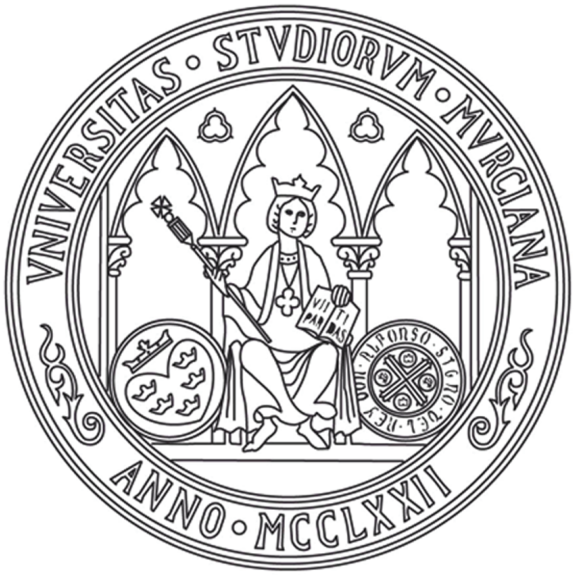

UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Functional Characterization of Purinergic Receptors in the Differentiation and Activation of Macrophages

Caracterización Funcional de Receptores Purinérgicos en la Diferenciación y Activación de Macrófagos

Dña. Maria Barberá Cremades

2015

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Index

List of figures

List of tables

Introduction

1. Purinergic signaling

1.1. Historic perspective of purinergic signaling pathways

Back in 1929, Drury and Szent-Gyorgyi described for first time the potent actions of the extracellular application of adenine compounds on the heart: they slow the rate of beating, impair conduction from auricle to ventricle, and arrest experimentally produced auricular fibrillation; they shorten the absolute refractory period of and improve slowed conduction in the auricle due to high rates of beating; they also dilate the coronary vessels. All these actions has as final symptom the lower general arterial pressure, this is due in part to the cardiac slowing and in part to a general arterial dilatation (Drury and Szent-Györgyi, 1929). Four decades later, adenosine triphosphate (ATP) was proposed by Burnstock as the transmitter responsible for non-adrenergic, non-cholinergic transmission in the gut and bladder (Burnstock et al., 1970). Was also Burnstock who introduced in 1972 the concept of purinergic signaling (Burnstock, 1972), in which extracellular purines (most notably ATP and adenosine) act as extracellular signaling molecules. Now we know that both, pyrimidine and purine nucleotides, are released from cells through several physiologically relevant mechanisms, including diffusion through membrane hemichannels, activation of membrane transporters and vesicular exocytosis

(Abbracchio et al., 2009; Burnstock, 2007a; North and Verkhratsky, 2006; Pankratov et al., 2006). In addition, purines and pyrimidines are released from dying cells: this being an early indicator for cell damage (Burnstock, 2007a; Burnstock, 2008). Upon release, ATP (and other nucleotides) are enzymatically degraded within seconds by an extended family of ectonucleotidases (Zimmermann, 2006). This process is physiologically relevant as ATP metabolites are also agonists of different purinergic receptors (Figure 1). These purinergic signaling molecules activate two receptor classes: (i) metabotropic P1 receptors, which are activated by adenosine; and (ii) the P2 family of nucleotide receptors which are subdivided into metabotropic P2Y and ionotropic P2X sub-classes (Figure 1) (Abbracchio et al., 2006; Abbracchio et al., 2009; Burnstock, 1976; Burnstock, 2007b; Burnstock and Kennedy, 1985; Ralevic and Burnstock, 1998).

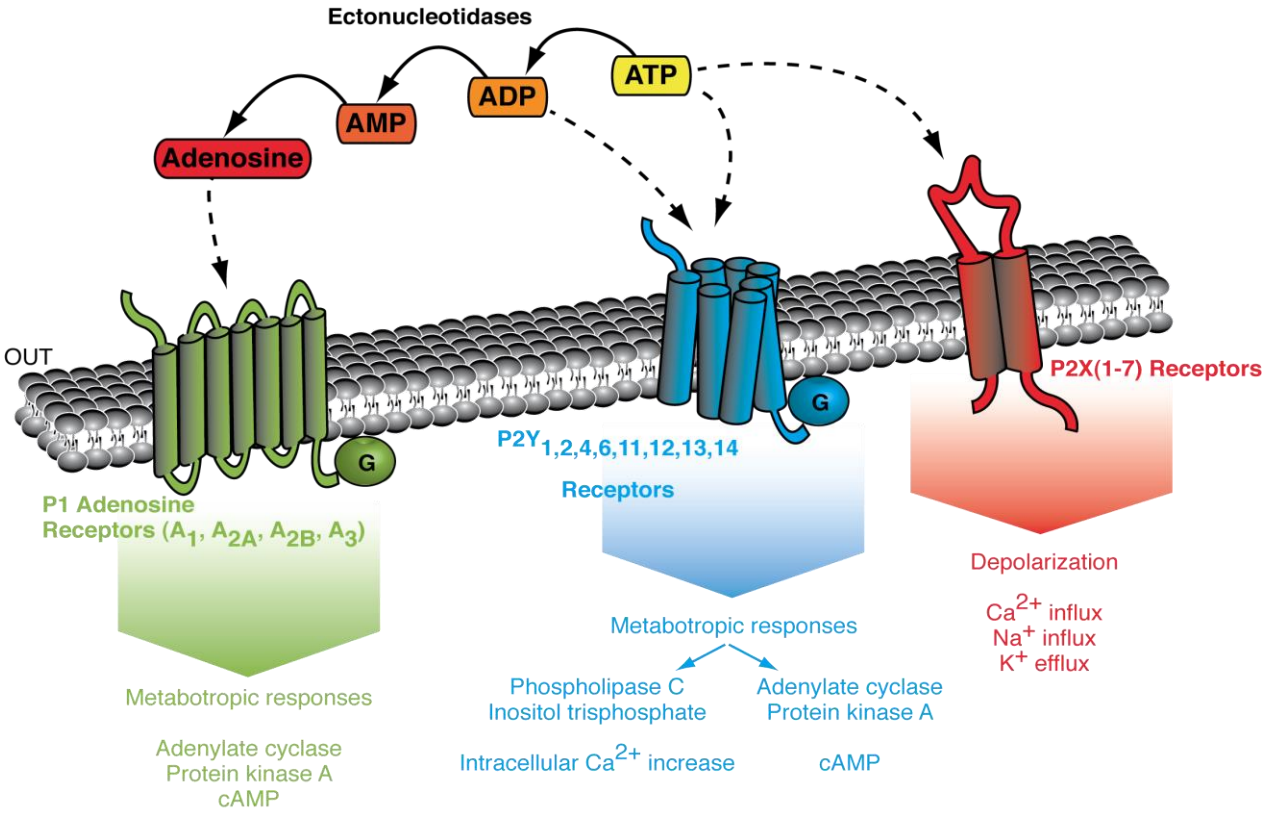

Figure 1. Simplified illustration of extracellular purinergic signaling mediated by ATP and other nucleotides. These purinergic signaling molecules activate metabotropic P1 receptors, activated by adenosine, and the P2 family of nucleotide receptors, with the sub-classes of P2Y metabotropic and P2X ionotropic receptors (ilustration adapted from (Baroja-Mazo et al., 2013b).

There are analogues of purinergic receptors in ancestral green algae and early in the fungi lineage (Fountain and Burnstock, 2009; Fountain et al., 2007), therefore the purinergic signaling is a primitive system probably because ATP is an ancient and fundamentally important biological molecule. Purinergic signaling is implicated in many neuronal and non-neuronal mechanisms, including exocrine and endocrine secretion, immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation (Burnstock, 2006; Burnstock and Knight, 2004). In addition, purinergic signaling processs are implicated in mediating cell proliferation, differentiation and death (Abbracchio and Burnstock, 1998; Burnstock, 2002). ATP-mediated signaling has been identified in virtually all tissues and cell types and appears to be the most widespread and omnipresent of all known extracellular signaling molecules.

In this Thesis, purine and pyrimidine nucleotides are studied as ligands of purinergic receptors, however they also serve as structural basis for RNA and ADN moleculas and to carry packets of energy within the cell in the form of the nucleoside triphosphates as ATP, playing a central role in cell metabolism. A nucleotide is made of a nucleobase, a five-carbon sugar, ribose or 2-deoxyribose (depending on if it is RNA or DNA respectively), and one or more phosphate groups. Without the phosphate group, the nucleobase and sugar compose a nucleoside (Alberts et al., 2002b).

1.2. P1 or adenosine receptors

The P1 class of purinergic receptor was first described in 1989, there are four adenosine receptors among vertebrates, which have been denoted: A_1, A_{2A}, A_{2B} and A_3 (Figure 1) (Cobb et al., 2003; Fredholm et al., 2001; Libert et al., 1989; Olah and Stiles, 2000; Yaar et al., 2005; Zhou et al., 1992). The nomenclature used in this Thesis follows the Alexander et al. guide (Alexander et al., 2011). In common with other G protein-coupled receptors, they contain seven transmembrane (TM) domains of approximately 21 to 28 hydrophobic amino acids composing an α-helix structure. The amino-terminus of the protein is present on the extracellular side of the plasma membrane, whilst the protein's

carboxy-terminus lies on the cytoplasmic side of the membrane. A pocket for the ligand-binding site is formed by the three-dimensional arrangement of the α-helical TM domains, and the agonist is believed to bind within the upper half of this pore (Ralevic and Burnstock, 1998). Binding of the agonist to the receptor gives rise to a stable configuration as has been shown by x-ray crystallography with the A_{2A} receptor (Xu et al., 2011). The TM domains are connected by three extracellular and three cytoplasmic hydrophilic loops (Ralevic and Burnstock, 1998). P1 receptors are coupled to adenylate cyclase signaling pathways in which A_1 and A_3 receptor activation have inhibitory effects on adenylate cyclase through the $G_{i/o}$ protein α-subunits. Activation of the A_{2A} and A_{2B} receptors stimulates the production of cyclic adenosine monophosphate (cAMP) *via* adenylate cyclase through the G_s protein (Abbracchio et al., 2009; Abbracchio and Ceruti, 2007; Fredholm et al., 2001; Reshkin et al., 2000).

A number of selective agonists and antagonists have been identified for several of the P1 adenosine receptor subtypes including agonists for the A_1, A_{2A} and A_{2B} receptors, and antagonists for the A_1, A_{2B} and A_3 receptors (Ralevic and Burnstock, 1998). One of the most used adenosine receptor agonist is the 5'-N-ethylcarboxamidoadenosine (NECA) which is a highly potent and general adenosine receptor agonist (Fig. 1.5-5A).

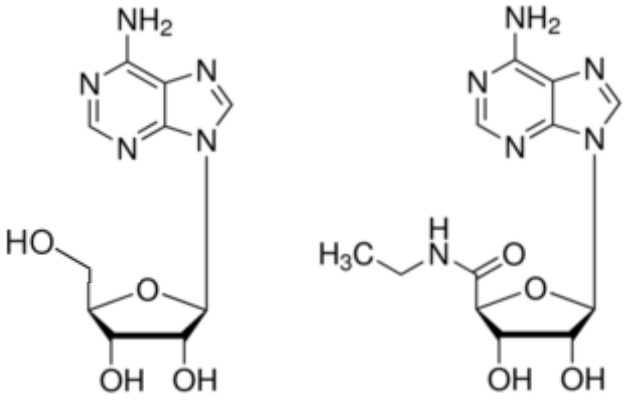

Figure 2. Chemical structure of adenosine (left) and 5'-N-ethylcarboxamidoadenosine (NECA, right), a high affinity P1 adenosine receptor agonist.

1.3. Purinergic P2Y receptors

P2Y receptors mediate an astounding array of biological functions including platelet aggregation, immune regulation, regulation of ion fluxes in airway epithelia and smooth muscle cell proliferation (Burnstock and Williams, 2000). The first P2Y receptor was cloned in 1993 (Lustig et al., 1993; Webb et al., 1993), and since then several other subtypes have been isolated. Eight human P2Y receptor subtypes have so far been identified, the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (Figure 1 and Table 1). The missing numbers in the nomenclature of P2Y receptors represent either non-mammalian orthologs or receptors having some sequence homology to P2Y receptors but for which there is no functional evidence of activation by nucleotides (Abbracchio et al., 2003; Abbracchio et al., 2006).

Molecular studies have shed light upon the mechanisms of receptor activation. Site-directed mutagenesis of $P2Y_1$ and $P2Y_2$ receptors has shown that some positively charged residues in TM3, TM6 and TM7 are crucial for receptor activation by nucleotides (Erb et al., 1995; Jiang et al., 1997). Furthermore, four cysteine residues in the extracellular loops, which are conserved in P2Y receptors, are known to be essential for proper trafficking of the receptor to the cell surface (Hoffmann et al., 1999). The various P2Y receptors are functionally coupled to distinct G proteins and therefore their downstream signaling varies among subtypes. $P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_4$, $P2Y_6$R and $P2Y_{11}$ receptors principally use $G_{q/11}$ family of G proteins to activate the phospholipase C/inositol trisphosphate (PLC/IP_3) pathway and release intracellular calcium, the $P2Y_{11}$ receptor can also bind to G_s family. On the other hand $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors almost exclusively couple to members of the G_i/G_o family of G proteins and activate the cAMP as second messenger system. (Abbracchio et al., 2006).

The lack of potent and selective agonists and antagonists for many of the P2Y receptor subtypes has limited research in this field to focus upon studying $P2Y_1$, $P2Y_4$, $P2Y_{13}$ and $P2Y_{14}$ receptors. The most striking difference between the P2Y receptor subtypes is their ability to use different natural nucleotides as agonists (Figure 3). $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors

preferentially utilize adenosine diphosphate (ADP), whilst $P2Y_{11}$ receptor has a high affinity for ATP. $P2Y_2$ and $P2Y_4$ receptor utilize uridine triphosphate (UTP) whilst uridine diphosphate (UDP) is the agonist for $P2Y_6$ receptor. Finally UDP-sugars activate $P2Y_{14}$ receptor (Table 1) (Abbracchio et al., 2006). Suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) are non-selective antagonists of the P2Y receptors, but also antagonize the P2X receptors (see later section 1.4). The PLC inhibitor, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122), is commonly used to block the intracellular calcium rise associated with P2Y receptor mediated downstream signaling (von Kügelgen, 2006). Recently, selective antagonists for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors have been developed by the pharmaceutical industry to treat platelet aggregation (Abbracchio et al., 2006; Hoffmann et al., 2009).

RECEPTOR	LIGAND	SIGNALING	TISSUE DISTRIBUTION
$P2Y_1$	ADP	PLC↑ Ca^{2+}↑ PKC↑	Wide, including platelets, brain, epithelial and endothelial cells, immune cells, osteoclasts
$P2Y_2$	ATP/UTP	PLC↑ Ca^{2+}↑ PKC↑	Wide, including immune cells, epithelial and endothelial cells, kidney, brain, osteoclasts
$P2Y_4$	UTP	PLC↑ Ca^{2+}↑ PKC↑	Placenta, lung, endothelial cells
$P2Y_6$	UDP	PLC↑ Ca^{2+}↑ PKC↑	Wide, including lung, brain, T-cells, placenta, spleen
$P2Y_{11}$	ATP	PLC↑ Ca^{2+}↑ PKC↑ AC↓ cAMP↓	Spleen, intestine, dendritic cells, graunolocytes
$P2Y_{12}$	ADP	AC↓ cAMP↓	Platelets, brain
$P2Y_{13}$	ADP	AC↓ cAMP↓	Brain, spleen, lymph nodes, bone marrow
$P2Y_{14}$	UDP-glucose	AC↓ cAMP↓	Wide, including brain, adipose tissue, placenta, intestine, mast cells, hematopoietic cells

Table 1. P2Y receptor family characterization and tissue distribution (Abbracchio et al., 2006). Abbreviations: PKC, protein kinase; AC, adenylate cyclase.

