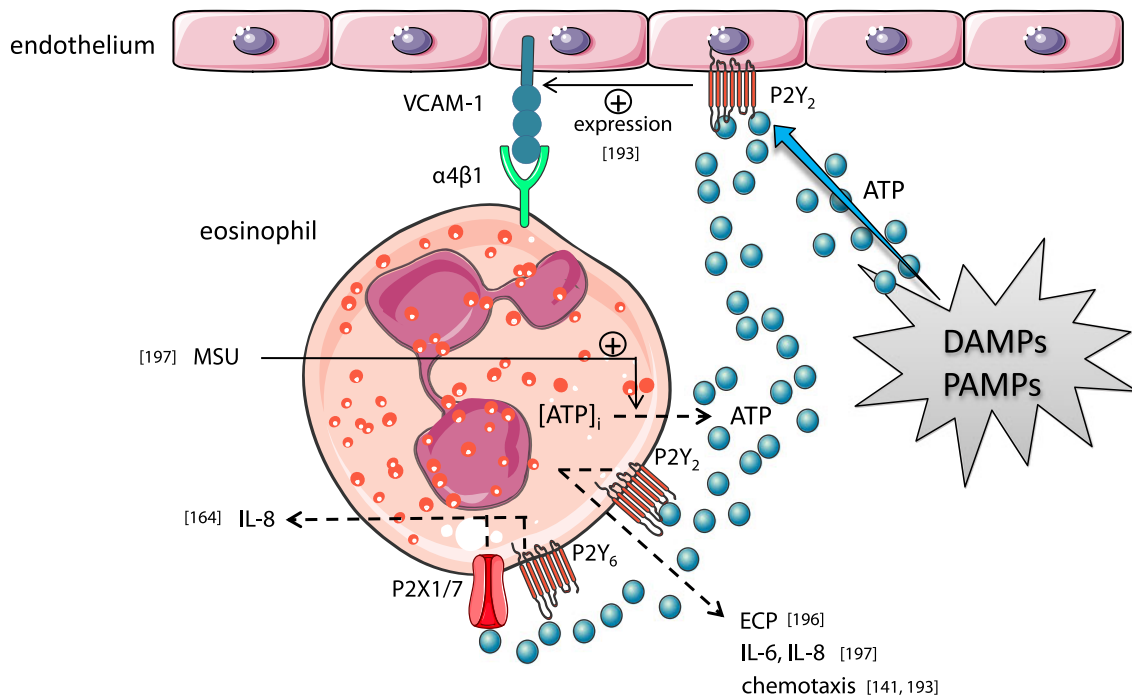
### NK cells

NK cells are reported to express P2X1, P2X4-7, and all P2Y receptor subtypes on mRNA level [183]. Schmidt et al. were the first to study the functional effect of ATP on NK cells showing that the treatment of human NK cells with ATP resulted in a strong inhibition of their cytotoxic activity on K562 target cells [184], a result that was corroborated by others [185, 186]. Miller et al. equally confirmed that ATP and related adenine nucleotides play a role in decreased NK cell activity [187] but in contrast to the previous studies, the authors reported that this was in fact due to an inhibition of proliferation because despite a reduced thymidine incorporation induced by extracellular adenine nucleotides, NK cells maintained their capacity to lyse K562 cells in their experiments [187]. Extracellular ATP furthermore regulates NK cell cytotoxicity via P2Y<sub>11</sub> receptor activation; NK cells stimulated with ATP increased chemokinesis and inhibited NK chemotaxis in response to CX3CL1 chemokine [183].

In contrast to the reduced cytotoxic activity of NK cells by ATP in the reports above, Beldi et al. described limited tissue damage mediated by NK cells during an experimental model of hepatic ischemia/reperfusion injury in CD39-null mice as a consequence of increased peri-cellular ATP levels on NK cells [188]. As these peri-cellular ATP levels are required for regulated IFN- $\gamma$  secretion by NK cells, alterations in the levels of ATP by CD39 deletions therefore modulate tissue injury. Indeed, IFN- $\gamma$  secretion by NK cells in response to interleukin-12 and interleukin-18 was inhibited by ATP $\gamma$ S in vitro [188].