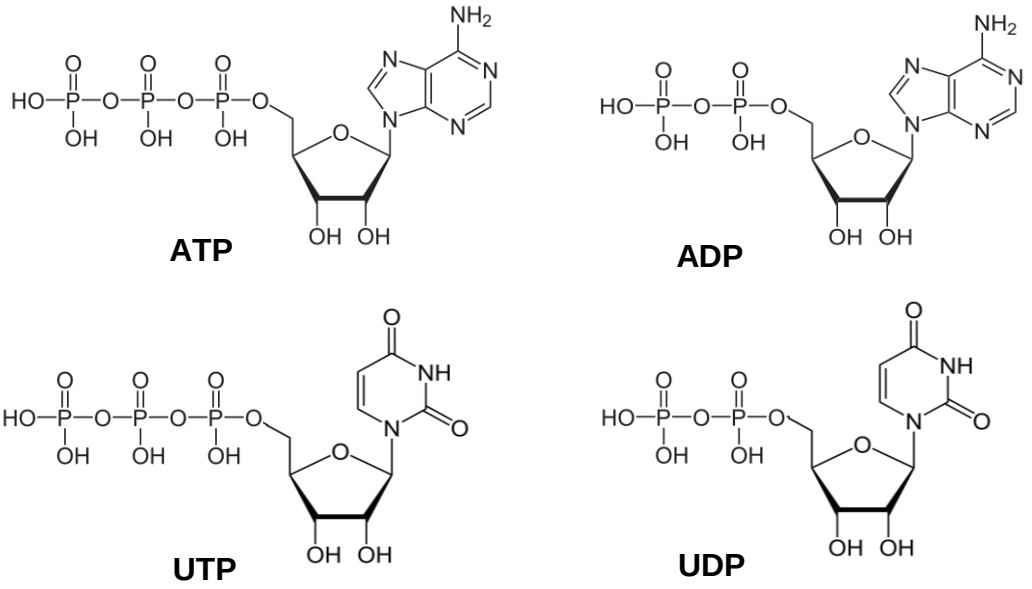

Figure 3. Chemical structure of the main agonists of P2Y receptors (ATP, ADP, UTP and UDP).

1.4. Purinergic P2X receptors

The ionotropic P2X receptors are ATP-gated non-selective cation channels, upon activation they allow passage for Na^+, K^+ and Ca^{2+} ions (North, 2002). The first cDNA encoding a P2X receptor (P2X1) was isolated in 1994 (Brake et al., 1994; Valera et al., 1994) and since then, seven different P2X receptor subunits have been identified (P2X1 to P2X7) (Abbracchio et al., 2009; North, 2002; Surprenant and North, 2009).

The members of this receptor family show conserved subunit topology. They comprise of two TM spanning regions and possess intracellular amino- and carboxy-terminus (Figure 1), which contain consensus-binding motifs for protein kinases. A large extracellular loop exits which contains 10 conserved cysteine residues and is capable of forming a series of disulfide bridges. The first TM is involved in channel gating whilst the second lines the ion pore in the membrane (Egan et al., 2006; Kawate et al., 2009; North, 2002; Roberts et al., 2006; Stojilkovic et al., 2005). P2X receptor subunits form functional homomeric and heteromeric trimers. In comparison to P2Y receptors, the P2X receptors have a significantly lower affinity for ATP but their ability to respond to ATP is influenced by the ionic conditions. In the case of P2X7 receptor (P2X7R),

replacement of Na^+ with K^+ greatly increases the responsiveness to ATP suggesting a physiological role in damaged tissues with altered ionic conditions.

1.4.1. Homomeric P2X receptors

All P2X receptors are able to form functional homomeric subtypes. The P2X1, P2X2, P2X3, P2X4 and P2X7 are the most studied P2X receptors subunits. P2X1 and P2X3 homomeric channels show a fast desensitization in contrast to the homomeric P2X2, P2X4 and P2X7 receptors (P2X4R and P2X7R). Whilest α,β-methylene ATP (αβ-meATP) is a potent agonist for P2X1 and P2X3 receptors, there are no specific ligands for the P2X2 receptor (Coddou et al., 2011; Lynch et al., 1999; Valera et al., 1994). Suramin, PPADS and 2',3'-O-(2,4,6-Trinitrophenyl) (TNP)-ATP can be used as non-selective P2X antagonists (Lewis et al., 1995), but specific antagonists for P2X1 and P2X3 receptors now exist (Coddou et al., 2011; Lynch et al., 1999). Homomeric P2X4Rs are activated by ATP but not by α,βme-ATP. ATP-evoked currents at P2X4R are potentiated by ivermectin, cibacron blue and zinc (Coddou et al., 2011; Soto et al., 1996). Prolonged ATP application of several seconds, to homomeric P2X2 receptor or P2X4R channels results in an increasing permeability to large organic cations such as N-methyl-D-glucamine (NMDG) (Nicke et al., 2005). This increase in permeability is also the main feature of the homomeric P2X7R, inducing the opening of a large pore (aprox 900 Da) that allows relatively large dye molecules, such as ethidium bromide or YO-PRO-1, to enter the cell. Among all the P2X receptors, P2X7R stimulation induces specific structural changes in the cell such as microvesicle shedding, membrane blebbing, activation of MAPK and ultimately cell death (Gan et al., 1998; Roger et al., 2008; Virginio et al., 1999).

A potent agonist for the P2X7R is 2',3'-O-(benzoyl-4-benzoyl)-ATP (BzATP) a modified ribose derivative (Figure 4), that it is also a effective agonist at similar or lower concentrations at other P2X receptors (table 2), but does not activate P2Y receptors, with the exception of $P2Y_{11}$ and $P2Y_{13}$ receptors (Coddou et al., 2011; North, 2002).

RECEPTOR	LIGAND	TISSUE DISTRIBUTION
P2X1	αβ-meATP, ATP	Platelets, cerebellum, smooth muscle
P2X2	ATP	Smooth muscle, brain, retina, pancreas
P2X3	αβ-meATP, ATP	Neurons
P2X4	ATP, BzATP	Wide, including endothelia cells, microglia, testis, colon
P2X5	ATP	Proliferating cells in skin, thymus, heart, bladder, adrenal medulla
P2X7	BzATP, ATP	Macrophages, mast cells, miroglia, pancreas, skin, endocrine organs

Table 2. P2Y receptor family characterization and tissue distribution adapted from (Coddou et al., 2011).

1.4.2. Heteromeric P2X receptors

Heteromeric receptors were first described after the coexpression of different P2X receptor subunits in *Xenopus* oocytes and subsequent functional analysis using voltage patch clamp. Later they have been identified in mammalian cells. The most physiologically relevant of these heteromeric receptors are the P2X1/2, P2X2/3, P2X1/4 and P2X1/5 receptors. P2X1 and P2X2 receptors co-expression yielded a mixed population of homomeric and heteromeric receptors. The heteromeric P2X1/2 ion channels showed a novel sensitivity to extracellular pH (Brown et al., 2002). It has been claimed that trimeric P2X1/2 receptors incorporate one P2X1 and two P2X2 subunits (Aschrafi et al., 2004; Brown et al., 2002; Calvert and Evans, 2004). P2X2/3 heteromeric channels are characterized by a sustained current elicited by αβ-meATP (Calvert and Evans, 2004; Coddou et al., 2011; Spelta et al., 2002). They also share some properties with homomeric P2X2 receptor in that they are potentiated by low pH, and they desensitize slowly (Spelta et al., 2002; Spelta et al., 2003). Voltage clamp experiments revealed functional P2X heteromeric receptors with kinetic properties resembling that of homomeric P2X4R, and a pharmacological profile similar to homomeric P2X1 receptor (Nicke et al., 2005). The defining phenotype of the P2X1/5 heteromer is a sustained current evoked

by αβ-meATP and a higher sensitivity to ATP. This is not seen in the P2X1 and P2X5 homomeric channels (Burnstock, 2007a; Coddou et al., 2011; Surprenant et al., 2000). P2X5 and P2X2 subunits associate in a new heteromeric P2X2/5 receptor, which is endogenously expressed within specific neuronal populations, and displays functional properties that were previously thought to be unique to the P2X7R (Compan et al., 2012).

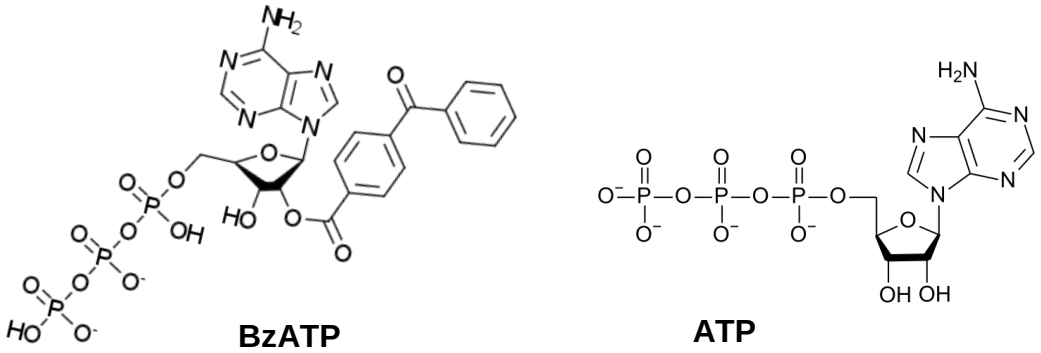

Figure 4. Chemical structure of the main agonists of P2X7R, BzATP (left) and ATP (right).

1.5. P2X7 receptor

P2X7R is one of the main receptors studied in this Thesis. The overall structure of P2X7 is similar to that of the other (ATP)-gated ion channel P2X family members except for the C-terminus. P2X7R present a longer C-terminal sequence (239 amino acids) that is unique among P2X family members and encompasses several other protein and lipid recognition motifs as well as a cysteine-rich domain. This domain also appears to be important for interactions with lipopolysaccharides (LPS) because it includes a conserved LPS-binding domain. *In vitro* experiments revealed that the ability of LPS to activate extracellular kinases is blocked by the presence of peptides derived from this P2X7 domain. These data suggest that the P2X7R C-terminus is able to coordinate events related to signal transduction during LPS activity. Also P2X7R shows an opening mechanism, while the ATP is undocked, the ion channel gate is totally closed, blocking the passage of any ion or even solvent. The initial open gate of P2X7 has a diameter of 7.6 Å, which allows the passage of cations (Costa-Junior et al., 2011; Denlinger et al., 2001; Pelegrin

and Surprenant, 2006). The prolonged stimulation of P2X7R couples to different cell-permeation pathways by oppenning different hemichannels o by dilating P2X7 ion channel pore itself. The binding of ATP to P2X7R opens the ion channel pore within milliseconds. With the time ATP allows the dilation of P2X7 receptor pore to a high conductance state permeable to N-methyl-D-glucamine. This new conformation of P2X7R receptor allows the opening of different hemichannel pores usually measured through dye uptake experiments (reviewed on (Pelegrín, 2011c). Opening of the different permeation pores allows diffusion of molecules from inside the cell to the extracellular space and from the extracellular space to the intracellular space (represented as dotted arrows in the Figure 5). Pannexin-1 channels are partially involved in P2X7 receptor induced cell permeabilization and are responsible for different downstream signaling events, as the activation of the inflammasome and caspase-1, releasing IL-1β in different cell types such as neurons, astrocytes, monocytes and macrophages (Pelegrín, 2011b).

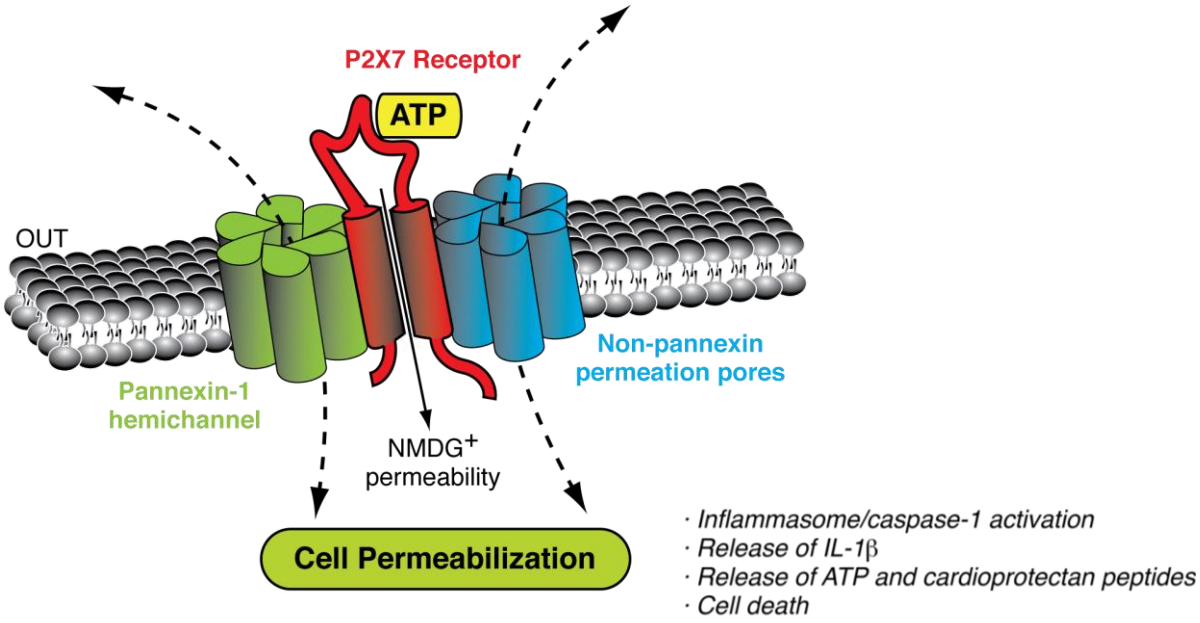

Figure 5. Schematic representation of the protein structure of the P2X7R, the different conformations and the diffusion of molecules through (ilustration adapted from (Baroja-Mazo et al., 2013b).

Due to P2X7R importance in patology, some selective antagonists for P2X7R have been developed by pharmaceutical companies as potential anti-inflammatory drugs (Pelegrin, 2008). In the differents experiments of this Thesis

we used two P2X7R antagonists, the 3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl] pyridine hydrochloride (A438079) and the N-[2-[[2-[(2-Hydroxyethyl)amino] ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7] dec-1-ylacetamide dihydrochloride (AZ10606120, Figure 6).

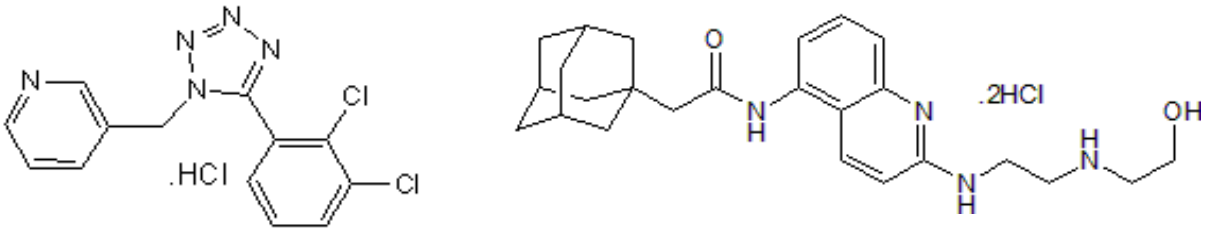

Figure 6. Chemical structure of both main P2X7R antagonists used in this Thesis, A438079 (left) and AZ10606120 (right).

2. The innate immune system

The immune system is the collection of cells, tissues and molecules that protects the body from pathogenic microbes and toxins presents in the environment. The immune sytem is divided into two general types of response: the innate and the adaptive immunity. Each one differs with respect to how quickly it responds and for how long it responds to pathogens, its central effector cell types and its specificity for different classes of microbes (Figure 7). Through contact, ingestion, or inhalation, vertebrate organisms are exposed to millions of potential pathogens and the ability to avoid infections depends on the correct coordinatrion of innate and adaptive immune systems. For example, the adaptive immune responses, are slow to develop on first exposure to a new pathogen, it can take a week or so before the responses are effective, but a bacterium can produce a full-blown infection in a single day. Therefore, during the first critical hours and days of exposure to a new pathogen, the innate immune system plays a central protection role against infection (Alberts et al., 2002a; Chaplin, 2010; Delves and Roitt, 2000).

The innate immune system, also known as the nonspecific immune system, is an important subsystem for immunity that comprises the cells and mechanisms to defend the host from infections. Innate immune cells comprise

populations of white blood cells such as circulating dendritic cells, neutrophils, natural killer cells, monocytes, eosinophils, and basophils, along with tissue-resident mast cells and macrophages (Alberts et al., 2002a). In the last years, important discoveries changed the way of looking at the innate immune system. Features as specificity and memory, the main traits of the adaptive immune system, are now also considered to some extent for innate immunity. Based on this information a new term has been proposed, trained immunity, which refers to the adaptive characteristics that can display the innate immune cells such as natural killer (NK) cells, monocytes and macrophages (Blok et al., 2015; Sun et al., 2014). The discovery of patter recognition receptors (PRRs) has introduced the concept of specificity in innate recognition, although not in the highly specific fashion characterising adaptive immune recognition. The existence of different classes of innate receptors, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs), and retinoic acid-inducible gene I (RIGI) helicases, allows innate immune cells to identify different pathogenic microorganisms based on the recognition of specific pathogen-associated molecular patterns (PAMPs). The cells of the innate immune system, such as monocytes, macrophagues and NK cells recognize and respond to pathogens via the PRRs (Töpfer et al., 2015). The main components of the innate immune system include physical barriers, as epithelial cell layers that express tight cell-cell contacts (tight junctions, cadherin-mediated cell interactions, and others), the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal and genitourinary tracts, and the epithelial cilia that sweep away this mucus layer permitting it to be constantly refreshed after it has been contaminated with inhaled or ingested particles. The innate response also includes soluble proteins and bioactive small molecules that are either constitutively present in biological fluids (such as the complement proteins, defensins, and ficolins) or that are released from cells as they are activated (including cytokines that regulate the function of other cells, chemokines that attract inflammatory leukocytes, inflammatory lipid mediators (eicosanoids), reactive free radical species, and bioactive amines and enzymes that also contribute to tissue inflammation). Lastly, the innate immune system includes membrane bound receptors and cytoplasmic proteins that bind specific molecular patterns

expressed on the surfaces of invading microbes or damage associated molecular patterns (DAMPs). Some aspects of the innate host defenses are constitutively active (such as the mucociliary blanket overlying many epithelia), and others are activated following interactions of host cells or host proteins with chemical structures that are characteristic of invading microbes but that are absent from host cells (Alberts et al., 2002a; Chaplin, 2010).

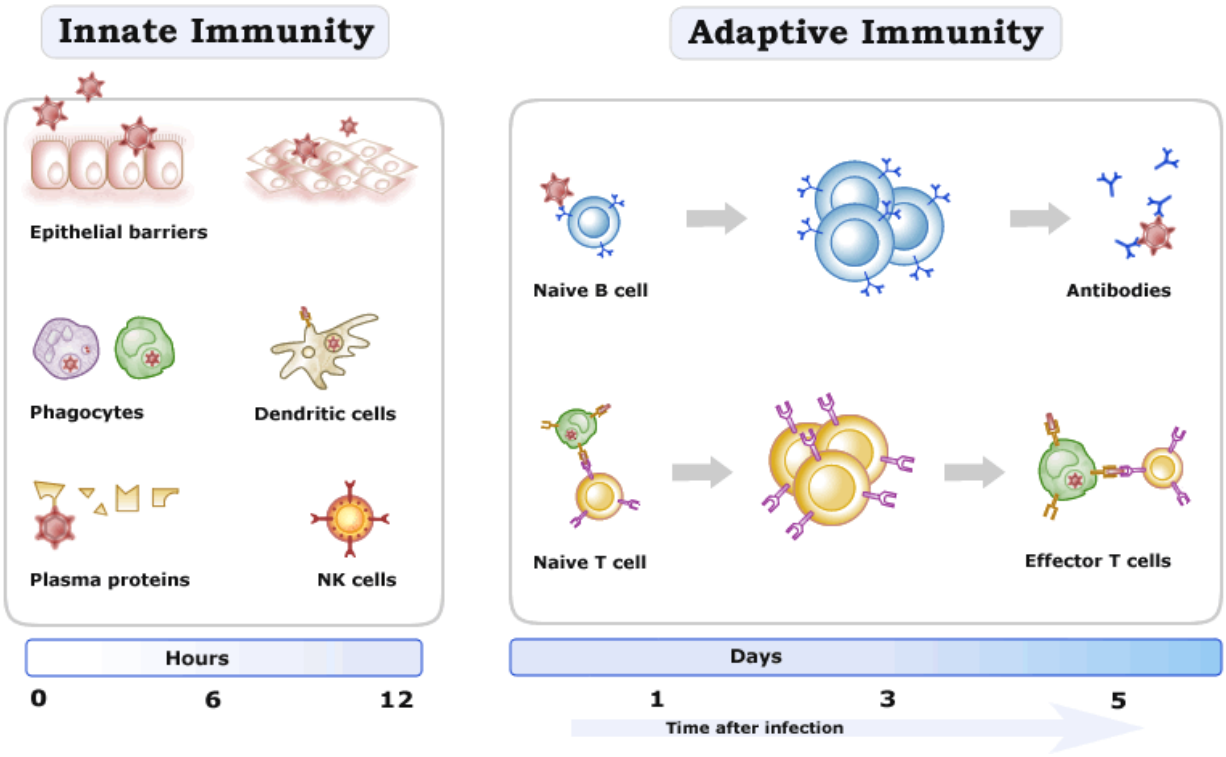

Figure 7. The illustration below is divided into the main components of the innate and adaptive immune systems. Ilustration adapted from Alberts et al., 2002a.

For this Thesis, two major functions of the innate immune system are relevant and will be further describe: the regulatory molecules and the macrophages as effector cells.

2.1. Regulatory molecules of the innate immune response

The innate immune response is regulated by the production of different molecules that serve to coordinate different cells of the innate and adaptive immunity (i.e. cytokines), to sensitize pain receptors (i.e. prostaglandins), to increase vascular permeability and cause vasodilation of the blood vessels (i.e.

leukotrienes) (Montero-Vega, 2012), or to promote healing of damaged tissue (Fibroblast growth factors (FGF) 1 and 2) (Demidova-Rice et al., 2012).

2.1.1. Cytokines

Cytokines are small proteins produced by activated immune cells, including dendritic cells, macrophages, and neutrophils, at the site of infection or injury such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) that promote many different functions, as promoting leukocyte extravasation by increasing the levels of leukocyte adhesion molecules on endothelial cells. Therefore, the synthesis and release of cytokines must be tightly regulated, and temporally orchestrated for the appropriate function of the immune system. Dysregulation of cytokine production leads to different pathologies, including atopic dermatitis asthma (Lambrecht and Hammad, 2013), rheumatoid arthritis (Krasselt et al., 2013), atopic dermatitis (Boguniewicz and Leung, 2011), or monogenic autoinflammatory diseases (Sanchez et al., 2013). However, even with the overwhelming and detailed literature on cytokine actions, just how cytokines are released or secreted by innate immune cells remains a significant “black box” in immunology. Most diagrams in textbooks and reviews show a simple arrow indicating cytokine release from a given cell type after activation of a signaling cascade in response to receptor stimulation. Surprisingly, the precise mechanisms of cytokine trafficking and release remain largely unknown in most cell types (Alberts et al., 2002a; Lacy and Stow, 2011; Newton and Dixit, 2012).

The production and release of cytokines from innate immune cells are critical responses to inflammation and infection in the body. Residing at the frontline of defense in immunity, these cells control opportunistic invasion by a wide range of pathogens, in part by releasing plenty of cytokines and chemokines to communicate with other cells and thereby to orchestrate immune responses. Two major innate cytokines studied in this Thesis are TNF-α and IL-1β, which are the key for the correct activation of the organism immune response (Alberts et al., 2002a; Lacy and Stow, 2011; Newton and Dixit, 2012)

IL-1, one of the first cytokines described back in the 1980s, is a potent mediator of fever, pain and inflammation, and is experiencing a revival in biology and medicine (Contassot et al., 2012; Dinarello, 2009; Dinarello et al., 1986; Dinarello et al., 2012; Lee et al., 2015). The IL-1 family of cytokines is a group of 11 different cytokines, which includes activator and inhibitor cytokines (Dinarello, 2005). From them, the IL-1β is the most studied of the family. IL-1β expression is controlled by transcription factor nuclear factor-k B (NF-κB) after activation of TLRs in innate immune cells by DAMPs or PAMPs, for example, after exposure of macrophages to LPS, and this priming consltutute the first signal to regulate IL-1β production (Ghonime et al., 2014; Lamkanfi and Dixit, 2014; Mosser and Zhang, 2008; Netea et al., 2009a; Thinwa et al., 2014). After TLR activation, IL-1β is synthesized as an inactive intracellular precursor and to gain the ability to bind to IL-1 receptor type I and exert biological activity, the IL-1β precursor has to be post-translational cleaved by different proteases, including the cysteine protease caspase-1, the serine protease proteinase 3, elastase, cathepsin G, granzyme A, or chymase (Schumann et al., 1998; Tsutsui et al., 2015). However, maturation of IL-1β by caspase-1 is the best described pathway and responsible for the majority of mature IL-1β found upon infection (Lamkanfi et al., 2007; Lopez-Castejon and Brough, 2011). Activación of caspase-1 requires an additional signal that induces the formation of multiprotein aggregates called inflammasomes, formed by intracellular patter recoginition receptors of the NLR family (Broz and Monack, 2011; Latz et al., 2013). Inflamasomes are assembled in response to infections, PAMPs and DAMPs and therefore the production of mature bioactive IL-1β requires two regulative steps. Another third layer of IL-1β production is its release, that follow an unconventional release pathway after being processed in the cytosol of the cell. It has been described that IL-1β could follow a vesicular release pathway via lysosomal exocytosis, production of exosomes and microvesicles loaded with the cytokine, or being release by direct plasma membrane destabilization (Monteleone et al., 2015). Recent insights, suggest that under acute stimulation, IL-1β release is an all or nothing event, controlled by cell death triggered by the second signal and further amplified by the activation of caspase-1 (Bergsbaken et al., 2011; Broz and Monack, 2011; Csak et al., 2011; Dinarello, 2005). Furthermore, the potent actions of mature IL-1β are regulated

by the IL-1 receptor type II (a decoy rececptor for IL-1β) and by the IL-1 receptor antagonist that binds to IL-1 receptor type I and blocks its signaling (Dinarello, 2010; Garlanda et al., 2013). IL-1 receptor type I is present in many tissues and cells and its engagement activates NF-kB signaling, therefore enhancing the production of proinflammatory cytokines (Contassot et al., 2012; Dinarello, 2010; Sahoo et al., 2011).

The TNF family is a group of cytokines that, upon activation by the immune system, are able to exert significant cytotoxicity on many tumor cell lines and to cause tumor necrosis in certain animal model systems via signaling through the TNF receptor I (Bertok et al., 2011; Wajant et al., 2003). On the contrary, signaling via the TNF receptor II results in a contrary phenotype that inlcludes cell proliferation and regeneration (Faustman and Davis, 2013). The TNF family consists of nineteen proteins, being TNF alpha (TNF-α) the family member in which we deepen throughout this Thesis and one of the best studied cytokines of this family. TNF-α is mainly a monocyte-derived cytokine that has been implicated in tumor regression, septic shock and systemic inflammation, cachexia, the acute phase reaction and the regulation of a wide range of immune cells (Croft et al., 2013; Mandrup-Poulsen, 2012; Ware, 2011). TNF-α can be also produced by many other cell types such as lymphocytes, NK cells, neutrophils, mast cells, eosinophils, or neurons (Alexander and al., 2009; Ware, 2011; Watts, 2005). TNF-α is produced upon NF-kB activation as a transmembrane protein following the classical endoplasmic reticulum-Golgi route of secretion and arranged in stable homotrimers at the plasma membrane, where it can exert activation via cell to cell interactions (Liang et al., 2004; Tak and Firestein, 2001). Also, from this membrane-integrated form the soluble homotrimeric TNF-α could be released via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE) (Black, 2002; Wajant et al., 2003). Different additional pathways could controls TNF-α release, for example p38 MAPK controls TACE activity and cathepsin B controls the trafficking of TNF-α vesicles to the plasma membrane, resulting in a fine tuning production of soluble TNF-α (Lu et al., 2015; Newton and Dixit, 2012; Wajant et al., 2003).

2.1.2 Eicosanoids

Eicosanoids, one of the most remarkable kind of autacoids, are lipid mediators derived from the oxidation of arachidonic acid that play an important role as signaling molecules in the regulation of the immune response. There are different subfamilies of eicosanoids, in this Thesis we will delve into prostaglandins (PGs), thromboxanes, and leukotrienes, potent enhancers of innate and adaptive immune activity and implicated in numerous inflammatory disorders, such as rheumatoid arthritis; inflammatory colitis; cystic fibrosis; allergic asthma (Ricciotti and FitzGerald, 2011; Rogerio et al., 2015). Eicosanoid production is spatially and temporally regulated by the sequential actions of eicosanoid-synthesizing enzymes. In particular, their synthesis starts with the translocation of cytosolic phospholipase A2 (cPLA2) to the membranes of the nuclear envelope, endoplasmic reticulum, and Golgi apparatus. On the membranes, this enzyme hydrolyzes phospholipids and release arachidonic acid. Then cyclooxygenases (COXs, such as COX-1 and COX-2) metabolize arachidonic acid to produce an unstable endoperoxide intermediate, which is further metabolized to differents prostaglandins (PGD2, PGE2, PGF2a, PGI2), or thromboxane A2 (TXA2) by terminal enzymes (Kihara et al., 2014; Norris et al., 2014; Norris et al., 2011; Ricciotti and FitzGerald, 2011). Prostanoids is a subclass of eicosanoids which includes PG and TX, its production depends on the activity of the two COX isoenzymes within cells. COX-1 is constitutively present in most cells, whereas COX-2 expression is low or undetectable in most cells but its expression increases dramatically upon stimulation, particularly in cells of the immune system (Cimino et al., 2008; Ricciotti and FitzGerald, 2011). Similarly to COXs, the lipoxygenases (LOXs, such as 5-LOX and 12/15-LOX) metabolize arachidonic acid to produce hydroperoxyeicosatetraenoic acids, which are converted into leukotrienes (such as LTA4 and LTB4) and hydroxyeicosatetraenoic acids by enzymatic and nonenzymatic reactions (Catalano and Procopio, 2005; Ding et al., 2003; Gerstmeier et al., 2015).

PGs present diverse hormone-like effects in vertebrates and have been found in almost every tissue in humans and other animals (Flower, 2006; Ricciotti and FitzGerald, 2011). PGE2 is one of the most abundant PGs

produced in the organism, is most widely characterized in animal species, and exhibits versatile biological activities. Under physiological conditions, PGE2 is an important mediator of many biological functions, such as regulation of immune responses, selectively suppresses effector functions of macrophages and neutrophils and the Th1-, cytotoxic T lymphocytes (CTL-) and NK cell-mediated type-1 immunity, but promotes Th2 response. PGE2 modulates chemokine production, inhibiting the attraction of pro-inflammatory cells, while enhancing local accumulation of regulatory T cells and myeloid-derived suppressor cells. Targeting the production, degradation and responsiveness to PGE2, provides tools to modulate the patterns of immunity in a wide range of diseases, from autoimmunity to cancer, such as promoting angiogenesis or having divergent effects (protective/suppressive) over NK and dendritic cells (Harizi, 2013; Kalinski, 2012; Zhang and Daaka, 2011). Dysregulated PGE2 synthesis or degradation has been associated with a wide range of pathological conditions, such as rheumatoid arthritis, fibrotic lung disease, fever (see section 2.3 below) (Fattahi and Mirshafiey, 2012; Saper et al., 2012). In inflammation, PGE2 is of particular important because induces redness, swelling and pain. Redness and edema result from increased blood flow into the inflamed tissue through PGE2-mediated augmentation of arterial dilatation and increased microvascular permeability. Pain results from the action of PGE2 on peripheral sensory neurons and on central sites within the spinal cord and the brain. Fever is a hallmark of inflammatory and infectious diseases, the febrile response is triggered by prostaglandin E2 synthesis mediated by induced expression of the enzymes COX-2 and microsomal prostaglandin E synthase 1 (mPGES-1) (Christie and Henderson, 2002; Ricciotti and FitzGerald, 2011; Simmons et al., 2004; Wilhelms et al., 2014; Yagami et al., 2015). COX-2 is implicated in fever production, its expression is selectively enhanced in brain after peripheral exogenous (i.e. LPS) or endogenous (i.e. interleukin-1) pyrogen administration, while selective COX-2 inhibitors suppress the fever induced by these pyrogens (Li et al., 1999). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used agents to suppress inflammation. One important NSAID target is COX, specially the selective inhibitors of the COX-2, whose main function is to block the production of the prostaglandins and the acute tissue inflammation. These inhibitors have analgesic, antithermal and antiinflammatory effects

similar to traditional NSAIDs; they are prescribed for example to diminish pain and inflammation in patients with rheumatoid arthritis and osteoarthritis (Carrillo Gutierrez et al., 2007; Chang et al., 2014).