### Eosinophils

Eosinophils (Fig. 5) are granulocytic leukocytes implicated in the pathogenesis of diverse inflammatory processes such



**Fig. 5** Human eosinophils release eosinophil cationic protein (*ECP*) [196] IL-6 and IL-8 [197] by P2Y<sub>2</sub> receptor stimulation, while P2X<sub>1</sub>, P2X<sub>7</sub>, and P2Y<sub>6</sub> receptors might also be implicated in the release of IL-8 [196]. The P2Y<sub>2</sub> receptor subtype expressed on eosinophils is also suggested to be implicated in eosinophil chemotaxis [141, 193]. Also endothelial P2Y<sub>2</sub> receptors can affect eosinophil migration by regulating the expression of endothelial VCAM-1 which mediates migration

and adhesion of eosinophils via the α4β1 integrin [193]. Monosodium urate (*MSU*) crystals induce the release of autocrine ATP from eosinophils which provides a pivotal positive feedback signal via P2 receptors on eosinophils [197]. *DAMP* damage-associated molecular patterns, *PAMP* pathogen-associated molecular patterns, *VCAM-1* vascular cell adhesion molecule 1. Values between brackets in the figure represent reference numbers

as parasitic, bacterial and viral infections, tissue injury, tumor immunity, and allergic diseases. In response to a set of diverse stimuli, eosinophils are recruited from the circulation into inflammatory foci where they modulate immune responses [189].

The first notion for a functional effect of purines on eosinophils comes from experiments showing that the ATP released from the granules of platelets can activate eosinophilic granulocytes and induces chemotaxis, a mechanism mimicked by exogenously added ATP [190].

To date human eosinophils are reported to express mRNA for P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>14</sub>, P2X<sub>1</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> purinergic receptor subtypes [10, 19, 191, 192] (Table 1).

Both the P2X and P2Y receptor family members are implicated in eosinophil chemotaxis, but P2Y receptors are reported to be more potent than P2X receptors in doing so [192]. Especially, the purinergic P2Y<sub>2</sub> receptor subtype stood recently in the spotlights as it was reported to be a potential therapeutic target for the treatment of asthma because of its implication in the chemotaxis of dendritic cells and eosinophils in allergic lung inflammation [141, 193]. The functional expression of P2Y<sub>2</sub> receptors in human and murine eosinophils revealed that the migratory response to

ATP was absent in eosinophils derived from P2Y<sub>2</sub>-deficient animals. The hypothesis that P2Y<sub>2</sub> receptor signaling might contribute to the pathogenesis of asthma was further supported by the observation that eosinophils from asthmatic subjects showed a higher expression of P2Y<sub>2</sub> receptor compared with healthy individuals [141]. At the same time, also Anderstocken et al. demonstrated that allergic eosinophilic lung inflammation is decreased in animals lacking the P2Y<sub>2</sub> receptor [193]. The authors observed that eosinophil accumulation was defective in OVA-challenged P2Y<sub>2</sub>-deficient mice compared with OVA-treated wild type animals. In contrast, comparable infiltration of macrophages and neutrophils in the BALF of LPS-aerosolized P2Y<sub>2</sub><sup>+/+</sup> and P2Y<sub>2</sub><sup>-/-</sup> mice were observed. In the same study, the P2Y<sub>2</sub> receptor was defined as a regulator of endothelial membrane and soluble forms of VCAM-1 mediating the adhesion and migration of eosinophils that express the α4β1 integrin which binds to VCAM-1 [193].

Besides its implication in chemotaxis, ATP has been shown to be involved in the induction of ROS production, CD11b upregulation, and cytokine production in eosinophils [19, 141, 192, 194, 195]. These effects occur through Ca<sup>2+</sup> mobilization [19, 194, 195]. ATP can either interact directly with P2X ion channels or indirectly activate

transmembranous  $\text{Ca}^{2+}$  fluxes following G protein activation. ATP is reported to induce actin polymerization and production of ROS via pertussis toxin-sensitive Gi proteins (P2Y) in eosinophils, while the upregulation of integrin CD11b by ATP occurs directly via  $\text{Ca}^{2+}$  transients (P2X) [194] although the latter has also been reported to occur equipotently by both P2Y and P2X receptor agonists [192].