The two major TXs are TXA2 and TXB2, because of its important participation in the innate immunity we will focus on TXA2 (Christie and Henderson, 2002). TXA2 formation is stimulated by most platelet agonists through transmembrane receptors that activate phospholipases either directly or as a consequence of raised intracellular Ca^{2+}. However, TXA2 formation is also dependent on secondary signaling that occurs following fibrinogen-GPIIb/IIIa interactions and subsequent platelet aggregation. Indeed, there is evidence that at least some antagonists of the platelet GPIIb/IIIa suppress TXA2 formation *in vivo* (Byrne et al., 1997; Yomo et al., 2000). The same effect is also achieved by ADP stimulating $P2Y_{12}$ in platelets, being two existing compounds that targets $P2Y_{12}$ clinically useful as antiplatelet activity (Dorsam and Kunapuli, 2004). $P2Y_{12}$ activation causes platelets to undergo shape change, release of granule contents, and aggregate, ultimately hydrolyzing arachidonic acid from phospholipid and convert it into TXA2 by sequential oxygenation via COX and TXA2 synthase (Tang et al., 2015). Apart from its role in platelet aggregation TXA2 plays a key role in neutrophil-mediated T-cell immune responses to infections and vaccinations (Kabashima et al., 2003; Yang and Unanue, 2013; Yang et al., 2014). Furthermore, TXA2 receptor signaling regulates negatively dendritic cell/T-cell interaction and modulates adaptative immunity (Tilley et al., 2001). Interaction between TXA2 with its receptor in thymic microenvironment may be important in the apoptosis of activated T-cells and TXA2 receptor signaling plays essential roles in early B-cell development (Yang et al., 2014).

Leukotrienes are lipid mediators produced in leukocytes derived by the oxidation of arachidonic acid by the enzyme arachidonate 5-LOX (Hedi and Norbert, 2004). As their name implies, leukotrienes were first discovered in leukocytes. Leukotrienes use lipid signaling to convey information to either the cell producing them (autocrine signaling) or neighboring cells (paracrine signaling) in order to regulate immune responses (Patnode et al., 2014). Leukotriene production is usually accompanied by the production of histamine

and prostaglandins (Lee et al., 2003). Leukotrienes act principally on a subfamily of G protein-coupled receptors, and they may also act upon peroxisome proliferator-activated receptors, receptors widely expressed on non-myeloid cells, such as vascular smooth muscle and endothelial (Back et al., 2005; Ricciotti and FitzGerald, 2011; Sala et al., 1998). Leukotriene family include the cysteinyl leukotrienes and leukotriene B4 (LTB4) (Crooks and Stockley, 1998; Christie and Henderson, 2002). LTB4 is synthesized from leukotriene A4 (LTA4) by the enzyme LTA4 hydrolase, the expression of this key enzyme is increased by different molecules including bacterial endotoxin, complement fragments, TNF-α or ILs (Fukasawa et al., 1999; Ricciotti and FitzGerald, 2011). LTB4 have a chemotactic and activation effect on neutrophils, monocytes and eosinophils to areas of tissue damage, promoting the production of inflammatory cytokines and other mediators such as histamine (which stimulates smooth muscle cell contraction, vasodilatation and increased venular permeability and further mucus secretion) or TNF-α (which induces adhesion molecules on endothelial cells and subsequent transmigration of inflammatory leucocytes, maintainig tissue inflammation) (Freire and Van Dyke, 2013; Hart, 2001).

2.2. Macrophages; effector cells of the innate immune system

From the leuckocites involved in innate immunity the macrophages are far the most plastic cell type, they derive either from tissue resident progenitor setm cells or from blood monocytes, and Together with neutrophils, they constitute the first line of defense against infections (Alberts et al., 2002a). Briefly, the other innate leuckocites are: 1) natural killer cells, which are ready to kill as soon as they are formed and recognize and kill infected or cancerous cells (Alberts et al., 2002a; Chaplin, 2010; Delves and Roitt, 2000); 2) neutrophils, that are the most common type of leuckocytes in the blood and they are very efficient phagocyting bacteria and other foreign cells (Alberts et al., 2002a; Chaplin, 2010; Delves and Roitt, 2000); 3) eosinophils, that attacht to foreign cells that are too large to ingest and thus immobilizing and killing parasites, they are also involved in inflammation and allergic reactions (Alberts

et al., 2002a; Chaplin, 2010; Delves and Roitt, 2000); 4) basophils are cells that release granules filled with histamine, and therefore are involved in allergic reactions; and finally 5) dendritic cells that are antigen-presenting cells, which main function is to process antigen material and present it on the cell surface to the T cells of the immune system (Alberts et al., 2002a; Chaplin, 2010; Delves and Roitt, 2000).

Tissue resident macrophages are the responsible to initiate innate immunity in response to infection or damaged cells. The number of macrophages infiltrating the affected tissue incrase after blood monocytes migration and differentiation. This differentiation involves the enlargement and production of granules within a period of 8 hours, after this time monocytes become macrophages. The macrophage granules are filled with enzymes and other substances that help in the killing and digesting bacteria and other foreign cells inviding the tissues. In affected tissues, macrophages secrete large amounts of eicosanoids, cytokines and chemokines, that attract other leukocites to the site of the infection or injury. It is important to know that beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of anti-inflammatory cytokines (Chaplin, 2010; Delves and Roitt, 2000). Therefore, macrophages can activate and resolve innate immune activation. Pro-inflammatory macrophages are called M1 macrophages, whereas those that decrease inflammation and promotes tissue repair are called M2 macrophages (see section 2.2.3 below) (Italiani and Boraschi, 2014). Mature macrophages can be detected by specific membrane proteins, such as CD11b, F4/80 (only in mice) and MAC-1/MAC-3 (Alberts et al., 2002a; Hirsch et al., 1981; Hume, 2006).

2.2.1. Macrophage differentiation and development

The mononuclear phagocyte system is a grouped lineage-committed bone marrow precursors, circulating monocytes, macrophages, and dendritic cells. The development, homeostatic maintenance, proliferation, differentiation, and function of these phagocytes are regulated by the growth factor colony-

stimulating factor (CSF)-1. In some circumstances, macrophages and dendritic cells have separate lineages and functions. In fact, macrophages and myeloid dendritic cells (because there is also a lymphoid dendritic cells line) represent alternative differentiation options of bone marrow progenitors and blood monocytes, with overlapping functions and marker expression (Geissmann et al., 2010; Italiani and Boraschi, 2014)

Monocytes are a group of cells circulating in the blood, but also present in the bone marrow and spleen. Monocytes constitute around 10 % of the total leukocytes in humans and only 2 – 4 % in mice. Monocytes can remain in the circulation for up to 1 – 2 days, after that time, if they have not been recruited into a tissue for facing a danger, they die and are removed (Italiani and Boraschi, 2014). Monocytes originate in the bone marrow from hematopoietic stem cells and develop through a series of sequential differentiation stages: the common myeloid progenitor (HSC); the granulocyte-macrophage progenitor (GMP); the common macrophage and dendritic cells precursor (MDP); and finally the committed monocyte progenitor (Figure 8). Monocytes are considered as the systemic reservoir of myeloid precursors for renewal of macrophages and dendritic cells. Tissue resident macrophages are heterogeneous and versatile cells found in virtually all tissues of adult mammals, where they can represent up to 10 – 15 % of the total cell number in quiescent conditions, but this number can increase further in response to infection or tissue injury. The specialization of macrophages in particular microenvironments explains this heterogeneity, and recently it has been shown that the majority of adult tissue-resident macrophages in liver (Kupffer cells), brain (microglia), epidermis (Langerhans cells) and lung (alveolar macrophages) originate from tissue resident erythro-myeloid progenitors distinct from monocytes (Epelman et al., 2014; Geissmann et al., 2010; Gomez Perdiguero et al., 2015; Hume, 2006; Rees, 2010).

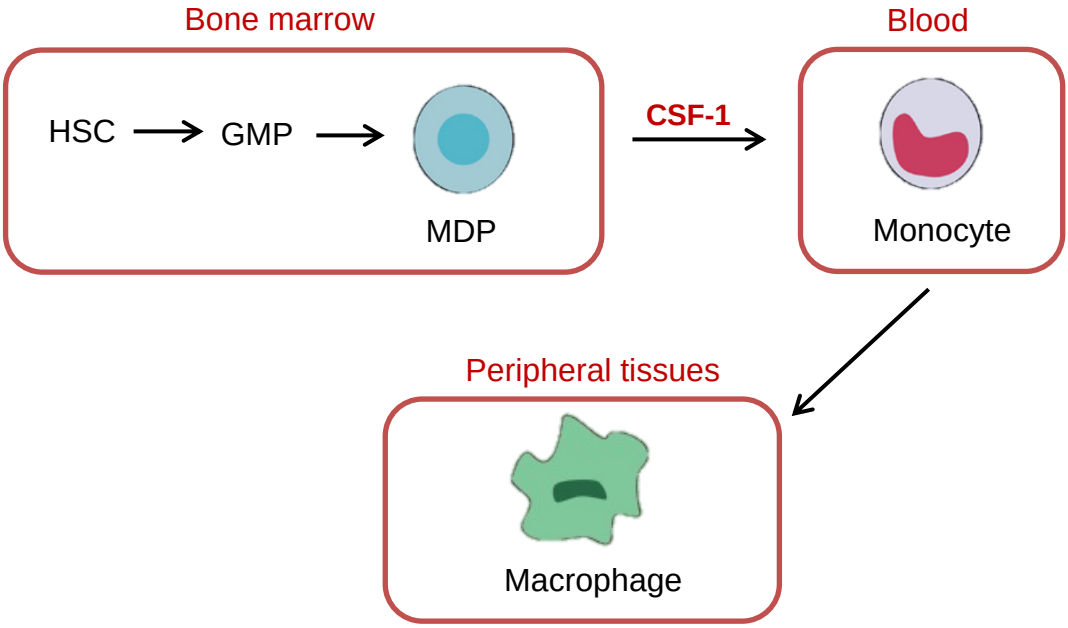

Figure 8. Schematic of development path of macrophage lineage from hematopoietic stem cells. The bone marrow hematopoiesis occurs, giving rise to monocytes and to monocyte-derive macrophages. HSC, hematopoietic stem cell; GMP, granulocyte-macrophage progenitor; MDP, macrophage–dendritic cell progenitor (adapted from (Italiani and Boraschi, 2014).

When tissues are damaged following infection or injury, inflammatory monocytes ($Ly6c^+$ in mice and $CD14^+ CD16^-$ in humans) are recruited from the circulation and differentiate into macrophages as they migrate into the affected tissues. Homeostatic control of monocyte/macrophage development is mostly influenced by CSF-1 (also known as macrophage colony-stimulating factor, M-CSF), produced by stromal cells within the blood and in tissues. Mature mononuclear phagocytes in turn express CSF-1 receptors (CSF-1R) and remove circulating CSF-1, allowing a feedback loop responsible for monocyte proliferation decrease. Granulocyte-macrophage colony-stimulating factor (GM-CSF), another factor involved in the development of mononuclear phagocytes, is only produced during inflammation and not under homeostatic conditions (Geissmann et al., 2010; Hume, 2006; Italiani and Boraschi, 2014; Rees, 2010; Verschoor et al., 2012). However, it has been poorly investigated how purinergic signaling could potentially modulate macrophage differentiation.

In vitro is it possible to derivate bone marrow hematopoietic stem cell to functional mature macrophages, as a model system to study the function of this

highly diversified and complex cell type. Bone marrow derived macrophages (BMDM) differentiated *in vitro* from bone marrow myeloid progenitors have enabled functional studies of macrophages, providing an abundant source of primary cells that are otherwise difficult to obtain. *In vitro* addition of recombinant M-CSF stimulates the formation of macrophage colonies from bone marrow hematopoietic progenitor cells and induces proliferation of isolated macrophages, alternatively M-CSF could derive from L929 cells, a murine aneuploid fibrosarcoma cell line, which constitutively secrete M-CSF. That is the reason why conditioned medium of L929 cells is used frequently as a source of murine M-CSF to generate BMDM. With M-CSF added *in vitro* bone marrow macrophage progenitors will proliferate and differentiate into a homogenous population of mature BMDM suitable for different types of experimental manipulations (Fleetwood et al., 2007; Sorgi et al., 2012; Weischenfeldt and Porse, 2008; Yamazaki et al., 2008).

2.2.2. Macrophage receptors

The process of acute inflammation is initiated by tissue resident macrophages acting as the first line of defense against pathogens, tissue injury or environmental toxins, providing a link between innate and adaptive immune response. Macrophages express a wide range of receptors, including the PRRs that recognize molecules that are broadly shared by pathogens but distinguishable from host molecules (PAMPs). These PRRs also recognize endogenous danger signals or DAMPs, therefore allowing the macrophage to sense tissue injury. PRR include resceptors at the plasma membrane receptors such as the Toll-like receptors (TLRs) and intracellular receptors as the nucleotide-binding oligomerization domain receptors (NLRs). At the onset of an infection, burn, or other injuries, the macrophages undergo activation by enagemente of one of their PRRs to a PAMP and/or DAMP, inducing the release of innate immune mediators responsible for the clinical signs of inflammation.

The TLRs are a class of widely studied PRR in macrophages, since they have a key role in the correct function of the innate immune system. TLR are

single membrane-spanning, non-catalytic receptors that recognize structurally conserved molecules derived from pathogens (PAMPs) and from the host (DAMPs). In mammals there have been identified thirteen TLRs (named from TLR1 to TLR13), but TLR11, 12, and 13 are not expressed in humans (Hopkins and Sriskandan, 2005). It is interesting that TLRs are involved in the cytokine production and cellular activation in response to microbes, and that they do not play a significant role in the adhesion or phagocytosis of the microorganisms (Imani Fooladi et al., 2011). The Gram-negative bacterial coat component LPS, the main culprit behind toxic shock syndrome and sepsis, is a highly potent trigger of cytokine secretion through TLR4 engagement (Lacy and Stow, 2011).

Another emerging group of important PRR receptors are the NLRs. They are in the cytoplasm of macrophages and act as intracellular sensors of PAMPs that enter the cell via phagocytosis or pores and DAMPs that are associated with cell stress or tissue injury (Jang et al., 2015). NLR receptors present a common central nucleotide-binding domain and a C-terminal leucine-rich repeat domain and depending on the domain of the N-terminal are divided into 5 subfamilies: NLRA (with an acidic activation domain), NLRB (with a baculovirus inhibitor of apoptosis repeat), NLRC (with a caspase activating and recruitment domain and including NOD1 and NOD2 receptors), NLRP (with a pyrin domain) and the NLRX (with with no strong homology to the N-terminal domain of any other NLR subfamily member) (Ting et al., 2008). The most characterized members of these subfamilies are NOD1, NOD2, NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4. The characteristic nucleotide-binding domain of the NLRs is a domain bound to by ribonucleotide-phosphates and is important for self-oligomerization of the receptor (Franchi et al., 2009b; Kawai and Akira, 2010). The C-terminus leucine-rich repeat domain, is though to serve as a ligand-recognition domain significant for activation (as it is similar to the ligand-binding domain of TLRs) (Botos et al., 2011). Upon activation, some NLRs aggregates into macromolecular complexes leading to the formation of inflammasomes, plataforms for the activation of inflammatory caspases (Broz and Monack, 2011; Franchi et al., 2009a). From the different NLR studied, the NLRP3 is the only one shown to form active inflammasomes by different non-microbial stimulus, including the phagocytosis of crystalline structures (as cholesterol crystals,

amyeloid aggregates and monosodium urate crystals), the activation of the P2X7R by extracellular ATP, or the recognotion of hyaluronic acid, acidity or hyposmolarity (Baroja-Mazo et al., 2014; Franchi et al., 2009b; Hari et al., 2014). Furthermore, recently it has been described that oligomeric particles of NLRP3 inflammasome could be released from activated macrophages and could serve to activate the inflammasome in other macrophages (Baroja-Mazo et al., 2014). Therefore, NLRP3 is important to mediate inflammation in 'sterile' conditions. All stimulus leading to NLRP3 activation seems to share a requirement for low intracellular potassium concentrations and the production of reactive oxygen species, however it is not know what is the final 'ligand' to activate the NLRP3 (Guo et al., 2015; Latz et al., 2013; Pétrilli et al., 2007). However, since different bacterial toxins, virus or bacterial infections can also lead to NLRP3 activaiton, it was postulated that certain hemichannels oppening involved in NLRP3 activaiton grant access of toxins into the cell to directly bind to NLRP3, although this hypothesis requrie further experimental support (Di Virgilio, 2013; Pelegrín, 2011a).