With regard to the release of pro-inflammatory mediators, human eosinophils release eosinophil cationic protein (ECP) and IL-8 by P2 receptor-stimulation; the release of ECP was triggered by ATP, UTP, and UDP via a member of the P2Y receptor family, possibly P2Y<sub>2</sub> while IL-8 release is triggered by UDP, ATP,  $\alpha,\beta$ -meATP, and BzATP mediated by P2X<sub>1</sub>, P2X<sub>7</sub>, and P2Y<sub>6</sub> receptor subtypes [196]. Eosinophils cultured with monosodium urate (MSU) crystals also produce cytokines and chemokines; Kobayashi et al. found that MSU crystals induce the release of autocrine ATP from eosinophils which provides a pivotal positive feedback signal in eosinophils to release cytokines and chemokines (including IL-8, IL-6, CCL2-4, IL-1 $\beta$ , IL-10, IL-17, IFN- $\gamma$ , and TGF- $\beta$ ) via P2Y<sub>2</sub> receptors [197]. Others reported P2Y<sub>6</sub> receptors to mediate MSU-induced inflammation in both keratinocyte and monocytes and P2Y<sub>6</sub> antagonists to suppress neutrophil infiltration in both mouse air pouch and peritonitis models [198]. They also demonstrated MSU-induced inflammatory cytokine and chemokine production (including IL-1 $\alpha$ , IL-8, IL-6, IL-18, and to a lesser extent TNF- $\alpha$  and IL-1 $\beta$ ) by keratinocytes and monocytes. In contrast to the study of Kobayashi et al. [197], the authors could not confirm a MSU-induced release of ATP to be the mechanism of action explaining the involvement of the P2Y<sub>6</sub> receptors in the responses described in their study [198].

### Mast cells

Mast cells (Fig. 6) are key components of allergic and anaphylactic reactions. When mast cells are activated by allergens binding to IgE antibodies attached to Fc $\epsilon$ RI receptors, they produce cytokines, chemokines, leukotrienes, and eicosanoids and release secretory granules. Mast cells are also increasingly regarded to be involved in chronic inflammatory diseases during which they can be activated or modulated by diverse nonallergic triggers such as neuropeptides and cytokines [199, 200].

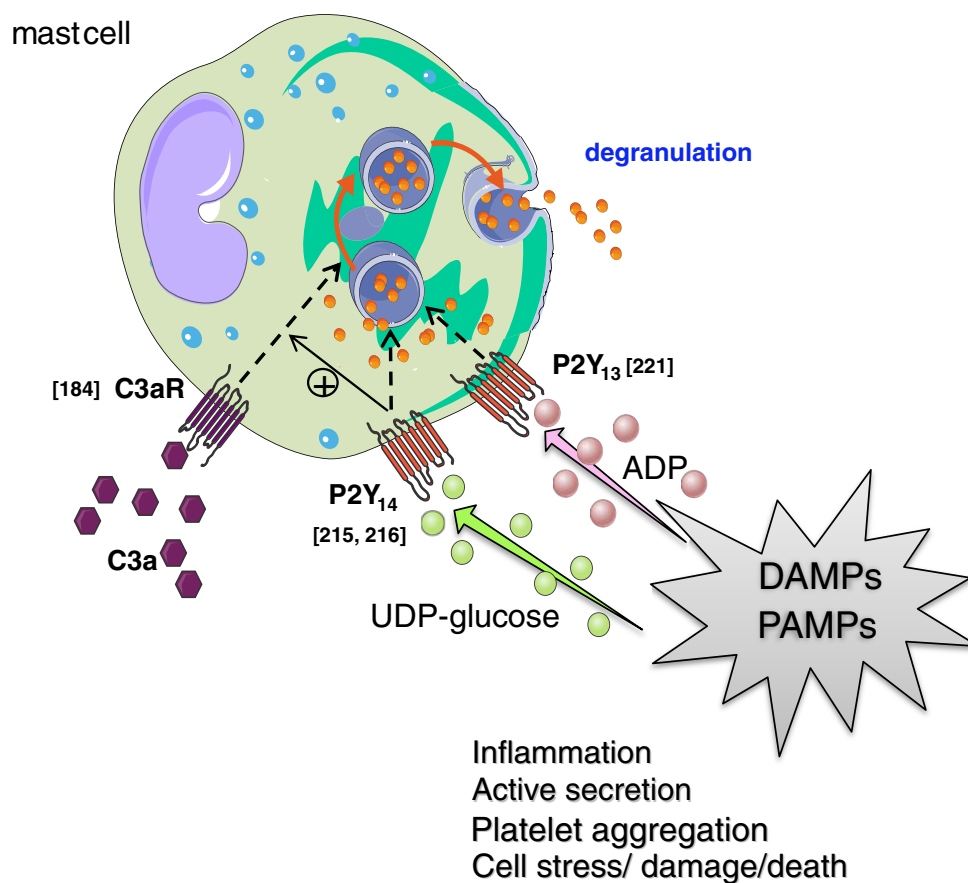
In the presence of  $\text{Ca}^{2+}$ , ATP is known to directly induce responses [199, 201–207] and/or modulate responses induced by other mediators such as compound 48/80 [208], anti-IgE [200], and ionophore A23187 [209–211] in mast cells, resulting in the release of histamine [199–204, 208, 209, 211, 212], PGD<sub>2</sub> [199] or phosphorylated metabolites and nucleotides [206, 207]. However, for most of above observations, no P2 receptor-mediated mechanisms were

reported; the events were described to be associated to either  $\text{Ca}^{2+}$ -dependent exocytotic degranulation [206, 210], permeability changes [199, 205–207], the metabolic state of the cells [202, 209–212], or morphological changes such as cell swelling [204].

The number of studies that investigated P2 receptor expression on mRNA and protein levels in human mast cells is scarce. Human lung mast cells (HLMC) were reported to express mRNA transcripts for P2X<sub>1</sub> and P2X<sub>4</sub> but not for P2X<sub>2</sub>, P2X<sub>3</sub>, and P2X<sub>5</sub>–P2X<sub>7</sub> [213] in a study that did not investigate the P2Y receptor family, while others found that of the 3 P2 receptor transcripts assessed in the study, P2Y<sub>1</sub> and P2Y<sub>2</sub> but not P2X<sub>7</sub> were expressed [200]. In contrast to the absence of the P2X<sub>7</sub> receptor in above mentioned studies, Wareham et al. reported its presence in HLMC next to P2X<sub>1</sub> and P2X<sub>4</sub> [214] (Table 1). Also, the human mast cell line HMC-1 was shown to highly express the P2X<sub>7</sub> receptor being further upregulated upon PMA (phorbol 12-myristate 13-acetate) or ionomycin stimulation [200], while Wareham et al. also confirmed the presence of P2X<sub>7</sub> receptors next to P2X<sub>1</sub>, P2X<sub>4</sub>, and P2X<sub>6</sub> in the LAD2 human cell line [214]. Among the P2Y receptors, the presence of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> subtypes were shown by RT-PCR on cord blood-derived human mast cells (hMCs) [23]. Recently, the presence of all P2Y receptors in mast cells was reported including P2Y<sub>4</sub> and P2Y<sub>14</sub> receptors as was shown in both murine RBL-2H3 and human LAD2 cell lines, the former being most highly expressed together with the P2Y<sub>11</sub> receptor [215, 216].

The application of ATP is known to evoke inward currents in the mast cells [214], yielding an increase in intracellular  $\text{Ca}^{2+}$  which is necessary for IgE-mediated and/or ATP-mediated/potentiated mediator release from mast cells [23, 195, 200, 202, 213, 217–220]. The first paper functionally linking P2 receptors to  $\text{Ca}^{2+}$ -dependent mediator release by mast cells was the work from Schulman et al. who reported that adenine nucleotides consistently enhanced anti-IgE-induced histamine release with a rank order of potency of ATP > 2mSATP >  $\alpha\beta$ meATP >  $\beta\gamma$ meATP [200]. Schulman et al. confirmed the presence of P2Y<sub>1</sub> and P2Y<sub>2</sub>, but not P2X<sub>7</sub> transcript in HLMC's as discussed above. Recent studies indicate that P2Y<sub>13</sub> and P2Y<sub>14</sub> might also be involved in mast cell degranulation, since these receptors induce  $\beta$ -hexosaminidase release in RBL-2H3 rat mast cells [215, 221] while P2Y<sub>14</sub> was also reported to potentiate C3a-induced  $\beta$ -hexosaminidase release in human LAD2 cells [216] (Table 1). Triggering of intracellular  $\text{Ca}^{2+}$  signaling in mast cells is also linked to cytokine expression and release. ADP and ATP inhibited the generation of TNF- $\alpha$  in response to the TLR2 ligand peptidoglycan, and blocked the production of TNF- $\gamma$ , IL-8, and MIP-1 $\beta$  in response to leukotriene D4 via P2Y receptors in hMCs for which the presence of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and

**Fig. 6** P2Y<sub>13</sub> and P2Y<sub>14</sub> receptor-activation directly induces mast cell degranulation [215, 221] while P2Y<sub>14</sub> receptors also potentiate C3a-induced degranulation [216]. DAMP damage-associated molecular patterns, PAMP pathogen-associated molecular patterns, C3a complement 3a, C3aR complement 3a receptor. Values between brackets in the figure represent reference numbers



P2Y<sub>13</sub> subtypes was confirmed on mRNA level [23]. Finally, ATP has also been reported to be liberated from mast cells itself which could diffuse to elicit responses in distant cells; this ATP release from mast cells is triggered either by FcεR cross-linking, by extracellular nucleotide application, or by mechanic stimulation and is independent on gap-junctional coupling [222, 223].