Macrophages also express different purinergic receptors to detect extracellular nucleotides, that although they are not considered PRR, their activation results in the release of cytokines, lipid mediators, proteases and in certain conditions activation of intracellular PRR (Coutinho-Silva et al., 2005; Desai and Leitinger, 2014; Taylor et al., 2005). In particular, macrophages express the P1 receptors A_1, A_2 and A_3, the P2X receptors P2X1, P2X4 and P2X7, and the P2Y receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ (Burnstock, 2007b; Coutinho-Silva et al., 2005). Extracellular concentrations of nucleotides are low under physiological conditions, following ischaemia, traumatic injury, infection or during inflammation, extracellular concentrations of nucleotides increase several fold over basal levels (Abbracchio and Ceruti, 2007; Burnstock and Williams, 2000). It is also known that extracellular ATP is degraded up to ADP, AMP and adenosine by action of ectonucleotidases (Mello et al., 2014; Sahota and Taylor, 2013; Wen and Knowles, 2003). Therefore an increase of ATP leads to a full pletora of purinergic P1 and P2 receptors. Among known adenosine receptors, A_2 receptors have properties that make them particularly well suited to serve as sensors of pro-inflammatory activities and to act as stop

signals of overstimulated macrophages (Abbracchio and Ceruti, 2007; Gordon, 2007). Signalling through A_1 and A_{2A} adenosine receptors can powerfully prevent the release of pro-inflammatory cytokines, thus inhibiting inflammation and reperfusion injury. However, signalling through the A_{2B} adenosine receptor has been linked to pro-inflammatory actions (Antonioli et al., 2015; Ryzhov et al., 2008). In macrophages, $P2Y_2$ plays a crucial chemotactic role to locate apoptotic cells that release ATP. On the other hand, microglial phagocytosis is triggered by UDP that is released by damaged neurons and is dependent on $P2Y_6$ receptors. Macrophages use the P2X7R to sense high concentrations of extracellular ATP, in such dangerous situations, P2X7R mediates the activation of the NLRP3 inflammasome and the caspase-1-dependent death of the macrophage via pyroptosis (Baroja-Mazo et al., 2013a; Desai and Leitinger, 2014; Pelegrin and Surprenant, 2011; Surprenant and North, 2009). Caspase-1 activation is also used by the macrophage to release proinflammatory cytokines of the IL-1 family, such as IL-1β and IL-18 (Denes et al., 2012; Raupach et al., 2006). Therefore, extracellular nucleotides acting on purinergic receptors is a fine tunning mechanism for the activation of macrophages (Chen et al., 2014; Gorini et al., 2013).

2.2.3. Macrophage polarization

Macrophages do not only play an important role in the initiation of the innate immune response, but they also play an important role during the resolution of inflammation by releasing potent anti-inflammatory mediators. The different activation states of macrophages, pro- or anti-inflammatory, is refered as macrophage polarization and occurs through different activation pathways. Therefore, macrophages are able to respond with appropriate functions in distinct contexts, and this functional diversity is a key feature of these cells. It has become commonplace to classify macrophages into one of two major subtypes, M1 and M2, based on their surface receptor expression and which mediators they produce (Italiani and Boraschi, 2014; Jha et al., 2015; Tian and Chen, 2015). Essentially, macrophages can modify their metabolic functions from a heal or growth promoting setting (M2 macrophages), to a killing or inhibitory capacity (M1 macrophages). The main difference between these cells

is that in M2 macrophages the arginine metabolism is shifted to ornithine and polyamines, while in M1 cells it is shifted to nitric oxide (NO) and citrulline (Kelly and O'Neill, 2015; Mills, 2015). M2-produced ornithine can promote cell proliferation and repair through polyamine and collagen synthesis, fibrosis and other tissue remodeling functions, while M1-produced NO is an important effector molecule with microbicidal activity and cell proliferation inhibitory capacity (Kelly and O'Neill, 2015; Mills, 2015). Throught this polarization process, different subsets of macrophages can adopt different functional phenotypes, the $Ly6C^+$ monocytes or monocyte derived macrophages in the tissue become M1 macrophages, and $Ly6C^-$ monocytes or tissue resident macrophages become M2 macrophages (Mills et al., 2015). It is postulated that resident macrophages maintain cytoprotective and reparative functions, whereas macrophages derived from circulating inflammatory monocytes perform mainly M1 type functions (Italiani and Boraschi, 2014). M1 and M2 macrophage activities do not need the presence of lymphocytes, but T-cell derived cytokines, such as IFNγ and IL-4, may amplify macrophage polarization (Italiani and Boraschi, 2014; Mills et al., 2015). *In vitro* studies show that macrophages are activated toward an M1 functional program by infectious microorganism-related molecules (as LPS) and by inflammation-related cytokines TNF-α or IFNγ, alone or in combination. M1 macrophages are characterized *in vitro* by an IL-12, IL-23, and IL-10 phenotype, as well as these macrophages are efficient producers of toxic effector molecules (radical oxygen species and NO). Inflammatory cytokines (IL-1β, TNF, IL-6) participate as inducers and effector cells in polarized Th1 response, they also mediate resistance against intracellular parasites and tumors (Lech and Anders, 2013; Martinez and Gordon, 2014; Trifunović et al., 2015). On the opposite, M2-like polarization has been observed *in vitro* in response to the Th2-related cytokines IL-4 or IL-13, to the concomitant triggering of fragment crystallizable gamma receptors and TLRs, to immune complexes, and to anti-inflammatory molecules such as IL-10, TGF-β, and glucocorticoids (Italiani and Boraschi, 2014; Murray and Wynn, 2011b; Trifunović et al., 2015). Cells of the monocyte/macrophage lineage exhibit extraordinary plasticity in response to endogenous as well as exogenous stimulation, which can reprogram the initial M1/M2-polarization

process, for example M2 polarized macrophages can be re-polarized into macrophages with M1 phenotype following exposure to TLR ligands or IFNγ or overexpression of miRNAs (like miR-155 and miR-146) (Mylonas et al., 2009; Stout et al., 2005), whereas M1 macrophages can be reprogramed to express various genes of M2 macrophage by treating macrophages with reagents that increase IL-10 level (Banerjee et al., 2013; Hyam et al., 2013; Jang et al., 2013; Murray and Wynn, 2011a; Wang et al., 2014). And, conversely, M1 macrophages could be forced to turn to M2 macrophages upon stimulation with IL-14 (López-Castejón et al., 2011; Pelegrin and Surprenant, 2009). Although macrophage taxonomy in M1 and M2 is used to categorize an extended variety of cell functions, the M1/M2 paradigm is a limited attempt to define the complexity and plasticity of mononuclear phagocytes found *in vivo*. Under *in vivo* physiological environment, macrophages can adopt a variety of functional phenotypes depending on subtle and continuous changes in the tissue microenvironment. So, the M1/M2 polarization of macrophage functions may be taken as a simplified conceptual framework which describes a continuum of diverse functional states, where M1 and M2 activation states only represent the extremes (Italiani and Boraschi, 2014; Mills, 2015; Mills et al., 2000; Verschoor et al., 2012; Wang et al., 2014; Wynn et al., 2013).

2.2.4. Role of macrophages in the purinergic response

As previously described, macrophages express different types of purinergic receptors. We now turn to the role of macrophages as important cells inducing and controlling the release and levels of extracellular nucleotides. In macrophages, cytosolic ATP can be secreted through the release of ATP-loaded vesicles, through the activation of large conductance channels or during pyroptotic cell death (Desai and Leitinger, 2014). After release, ATP could be degraded by different ecto-nucleotidases expressed in macrophages to ADP and adenosine, being able to signal through different purinergic receptors (Burnstock and Boeynaems, 2014). In pathological conditions, macrophages contribute to the high level of extracellular ATP that could be initially released from necrotic cells. Macrophages could induce the release of ATP by subsequent pyroptosis induced by P2X7R signaling to the inflammasome or by

non-death releated patways, such as the oppening of hemichannels (Figure 9) (Abbracchio and Burnstock, 1998; Bodin and Burnstock, 2001; Desai and Leitinger, 2014).

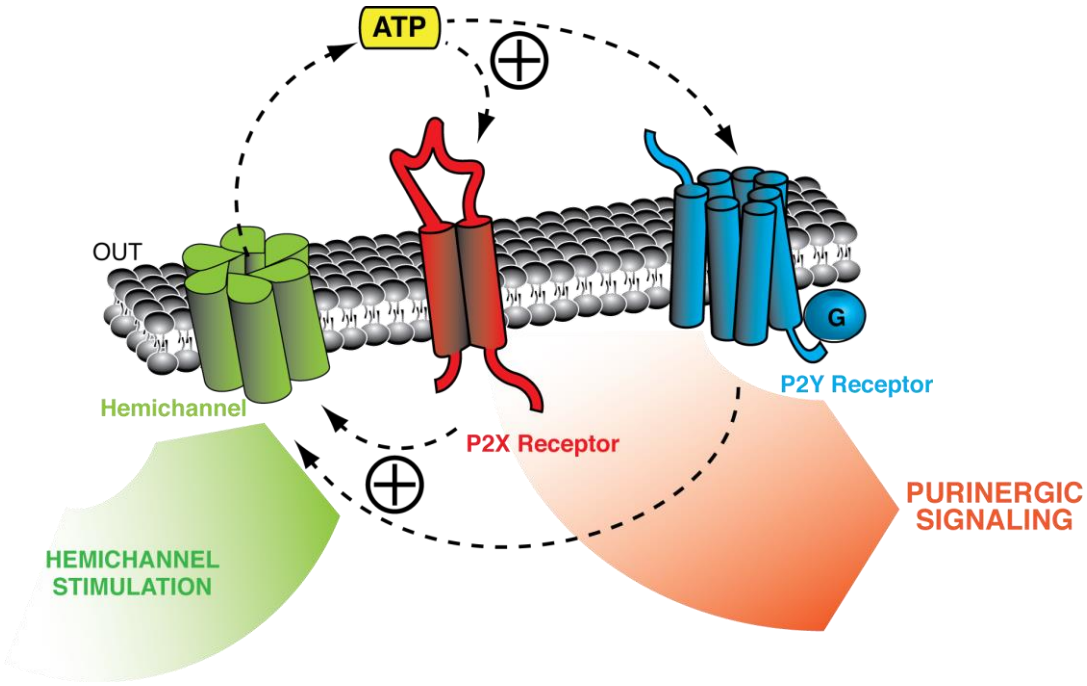

Figure 9. ATP-induced ATP release. Hemichannel activation represents a well-established pathway for the release of ATP, which serve as an agonist for purinergic receptors. Activation of different purinergic receptors (like P2X7 or P2Y receptors) leads to the opening of hemichannels (as Pannexin-1), generating a positive feedback-signaling loop (ilustration adapted from (Baroja-Mazo et al., 2013b).

2.3. Inflammatory response

Inflammation is one of the first responses of the immune system to infection or tissue damage. Inflammation is stimulated by chemical factors released by injured cells and serves to establish a physical barrier against the spread of infection, to finally promote the healing of any damaged tissue following the clearance of pathogens (Di Meglio et al., 2011). The initial inflammatory response is initiated and maintained by activated M1 polarized macrophages, eliminating invading microbes and inducing tissue damage via the production of cytotoxic mediators. Then, the resolution phase of the inflammation is initiated by M2 macrophages that eliminate injured cells, promote angiogenesis, cell proliferation, matrix deposition, and in general in

tissue remodeling (Italiani and Boraschi, 2014; Laskin et al., 2011). The mechanisms that account for macrophage deactivation via M2 polarization play a key role in maintaining tissue homeostasis and keeping the immune response under control (Abbracchio and Burnstock, 1998; Mills et al., 2015).

Inflammation is a process traditionally defined by the four Latin cardinal signs of acute inflammation attributed to Celsus: rubor (redness), calor (heat), tumor (swelling) and dolor (pain), all of which reflect the effects of cytokines and other lipidic mediators on the local blood vessels. Virchow, in 1871, incuded a 5^{th} cardinal point: loss of function, to denote the effects of chronic inflammation on tissues and organs (Horst, 1991). Pro-inflammatory cytokines produced by macrophages also have important effects on the adhesive properties of the endothelium, causing circulating leukocytes to stick to the endothelial cells of the blood vessel wall and migrate between them to the site of infection, to which they are attracted by chemokines (Dinarello, 2000). The main cell type observed in an inflammatory response in its initial phases are neutrophils, which are recruited into the inflamed infected tissue in large numbers. The initial influx of neutrophils is followed at later short times by monocytes that rapidly differentiate into macrophages (Alberts et al., 2002a). Once the initiating noxious stimulus is removed via phagocytosis, the inflammatory reaction can decrease and resolve. During the resolution of inflammation, granulocytes are eliminated and macrophages and lymphocytes return to normal pre-inflammatory numbers and phenotypes, with important process of tissue remodeling (Ricciotti and FitzGerald, 2011). If the inflammatory response of macrophages is not controlled, it can turn pathogenic and contribute to specific types of disease, as in chronic inflammatory and autoimmune disease (Ajuebor et al., 1999; Alberts et al., 2002a; Dinarello, 2000).

During inflammation extracellular ATP has a prominent role (Figure 10). For example, in the lungs of asthmatic patients, allergic challenges cause rapid accumulation of ATP and this has been modeled successfully in mice using experimentally induced asthma, showing that exogenous ATP potentiates airway inflammation (Idzko et al., 2007) and similar findings have demonstrated a role for purinergic signals in cigarette smoke-induced inflammation and

emphysema (Lucattelli et al., 2011). Furthermore, some studies have also implicated extracellular ATP in the development of intestinal inflammation in patients with Crohn's disease (Kurashima et al., 2012). In corresponding mouse models, blocking purinergic signaling greatly reduces the activation of intestinal mast cells and thereby blocks the subsequent rise in proinflammatory cytokines and leukotrienes (Kurashima et al., 2012). Furthermore, ATP released from airway-administered bleomycin-injured lung cells constitutes a major endogenous danger signal that engages the P2X7R/pannexin-1 axis, leading to IL-1β maturation and lung fibrosis (Riteau et al., 2010).

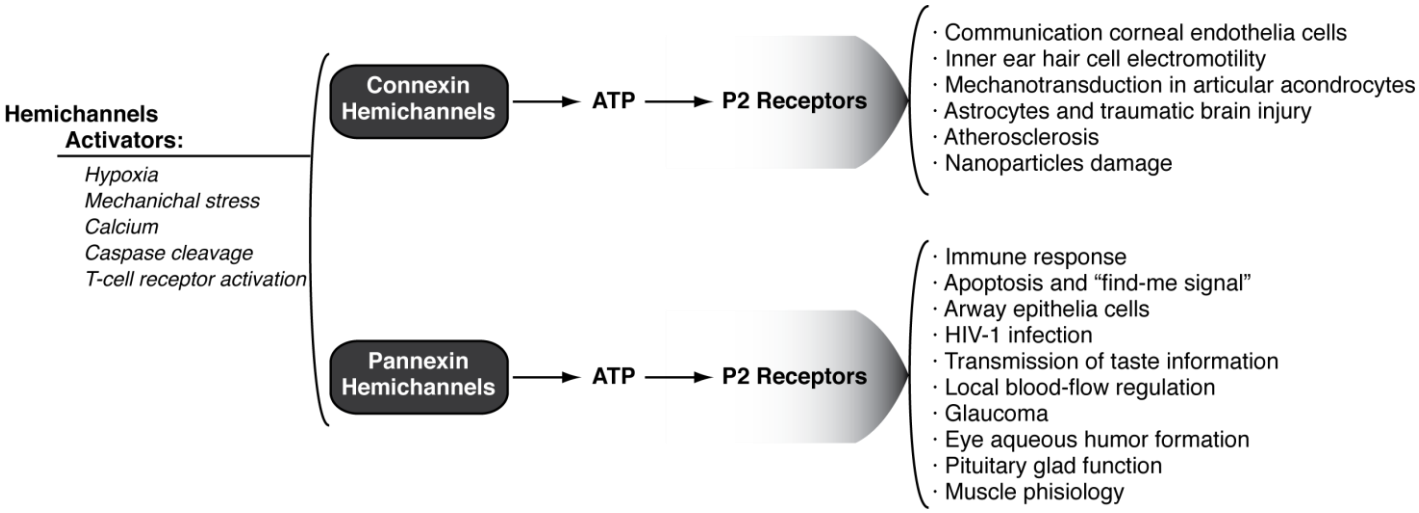

Figure 10. Pathological and physiological roles of ATP released through hemichannels. Hemichannels are activated in response to different stimuli (hypoxia, mechanical stress or extracellular calcium decrease), inducing the release of ATP which in turns activate purinergic signaling in a wide range of pathological and physiological situations (adapted from (Baroja-Mazo et al., 2013b).