Adenine nucleotides have also been reported to participate in the recruitment of mast cells, an event where also a role for Ca<sup>2+</sup> influx has been suggested in the process; ADP, ATP, and UTP are indeed reported to be effective chemoattractants for rat-bone-marrow-cultured mast cells [116].

### Therapeutic potential of P2 receptor targeting

While this review provides an overview of purinergic receptors implicated in various functions of different immune cells, based on the currently explored therapeutic interventions we may suggest that especially the P2X<sub>7</sub> receptor may have an important mechanistic role in inflammation. Indeed, several P2X<sub>7</sub> antagonists are found to be promising tools in the fight against chronic inflammatory conditions such as Crohn's disease, rheumatoid arthritis, psoriasis, allergic dermatitis, chronic pulmonary disease, and asthma [224–226]. Albeit not directly affecting inflammatory cells, also the

P2Y<sub>2</sub> receptor agonists Denufzol is currently under investigation in the treatment of cystic fibrosis (CF), improving lung function in CF patients with mildly impaired lung function by enhancing airway hydration and mucociliary clearance, and thus indirectly reducing the susceptibility to infection, inflammation and the resultant progressive airway damage [227].

While a possible therapeutic potential of other P2 receptor subtype selective drugs in inflammatory conditions cannot be excluded, more studies are required to confirm the importance of these receptors. Here, we discuss a few ideas regarding potential therapeutic possibilities on which was speculated in the literature: as Vaughan et al. [35] reported that ATP causes a substantial delay in neutrophil apoptosis which is mediated via P2Y<sub>11</sub> receptors, specific antagonizing P2Y<sub>11</sub> receptors could reduce the injurious effects of increased neutrophil longevity induced by ATP during neutrophilic inflammation while retaining their key immune functions such as chemotaxis and phagocytosis which are primary mediated via P2Y<sub>2</sub> receptors (see paragraph on neutrophils). The P2Y<sub>11</sub> receptor could thus be a novel potential therapeutic target for the amelioration of neutrophilic inflammation in a wide range of inflammatory diseases [35]. Another possible mechanism of action that involves to the inhibition of P2Y<sub>11</sub> receptors relates to DC migration; the observation that extracellular ATP at sites of

inflammation inhibits the migration of local DCs via P2Y<sub>11</sub> [125] makes P2Y<sub>11</sub> receptor-inhibition a strategy to improve the migration of antigen-loaded DCs from the vaccination site to lymph nodes as a result of a prolonged time of antigen encounter [125]. As also the P2Y<sub>2</sub> receptor appears to be involved in ATP-triggered migration of DCs and eosinophils, the latter showing increased expression of P2Y<sub>2</sub> in allergic asthmatics [141], P2Y<sub>2</sub> receptor targeting could also represent another potential therapeutic option for the treatment of allergic asthma and other allergic inflammatory conditions.

Besides inflammatory conditions, there is also an interest in the therapeutic potential of purinergic compounds in a wide range of other pathologies [228] including atherosclerotic vascular diseases [229], cancer [230, 231], ocular problems [232], lower urinary tract dysfunction [233], and neurodegenerative disorders including amyotrophic lateral sclerosis, multiple sclerosis and Alzheimer's disease [225]. To date only two P2 receptor antagonist are marketed: the irreversible P2Y<sub>12</sub> receptor antagonist clopidogrel (Plavix®), which is used as an antithrombotic agent in patients with atherosclerotic vascular disease [234] and another orally active but reversible antagonist of P2Y<sub>12</sub>, Ticagrelor (Brilinta®), which showed to even have a greater efficacy in reducing cardiovascular death compared with clopidogrel [235].

The diversification and the introduction of new clinical targets will intensify the ongoing effort to design more selective agonists and antagonists for the P2 receptors, both as pharmacological tools and as potential therapeutic agents [236, 237].

## Conclusions

Extracellular nucleotides are important players in regulating inflammatory responses through binding to purinergic P2 receptors present on various inflammatory cell types. All immune cells express a wide range of P2 receptors which modulate a diverse set of responses. Among the different P2 receptors expressed, it can be generally concluded that particularly the P2X<sub>4</sub>, P2X<sub>7</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub> receptor subtypes come into the picture when analyzing the effects of purines in immune responses. However, the picture of P2 receptors is still not final and further research is needed in order to gain a more complete overview of their role in inflammation. The recent development of P2 receptor-targeting drugs has provided potential candidates as therapeutics in inflammatory diseases. While currently several selective P2 receptor ligands are available, much more work is needed to fully cover the wide range of P2X and P2Y receptor subtypes with selective agonists and antagonists.

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