Fever is one of the most frequent clinical signs encountered in human pathology, especially during infections. When a microorganism invades a host, stimulation of leukocytes determine the synthesis and release of a group of endogenous molecules that can induce fever, refered as endogenous pyrogens (Blatteis, 2010; Netea et al., 2000). The classical model of induction of fever is mediated by the release of pyrogenic cytokines such as TNF, IL-1, IL-6, and IFNs into the bloodstream in response to exogenous (bacterial) pyrogens. When these pyrogenic cytokines reach the central nervous system they induce the production of central mediators such as prostaglandins (PGs), which are the

central mediators of the coordinated responses leading to temperatura increase (Gourine et al., 2005; Netea et al., 2000). However, there are multiple pathways that may be involved in the induction of fever by cytokines, such as local cytokine production leading to signaling through vagal fibers, release of cytokine induced circulating mediators at the tissue level, the use of membrane bound cytokines as cell-cell mediators, or the local release of cytokines in the hypothalamus by circulating activated monocytes. In addition, certain bacterial products can stimulate cytokine production directly at the level of hypothalamus, by activation of TLRs (Netea et al., 2000; Sternberg, 2006). However, how purinergic signaling could modulate the febrile response is not well studied. (Dinarello, 1999; Kozak et al., 1998; Mills et al., 2015; Netea et al., 2000). Fever as an upregulation of the hypothalamic temperature, is often difficult to differentiate from hyperthermic syndromes. Fever is a preserved phylogenetic response to a wide variety of infectious and non-infectious triggers, which induce, by different methods, upregulation of the thermostatic setpoint in the preoptic area of the hypothalamus, finally resulting in fever (Launey et al., 2011).

When infections are not controlled and spread thgough the organism there is a systemic inflammatory sindrome that could damage different organs leading to sepsis (Reinhart et al., 2012). Sepsis is generally caused by Gram-negative bacterial endotoxins or LPS, and is characterized by severe shock and multiple organ failure. Strong activation of PRR initially by PAMPs and later by DAMPs results in the up-regulation of inflammation. Many cellular aspects become dysfunctional in sepsis and may be characterized as either excessive activation or depressed function. Excessive activation refers to cells that are primed such that they respond in a very vigorous manner to a second stimulus. ATP is released from macrophages stimulated with various PAMPs and extracellular ATP is one of the most potent NLRP3 inflammasome activators (Gombault et al., 2013). IL-1β and HMBG1 are cytokines released upon NLRP3 that play an important role in sepsis (Vande Walle et al., 2011). Furthermore, it has been demonstrated that M1 macrophage induction is related to the severity of sepsis. In patients with severe sepsis, high concentrations of circulating M1-type cytokines are strongly correlated with mortality (Mehta et al., 2004).

Several years ago, the mortality associated to sepsis was directly related to the production of proinflammatory molecules, now we know that there are numerous immunopathologic alterations that account for the morbidity and mortality of sepsis (Cavaillon and Adib-Conquy, 2005; Hotchkiss and Karl, 2003; Kurmyshkina et al., 2015; Remick, 2007; Stearns-Kurosawa et al., 2011).

Objectives

1. To study how purinergic signaling affects macrophage differentiation.
2. To analyze the association between the activation of P2X7 in macrophages and the production of eicosanoids.
3. To identify how P2X7 receptor regulates TNF-α release during immune extenuation.

Material and Methods

1. Reagents

The main reagents and their sources were as follows: *Escherichia coli* LPS serotype 055:B5, *Salmonella enterica* LPS serotype Typhimurium, BzATP, adenosine, ADP, UTP, UDP, 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), brefeldin A, colchicin, cytokalasin B; N,N,N',N',-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), N-(benzyloxycarbonyl)leucinyl-leucinyl-leucinal (MG132), nigericin sodium salt, 5,8,11,14-eicosatetraynoic acid (ETYA), ivermectin, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), 1,9-pyrazoloanthrone (SP600125) and 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate (U0126) were purchased from Sigma-Aldrich; recombinant murine IL-1β, acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid-2,6-dimethylbenzoyloxymethylketone (Ac-YVAD-AOM), L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E-64), [L-3-trans-(propylcarbamoyl) oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (Ca-074Me), 1-[[4,5bis (4-methoxyphenyl)-2-thiazolyl]carbonyl]-4-methyl-piperazine hydrochloride (FR122047), 4-[(5-difluoromethyl-3-phenyl)-4-isoxazolyl]benzenesulfonamide (SC-791), N-(4-Biphenylylsulfonyl) phenylalanine (MMP9 Inhibitor I) and N-[(2R)-2-(Hydroxamidocarbonylmethyl)-

4-methylpentanoyl]-L-tryptophan Methylamide (GM6001) were from Calbiochem Merck-Millipore; L-Alaninamide,N-[2-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-3-(2-naphthalenyl)-L-alanyl-(9Cl) (TAPI-0) was from AnaSpec; Geneticin sulfate (G418) was from Acros; N-[2-[[2-[(2-Hydroxyethyl)amino] ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7] dec-1-ylacetamide dihydrochloride (AZ10606120), 3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), ionomycin free acid and NECA were from Tocris; and recombinant murine IL-4 from BD Pharmingen.

2. Buffers

The composition of the physiological buffer (Et) used in almost all experiments of this work to stimulate the cells was: 147 mM NaCl (Merck), 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, from Sigma-Aldrich), 13 mM D-glucose (Panreac), 2 mM $CaCl_2$ (Sigma-Aldrich), 2 mM KCl (Panreac) and 1 mM $MgCl_2$ (Sigma-Aldrich). In some experiments to block K^+ efflux after P2X7R activation, a high K^+ extracellular solution was used, where the 147 mM NaCl was reduced to 2 mM, and 145 mM of KCl was added. In some experiments, an Et buffer containing no added $CaCl_2$ and 1 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA, from Sigma-Aldrich) (Et-EGTA buffer) was used. In all these buffers, the pH was adjusted to 7.4 at room temperature.

The Fura 2-AM loading buffer for calcium measurements had the following composition: 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO_4 (Panreac), 1.2 mM $MgSO_4$ (Sigma-Aldrich), 5 mM $NaHCO_3$ (Panreac), 20 mM HEPES, 5.5 mM D-glucose and 1 mM EGTA. The pH was adjusted to 7.4 at room temperature.

The composition of the basal salt solution (BSS) used in the experiments to measure extracellular levels of ATP was: 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Na-HEPES (adjusted to pH 7.5 at room temperature), 5 mM D-glucose and 0.1 % bovine serum albumin (BSA, Sigma-Aldrich).

The cytometry buffer composition used in flow cytometry samples preparation contained 3 % foetal calf serum (FCS, Life Technologies) and 0.01 % sodium azide (Sigma-Aldrich) in 1x phosphate buffered saline solution (PBS, Fisher Scientific).

3. Human samples

The Clinical Institutional Review Board of the *Hospital Clínico Universitario Virgen de la Arrixaca* (Murcia, Spain) approved the use of the human samples used in this Thesis and all patients enrolled signed an informed consent.

Primary human peritoneal macrophages were obtained from peritoneal lavage performed with 6 ml of sterile saline buffer 0.9 % NaCl (Braun) before laparoscopy for benign ovarian cyst removal. Blood-contaminated peritoneal fluids were excluded. Human peripheral blood monocytes (HPBM) were isolated from healthy donors or from patients with severe sepsis of abdominal origin the first day they were recruited to the Intensive Care Unit (n = 8) (Table 3), by centrifugating at 400 xg during 30 min using Limphoprep (Axis-Shield).

Peritoneal cells and blood leukocytes were resuspended in RPMI 1640 medium (Lonza) with 10 % FCS, 2 mM GlutaMAX™ (COMPAÑIA) and 100 U/ml penicillin-streptomycin (Life Technologies), and plated into 12-well plates (Sigma-Aldrich) for 16 h at 37 ˚C with 5 % CO_2 in a HealFoce incubator. Blood monocytes and peritoneal macrophages were isolated by adherence. Purity was checked by flow cytometry using CD163-staining using a FACSCanto flow cytometer (Beckton-Dickinson Biosciences). For details about flow cytometry, please see section below. Peritoneal samples with an initial percentage (before adherence) of macrophages from 25 to 35 % were used for experiments.

Patients enrolled in the study	
Gender (male/female)	4/4
Age (years, mean ± SD)	62.5 ± 15.2
Acute Physiology and Chornic Health Evaluation score (mean ± SD)	18.0 ± 3.8
Sepsis-related Organ Failure Assessment score (mean ± SD)	10.0 ± 3.8
C-Reactive Protein in serum (mg/l, mean ± SD)	28.6 ± 16.6
Death (number, %)	2 (25 %)

Table 3. Demographic and main clinical characteristics of the septic patients enrolled in this Thesis.

4. Cell lines and transfection

HEK293T cells were cultured in DMEM:F-12 media (1:1) from Lonza, supplemented with 10 % of FCS and 2 mM GlutaMAX™ and were routinely tested for mycoplasma contamination with a Mycoplasma Detection Kit (Roche). Lipofectamine 2000 (Life Technologies) was used according to the manufacturer's instructions to transiently transfect a plasmid coding for human TNF-α (Pexmtn-F2) into HEK293T cells. Briefely, 1 µg DNA was added in 100 µl of Opti-MEM® (Lonza), this firt blend was mixed with 3 µl of lipofectamine diluted in 100 µl of Opti-MEM®, this mixture was incubated for 20 min at room temperature and then added drop by drop to the cells. The cells were incubated 48 h at 37 ˚C with 5 % CO_2 in a HealFoce incubator before using.

PmeLUC stable HEK293 cells (HEK293-pmeLUC) cells were a courtesy of Dr F. Di Virgilio (Pellegatti et al., 2008) and were cultured in DMEM:F-12 media (1:1), supplemented with 10 % of FCS, 0.2 mg/ml G418 sulfate and 2 mM GlutaMAX™ during 4 days at 37 ˚C with 5 % CO_2 to obtain cells in the logarithmic growing phase. After this time, the cells were enzymatically

dissociated using 4 ml of 0.25 % trypsin-EDTA (Sigma-Aldrich) at 37 ºC tapping gentely the culture flask during 1 min, then neutralized with 8 ml of HEK293-pmeLUC culture media. Finally the cells were washed with 10 ml of PBS, resuspended in PBS to a concentration of 2 x 10^7 cells/ml and used in the mice model to detect extracellular ATP *in vivo* as described below.

L929 fibroblast mouse cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) supplemented with 5 % of FCS and 2 mM GlutaMAX™ during 7 days at 37 ˚C and 5 % CO_2until confluence, to obtain a media enriched on macrophage-colony stimulating factor (M-CSF). The media was collected and filtred using Stericup and Steritop Filtration and Storage Systems (Millipore) provided with a 0.2 µm pore size low binding polyvinylidene difluoride (PVDF) membrane. The filtered media was frozen in aliquots at -80 ºC until use to differentiate macrophages from bone marrow precursors as will be detailed below.

5. Mice

C57 BL/6 (wild type, WT) mice were purchased from Harlan, two different P2X7R-deficient mice (*P2rx7*$^{-/-}$) were used in this Thesis, both in C57 BL/6 background, the *P2rx7*$^{-/-}$ generated by Glaxo was a generous gift of Annmarie Suprenant from the University of Manchester (Chessell et al., 2005; Solle et al., 2001). The P2X4R-deficient mice was also in C57 BL/6 background (*P2rx4*$^{-/-}$) and was a gift of Francois Rassendren from the Universtity of Monteplier (Ulmann et al., 2008).

For all experiments, mice between 8-10 weeks of age bred under specific pathogen free (SPF) conditions were used in accordance with the *Hospital Clínico Universitario Virgen Arrixaca* animal experimentation committee guidelines, and the Spanish national (RD 1201/2005 and Law 32/2007) and EU (86/609/EEC and 2010/63/EU) legislation. The mice were bred with a photoperiod established for 12 h of light and 12 h of darkness. The breeding set temperature was 23 °C, the ventilation system in the SPF clean room prevented temperature control within 1 °C, maintained at a slightly positive pressure, and

relative humidity was established at 65 %. The temperature-controlled air passed through HEPA filters and then entered the SPF clean room. Besides, the caging systems used for housing SPF mice are designed to provide the animals with environments isolated from outside contamination. This system is designed so that each cage has its own sterile food and water, and HEPA filtered-air supply. Mice had *ad libitum* access to water and a commercial diet. The cages were individually ventilated using ventilated cage rack and cages Sealsafe Next (Tecniplast).

5.1. Mice model for P2X7 receptor activity

AThis procedure was approved by the *Hospital Clínico Universitario Virgen Arrixaca* animal experimentation committee and approved by the *Servicio de Sanidad Animal, Dirección General de Ganadería y Pesca, Consejería de Agricultura y Agua Región de Murcia* (Health Animal Service, Murcia Fishing and Farming Council, reference C1310050308). For this experiment we follow the protocol described by Solle et al. (2001) using groups of male animals (22-24 g of weight): the control group was i.p. injected with 200 µl of sterile PBS and integrated with 3 C57 BL/6 and 4 *P2rx7*$^{-/-}$ mice (Solle et al., 2001); the LPS-treated group had 6 C57 BL/6 and 8 *P2rx7*$^{-/-}$ mice, which were injected i.p. with 200 µl of *E. coli* LPS serotype 055:B5 (0.05 mg/kg) in sterile PBS. 2 h after this LPS injection, half of the mice of each strain of the LPS-treated group were injected i.p. with either 0.5 ml of ATP (1.5 M/kg) prepared in sterile PBS, and the other half and the control group with 0.5 ml of sterile PBS (Solle et al., 2001). Mice were euthanized by CO_2 inhalation 120 min after ATP or PBS injection, and each peritoneal cavity was washed with 3 ml of sterile PBS. Individual lavages were centrifuged, supernatants were collected and tested by ELISA for the presence of IL-1β and TNF-α and cellular pellet was analized by flow cytometry, both techniques will be described below.

5.2. Mice model for fever

This procedure was approved by the same application than prevous model reference C1310050308. Ten male C57 BL/6 (WT) and 6 male *P2rx7*$^{-/-}$ mice (Solle et al., 2001) were injected i.p. with 200 µl of *Salmonella* or *E. coli* LPS (0.01 mg/kg), recombinant murine IL-1β (0.01 mg/kg) or saline vehicle. 100 µmol/kg of A438079 was injected i.p. 1 h before *Salmonella* LPS injection (0.01 mg/kg). The rectal temperature of all mice was measured with an electronic thermometer (Panlab Harvard Apparatus) immediately before injections, and 3 and/or 6 h post-injection. The increase in temperature was calculated comparing at each time the LPS-injected group with the saline-injected group.

5.3. Mice model to detect extracellular ATP in vivo

In vivo extracellular ATP levels during pathophysiological conditions were measured by bioluminescent imaging using HEK293-pmeLUC cells as previously described (Pellegatti et al., 2008). The University of Murcia animal experimentation committee approved this procedure (Pellegatti et al., 2008). Bioluminescence is the chemical emission of light by living organisms; this process yields photons as a consequence of an exergonic reaction catalyzed by a family of enzymes called luciferases, which oxidize a photon-emitting substrate. Luciferase (Luc) derived from the firefly *Photinus pyralis* was used in this Thesis to measure ATP. In the presence of magnesium ions, molecular oxygen and ATP, Luc catalyzes oxidation of the substrate D-luciferin (LH2) accompanied by light emission in the green to yellow region (λmax = 560 nm) (Figure 11) (Pellegatti et al., 2008). That reaction developes:

1. Formation of the intermediate Luc-D-luciferyl adenylate (LH2-AMP), with release of inorganic phosphate (PPi).

2. The intermediate complex Luc-D-luciferyl adenylate is oxidized by molecular oxygen with the formation of an excited enzyme-

oxyluciferin-AMP complex and the release of carbon dioxide (CO_2).

3. In the final step, energy loss from the excited complex produces photon emission and dissociation of the complex.

1	$Luc + LH2 + ATP \xrightarrow{Mg} Luc \cdot LH2\text{-}AMP + PPi$
2	$Luc \cdot LH2\text{-}AMP + O_2 \rightarrow Luc \cdot AMP \cdot Oxyluciferin^{\ast} + CO_2$
3	$Luc \cdot AMP \cdot Oxyluciferin^{\ast} \rightarrow Luc + AMP + Oxyluciferin +$ **Light**

Figure 11. Eschematic reaction of Luc derived from *Photinus pyralis*

The HEK293-pmeLUC were engineered to expresses a chimeric Firefly luciferase on the outer aspect of the plasma membrane (named pmeLUC). pmeLUC expression allows extracellular ATP monitoring in the extracellular compartment, notably in the close vicinity of the plasma membrane. The normal extracellular ATP concentration in tissues is negligible, around the low nanomolar range which is below pmeLUC detection limit, which makes this methot suitable to measure elevation of extracellular ATP in pathogenic conditions. Two groups of 9 WT male mice (C57 BL/6) were i.p. injected with sterile PBS or *Salmonella* LPS (0.01 mg/kg) diluted in sterile PBS. 2 h after this initial injection, 2 x 10^6 HEK293-pmeLUC cells (prepared as described above) were injected i.p. per mice and gently massaged the mice abdomen to allow cells distribution throught the peritoneum. After 2 h the mice were i.p. injected with 150 mg/kg D-luciferin (Xenogen Caliper Life Sciences). Ten minutes after luciferin injection, mice were anesthesized with isofluorane (Braun), 3 % for anesthesic induction and 1.5 % for anesthesic maintenance. Luminescence was captured for three minutes with large binning from mice ventral view using an ultra low noise high sensitivity cooled CCD camera mounted on a light-tight imaging chamber (IVIS 100 System^{TM}, Xenogen Caliper Life Sciences). After recording, mice were euthanized by CO_2 inhalation. Ligth emission was quantified as average radiance (p/s/cm^2/sr) in saline- and LPS-injected mice, respectively, using the same region of interest (ROI) in the peritoneum in all the animals.

6. Differentiation of macrophages from mouse bone marrow precursors

Ex vivo experiments were performed with cells from WT C57 BL/6, both *P2rx7*$^{-/-}$ mice (Chessell et al., 2005; Solle et al., 2001) or *P2rx4*$^{-/-}$ mice (Ulmann et al., 2008). Bone marrow was obtained from leg bones of mice (8–10 weeks of age) euthanized by CO_2 inhalation. Femurs and tibia were removed, and the bone marrow was flushed out and resuspended in DMEM supplemented with 25 % of L929 media containing M-CSF, 15 % FCS, 100 U/ml penicillin-streptomycin, and 2 mM GlutaMAX™, plated onto 150-mm dishes, and cultured at 37 °C in the presence of 5 % CO_2 (Figure 12). To study macrophage differentiation in some experients of the first section of *Results "Purinergic signaling during macrophage differentiation resultins in M2 alternative activated macrophages",* bone marrow cells were treated with purinergic agonists and/or antagonists (concentrations specified in figure legends), or LPS 10 µM from the first day of differentiation (day 0) and in other experiments these compounds were also supplemented at days 2, 5 and 7 as indicated in the figure legends. Cells were harvested at different days for analysis as indicated in figure legends. In the rest of experiments, bone marrow precursors were not trated with any compound and mature macrophages were used after 7 days of differentiation, the resulting bone marrow derived-macrophages (BMDMs) were detached with cold PBS, seeded into well plates at a confluence of 0.42 x 10^6 cells/cm^2, and used the following day for experiments. In some experiments on *Results section 1,* BMDM were left for further 7 days to study macrophage senescence.

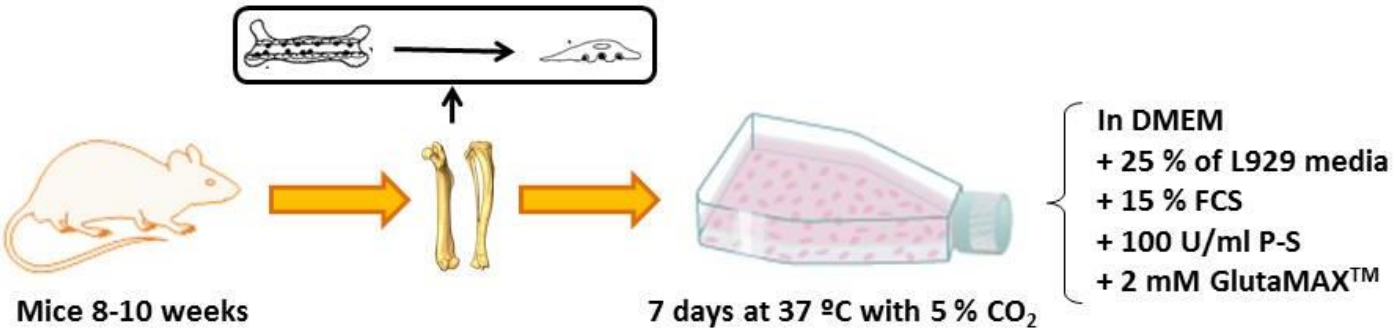

Figure 12. BMDM differentiation scheme. Abbreviation: P-S, penicillin-streptomycin

The macrophage purity of these preparations was usually higher than 90 % and was routinely checked by flow cytometry using an antibody that recognise the mouse F4/80 antigen, a 160 kD glycoprotein specifically expressed by murine macrophages (Austyn and Gordon, 1981) (see below for methodology with flow cytometry).

7. In vitro macrophage stimulation

The day of the experiment, the culture medium of macrophages (BMDMs or primary human) was replaced with fresh medium, and cells were primed for 4 h at 37 °C and 5 % CO_2 with different doses of *E. coli* LPS (as indicated in the figures legends), a combination of 100 ng/ml *E. coli* LPS and 20 ng/ml IFNγ (M1 macrophage polarisation) or with 20 ng/ml of IL-4 (M2 macrophage polarisation) or left in medium alone to obtain resting macrophages. Cells were then rinsed with pre-warmed Et-buffer and directly lysed for RNA extraction (see below) or incubated in Et-buffer at 37 ºC for different times and with various concentrations of nucleotides or with 5 µM nigericin for different times as stated in the text and figure legends. In some experiments of the section 3 of *Results "P2X7 receptor controls TNF-α release"*, macrophages were stimulated with ATP on the top of the LPS priming medium without washing the LPS. In other experiments as explained in the results, BMDMs were pretreated with various pharmacological compounds 10 min before and during nucleotide stimulation.

To study the effect of ion flux across plasma membrane in our experimental settings, macrophages were rinsed three times with Et-high K^+ buffer, as indicated in the figure legends (if no indication of buffer, cells were stimulated in normal Et-buffer) and incubated in Et-high K^+ buffer at 37 °C with 3 mM of ATP or 5 µM nigericin for different times as indicated in the text. The effect of extracellular Ca^{2+} was investigated stimulating the macrophages in Et-EGTA buffer as indicated in the text and figure legends.

To terminate macrophage stimulation, the entire volume of extracellular medium was transferred to a tube on ice, and cells were immediately lysed in lysis buffer: 50 mM Tris-HCl (Sigma-Aldrich) adjusted to pH 8.0, 150 mM NaCl,

2 % Triton X-100 (Sigma-Aldrich) and supplemented with 100 µl/ml of protease inhibitor mixture (Sigma-Aldrich) and incubated for 30 min on ice. Then cell lysates were centrifuged at 16000 xg for 10 min at 4 ºC to remove particulate matter. Soluble native protein lysates were keept at -80ºC for further analysis as described below. To obtain cell-free supernatants, recovered extracellular medium was clarified by centrifugation at 14000 xg for 30 s at 4 ºC to remove floating cells, followed by supernatant withdrawal and analysis as described below.

8. Enzyme Linked Immuno Sorvent Assay

Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay, is a biochemical technique developed to detect the presence of an antibody or an antigen in a sample. In simple terms, in ELISA, the substance of interest is measured by a specific antigen-antibody reaction, and the last antibody used is linked to an enzyme that convert a compound to a detectable signal, most commonly a colour change in a chemical substrate.

8.1. IL-1β and TNF-α ELISA

Amounts of human or mouse IL-1β and TNF-α released to the supernatant of cells or present in the peritoneal cavity of the animal models, were measured by commercial ELISA kits (R&D Systems) following the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the citokyne we want to measure was pre-coated onto a microplate. Standard curve (*E. coli*-derived recombinant human proteins IL-1β and TNF-α, 0-250 pg/ml and 0-1000 pg/ml respectively; *E. coli*-expressed recombinant mouse proteins IL-1β and TNF-α, 0-1000 pg/ml and 0-700 pg/ml respectively), and samples were dispensed into the wells and the citokyne present was allow to bound to the immobilized antibody by incubating the plate for 2 h at room temperature. *In vitro* derived samples of human origin were diluted 1:2 for IL-1β and 1:6 for

TNF-α detection and mouse *in vitro* derived samples were diluted 1:8 for IL-1β and 1:4 for TNF-α. For *in vivo* experiments mouse samples were diluted 1:4 for IL-1β and no dilution was made for TNF-α determination. After primary sample incubation, plate wells were washed five times to remove any unbound substances with the provided wash buffer of the kit. Next, a polyclonal antibody conjugated to horseradish peroxidase, specific for the citokyne, was added to the wells and incubated for 2 h at room temperature, followed by another five times washes to remove any unbound antibody-biotin reagent. Finally the substrate solution (hydrogen peroxide and chromogen tetramethylbenzidine) freshly mixed was added to each well and incubated for 30 min at room temperature in dark conditions. During this time, the enzyme reaction yields a blue product that turned yellow when the provided stop solution (composed of diluted hydrochloric acid) was added. The intensity of the final color was measured at λ = 450 nm with a dual correction by substracting the values obtained at λ = 540 and 570 nm in a Synergy Mx plate reader (BioTek), and the average of the sample values were then read off the standard curve. The absorbance at 450 nm was proportional to the amount of IL-1β or TNF-α present in the original sample and the concentration of target cytokine was determined comparing the mean absorbance for each standard on the y-axis against the concentration of recombinant protein on the x-axis using a linear fit equation: $y = a + bx$. Defining a standard curve by plotting the corrected absorvances vs. cytokine concentration and draw a best fit curve through the points on the graph, being “y” the absorbance, “x” the cytokine concentration (pg/ml), “a” the intercept of the regression line and “b” the slope. Then a regression line was performed with the standard values (pg/ml) versus the absorbance.

For human macrophages and monocytes experiments the concentration of IL-1β and TNF-α were normalized to the total amount of cellular lactate dehydrogenase (LDH, as determined below) and expressed as pg/ml/LDH values to allow for differences in cell concentration among the different samples, since although we plated a constant amount of cells, primary human monocytes and macrophages presented a differential adherence depending of the different donors that we could not control.

8.2. PGE2, TXA2 and LTB4 ELISA

After appropriate dilution of extracellular samples in assays dilution buffer supplied by the manufacturer (1:10, 1:10 and 1:6, respectively for PGE2, TXA2 and LTB4 determination), the concentration of lipidic mediators was measured in duplicate using specific metabolite ELISA competition system (Cayman Chemical Company, Assay designs or Arbor Assays), according to the manufacturer's instructions. Autacoid release from human macrophages and monocytes was normalized to total cellular LDH content as described for human IL-1β and TNF-α measurement.

ELISA competition assay is based on the competition between the metabolite (PGE2, TXA2 or LTB4) and the respectively conjugated metabolite called as Tracer (PGE2-acetylcholinesterase, TXA2-acetylcholinesterase and LTB4-acetylcholinesterase) for a limited number of respectively monoclonal antibody (anti-PGE2, anti-TXA2 or anti-LTB4) (Figure 13). The concentration of the Tracer was held constant while the concentration of the substance to mesure varied in the different samples. The amount of the Tracer that was able to bind to the monoclonal antibody was inversely proportional to the concentration of the substance in the well. The monoclonal antibody, the Tracer and our sample was incubated in the plate for 18 h at 4 ºC, covered with plastic film, to allow the formation of the antibody complex and their binding to the coated goat polyclonal anti-mouse IgG. Then the plate was washed five times with the provided wash buffer to remove any unbound reagents and the Ellman's Reagent (which contains acetylthiocholine, the colorimetric substrate that acetylcholinesterase degrades producing thiocholine) was added to the well for 60 min. The product of this enzymatic reaction has a distinct yellow color and the plate was readed at λ = 450 nm by substracting the values obtained with a dual correction at λ = 570 and 590 nm in a Synergy Mx plate reader. The sample values were then read off the standard curve, since the intensity of the color is proporcional to the amount of Tracer bound to the well, which is inversely proportional to the amount of free substance present in the well during the incubation by using the equation: $y = - a \cdot \ln(x) + b$, being "y" the percentage of the average of the values subtracted of basal values ($\%B/B_0$), "x" the PGE2,

TXA2 or LTB4concentration (pg/ml), “a” is the slope of the line and “b” is the “y” intercept. Then, a 4-parameter logistic fit was performed with standard values and the plot obtained represented the $\%B/B_0$ for standards 0-2000 pg/ml versus PGE2 concentration (pg/ml), using linear (y) and logaritmic (x) axes.

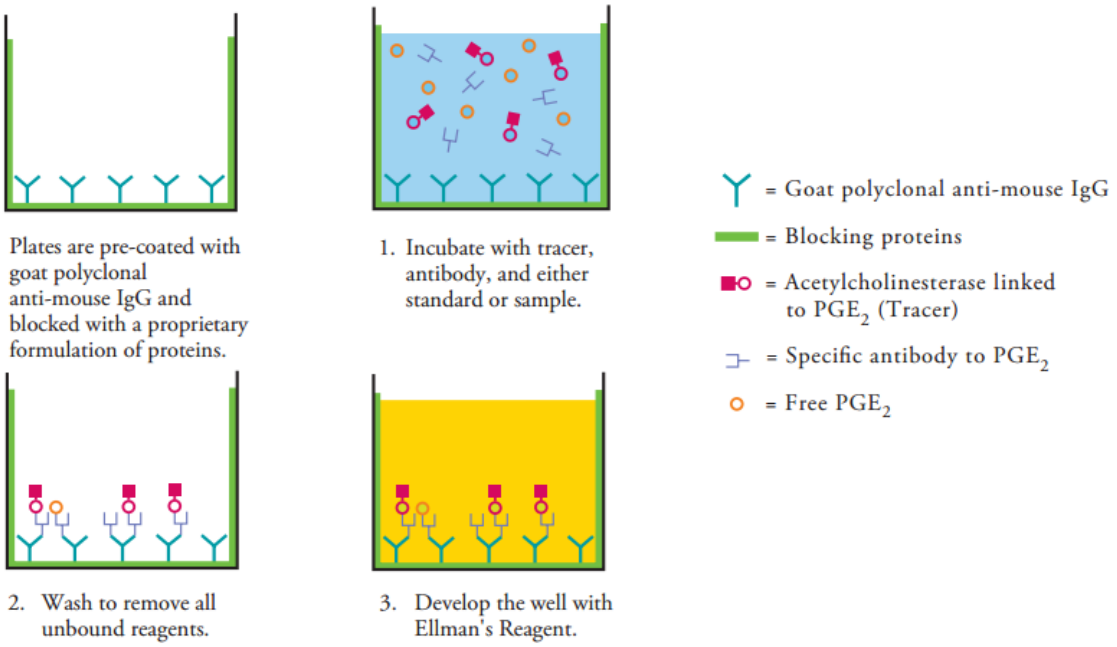

Figure 13. A schematic of PGE2 assay adapted from Cayman protocol.

9. Lactate dehydrogenase determination

Cell death was measured by the quantification of the enzyme LDH in cell-free supernatans and compared to the total amount of cellular LDH using the Cytotoxicity Detection kit (Roche) following the manufacturer’s instructions. LDH is a stable cytoplasmatic enzyme present in all cells. It is rapdidly released into the cell cuture supernatant upon damage of the plasma membrane. 25 µl of cell-free culture supernatant was incubated with 75 µl of the reaction mixture from the kit in dark conditions for 15 min. During this time the LDH reduces NAD^+ to $NADH/H^+$ by oxidation of lactate to pyruvate. Then, the catalyst (diaphorase) transfers 2 H^+ from $NADH/H^+$ to the tetrazolium salt 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium choride which is reduced to formazan (Figure 14).

The increase in the amount of LDH enzyme activity is directly correlated to the amount of formazan formed. The amount of red formazan salt produced was measured using a spectrophotometric method at λ = 492 nm in a Synergy Mx plate reader and corrected by substracting the values obtained at λ = 620 nm. Values were expressed as percentage of total cellular LDH content.

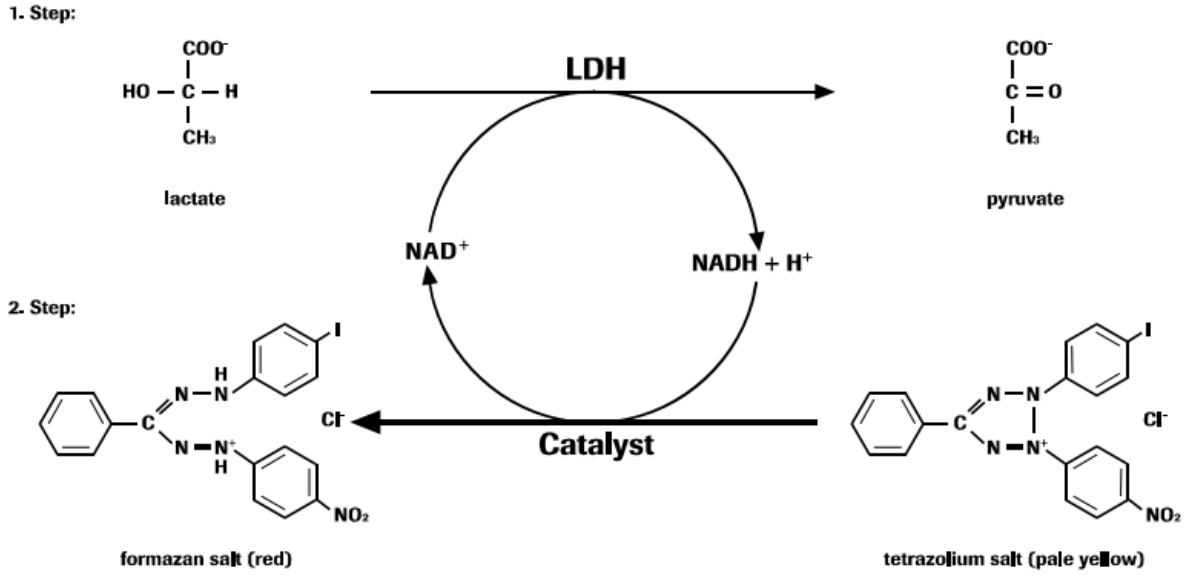

Figure 14. Chemical reaction of the LDH determination kit used in this Thesis. First step, LDH reduces NAD^+ to $NADH/H^+$ by oxidation of lactate to pyruvate. Second step, the catalyst transfers 2 H^+ from $NADH/H^+$ to the tetrazolium salt (yellow) which is reduced to formazan (red).

10. Cell viability assay

BMDMs cell viability was measured at 0, 2, 5, 6, 7 and 8 days of differentiation (as indicated in the figure legends) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After flushing and resuspended cells from bone marrow, this suspension was plated, in the same proportion as in 150 mm dishes, in 24 or in 96 well culture plates (Sigma-Aldrich), approximately 10^5 cells per well. Different nucleotides and antagonists were added to the growing cells: 50 μM, 100 μM or 1 mM of ATP; 10 μM A438079; 100 μM ADP; 100uM UTP; 100 μM UDP; 100 μM NECA; 100 μM adenosine; or left with differentiation media alone as control conditions. At the selected days of BMDM differentiation, the cells were incubated with DMEM

and 37.5 µl/well of MTT solution (1.9 mg/ml) was added to the wells and then the plate was incubated for 4 h at 37 °C. During this time the yellow MTT is reduced to purple formazan by metabolic active mitochondrial reductase of living cells that cleave the tetrazolium MTT ring yielding purple MTT formazan crystals which are insoluble in aqueous solutions and therefore the amount of purple formazan can be directly related to the number of viable living cells (Figure 15).

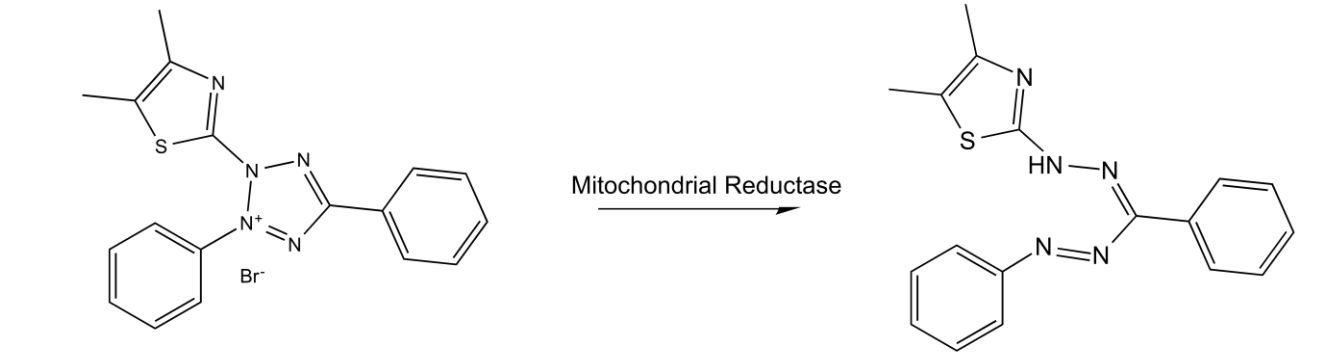

Figure 15. The MTT is reduced by mitochondrial reductase to formazan (purple).

Then, the medium was removed and purple formazan crystals were solubilized with 200 µl of DMSO per well in orbital agitation during 30 min at room temperature. Then 200 µl of the supernatant were transfer to a clear 96 well plate and optical density value was measured at λ = 570 nm using Synergy Mx plate reader and corrected by substracting the values obtained at λ = 620 nm. Cell survival was expressed as normalized percentage of cells respect control untreated macrophages.

11. Quantitative reverse transcriptase-PCR analysis

BMDMs and human macrophages or monocytes plated in 6 or 12-well plates, respectively, were stimulated as described above. Total RNA extraction was performed using the RNeasy Mini kit (Qiagen) from 10^7 cells that were disrupted in Buffer RLT and homogenized. Then ethanol was added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy

membrane. The sample was applied to the RNeasy Mini spin column. Total RNA was bound to the membrane, contaminants were washed away with Buffer RPE (a mild washing buffer), and high-quality RNA was eluted in RNase-free water. All bind, wash, and elution steps were performed by centrifugation in a microcentrifuge at 12000 xg and 4 ºC. The resulting RNA concentration was measured by spectrophotometry in 1 µl of each sample using NanoDrop 2000 and measuring absorvance at λ = 260, 280 and 230 nm. The purity of the RNA was determinated by the 260 nm/280 nm and 260 nm/230 nm ratio, which was always higher than 2.

Total RNA was reverse transcripted using the iScript cDNA Synthesis kit (BioRad) with a mix of oligo(dT) and random hexamer primers. The cDNA reverse transcription was performed using 1 µg of total RNA with 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase, and the final volume was adjusted to 20 µl using nuclease-free water. The reaction was performed at 25 ºC for 5 min, then at 42 ºC for 30 min, and finally at 85 ºC for 5 min. Reactions were holded at 4 ºC until sample storage.

The mix SYBR Premix ExTaq (Takara) was used for quantitative PCR using an iCycler MyiQ thermocycler (BioRad). The master mix composition was made as indicated on the Table 4:

Quantitative PCR MASTER MIX	
SYBR Premix ExTaq	10 µl
Nuclease-free water	7 µl
Primers	2 µl
cDNA	1 µl

Table 4. Quantitative PCR master mix composition

Specific primers were purchased from Qiagen (QuantiTech Primer Assays) to detect the expression of the mouse genes, using European Nucleotide Archive: *P2rx1; P2rx4; P2rx7; P2ry2; P2ry6; P2ry12; P2ry13; P2ry14; Chi3l3; Arg; Mrc1; Retnla; Il1b; Tnfa; Il6; Nos2; Adora2a; Adora2b;*

Ywhaz; Hprt1; Sdha; Gapdh or the human genes *P2RX4*, *P2RX7* or *GAPDH*. Only a single product was seen on melt curve analysis and for each primer set the efficiency of the reaction was > 95%. For the initial experiments using BMDMs, the expression of four housekeeping genes was evaluated (*Gapdh*, *Ywhaz*, *Hprt1,* and *Sdha*, Figure 24). No significant differences were observed in the normalized data to each of the four different housekeeping genes. The presented relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalizing to *Gapdh* expression levels for each treatment and the fold increase in expression was relative to the smallest expression level or to control basal levels (Livak and Schmittgen, 2001).

12. In vitro extracellular ATP degradation

Culture media from mature BMDM (day 8) or bone marrow precursors (day 0) plated in 24-well plate was replaced with BSS buffer. Then 3 mM of ATP was added to the wells and the degradation of the ATP was measured at 0, 5, 15, 30, 60 and 120 min using the ATP Bioluminescent Somatic Cell Assay kit (Sigma-Aldrich) following manufacturer's instructions. Free ATP on the supernatans was consumed and light was emitted because of the oxidation of D-luciferin by firefly luciferase catalization (as explained previously at *"Mice model for extracellular ATP in vivo"*). In this reaction, extracellular ATP is the limiting reagent, therefore the light emitted is proportional to the concentration of ATP present in the cell supernatatnts, as ATP was degraded by ectonucleotidases at differents times the light measured decreased. In a 96 wells white plate (Costar Corning Life Sciences) 3 µl of cell-free media samples or the ATP standard curve (4 µM – 0 µM, diluted in BSS buffer) were placed in triplicates and mixed with 97 µl of the provided reagents mix (luciferase, luciferin, $MgSO_4$, DTT, EDTA, BSA and tricine salts; Sigma-Aldrich) at the moment of the reading using the automatical inyectors system integrated in the Synergy Mx plate reader. Each sample was readed at endpoint three times with an integration time of 10 sec and a sensitivity level of 185.

13. Flow cytometry

Flow cytometry was used to determine different membrane markers at cell surface, cell death and cell cycle determination as detailed below:

13.1. Membrane marker quantification

To check maturation of BMDM in normal conditions or differentiated in the presence of nucleotides or LPS, the mature macrophage surface F4/80 antigen was stained. Macrophages were washed and detached using cold PBS supplemented with 2 mM EDTA (Sigma-Aldrich). Cells were first incubated with mouse seroblock-FcR antibody (AbD Serotec) and then stained with the antibody Alexa Fluor 488 conjugated anti-mouse F4/80 (Caltag Laboratories) for 30 min at 4 ºC in dark conditions. Finally, cells were washed twice with 2 ml of Citometry Buffer at 700 xg 3 min, and fixed with 1 % *p*-formaldehyde (PFA, COMPAÑIA?) in PBS. Fixed cells were subjected to flow cytometry analysis using a FACSCanto flow cytometer, keeping the flow rate under 400 events/sec and gating 10^4 events for BMDM cells based on FSC *versus* SSC parameters. Alexa Fluor 488 fluorescence was detected using FL1 channel, λ excitation = 488 nm, and λ emission = 530 ± 30 nm.

13.2. Cell death measurement

Cell death was determined in BMDMs at 2 h, 5 h and 8 days after treatment with 1 mM ATP or 0.1 mM adenosine *by using* the Live/Dead® Fixable Dead Cell Staining kit (Life Technologies) according to manufacturer's instructions. The assay is based on the reaction of a cell-impermeant fluorescent reactive dye with protein amines, in necrotic cells the reactive dye corss the plasma membrane and reacted with free amines both in the interior and on the cell surface, resulting in intense fluorescent staining. In contrast, only the cell-surface amines of viable cells were available to react with the dye, resulting in relatively dim staining (Figure 16).

BMDMs were first detached from the plate with cold PBS and then incubated with the fluorescent reactive dye for 30 min. Then, cells were washed twice with 2 ml of Citometry Buffer at 700 xg 3 min, fixed in 1 % PFA and subjected to flow cytometry using a FACSCanto flow cytometer software keeping the flow rate under 400 events/second and gating 5000 events based on FSC *versus* SSC parameters. Near-infra red fluorescence was detected using FL6 channel, λ excitation = 640 nm, and λ emission = 780 nm ± 60 nm.

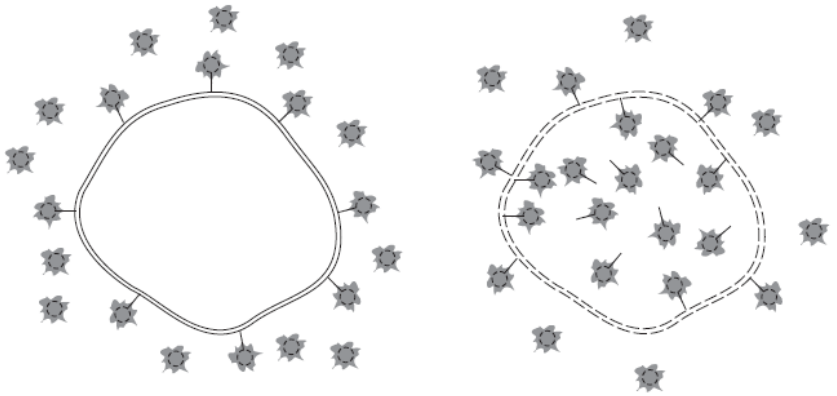

Figure 16. Live cells (left) react with the fluorescent reactive dye only on their surface to yield weakly fluorescent cells and cells with compromised membranes (right) react with the dye throughout their volume, yielding brightly stained cells

13.3. Cell cycle determination

Cell cycle was determined at 2 h and 8 days after treatment with 0.1 mM adenosine or 1 mM ATP. Cells were detached with cold PBS, then washed and fixed in ice-cold 70 % ethanol for 1 h at 4 ºC to fix and permeabilizate the cells. Then 10^6 cells were centrifuged at 400 xg for 5 min and then washed twice with 2 ml of Citometry Buffer at 700 xg 3 min. Cells were treated for 30 min at 37 ºC with 10 mg/ml ribonuclease A (Roche) and then stained for 30 min with 5 μg/ml propidium iodide (Sigma-Aldrich) before flow cytometry. Propidium iodide binds to the DNA and emit fluorescence at λ = 695 ± 40 nm after excitation at λ = 488 nm. Cells positive to propidium iodide were detected using FL3 channel in a FACSCanto flow cytometer keeping the flow rate under 400 events/second and gating 5000 events based on FSC *versus* SSC parameters. Cells in S phase of the cell cycle differentiate from cells in G1 or G2 phase by the amount of DNA

present in the cell, that is proporcional to the propidium iodide fluorescence (Darzynkiewicz et al., 2010).

14. TNF-α converting enzyme activity determination

To measure TNF-α converting enzyme (TACE) activity the 520 TACE Activity Assay Kit (AnaSpec) was used. Briefly, the assay contains a QXL™520/ 5-FAM Fluorescence Resonance Energy Transfer (FRET) substrate, derived from the sequence surrounding the cleavage site of TACE. In the intact FRET peptide, the fluorescence of 5-carboxyfluorescein (5-FAM) fluorophore is quenched by QXL™520. Active TACE cleaves FRET substrate into two separate fragments resulting in an increase of 5-FAM fluorescence as can be seen in Figure 17:

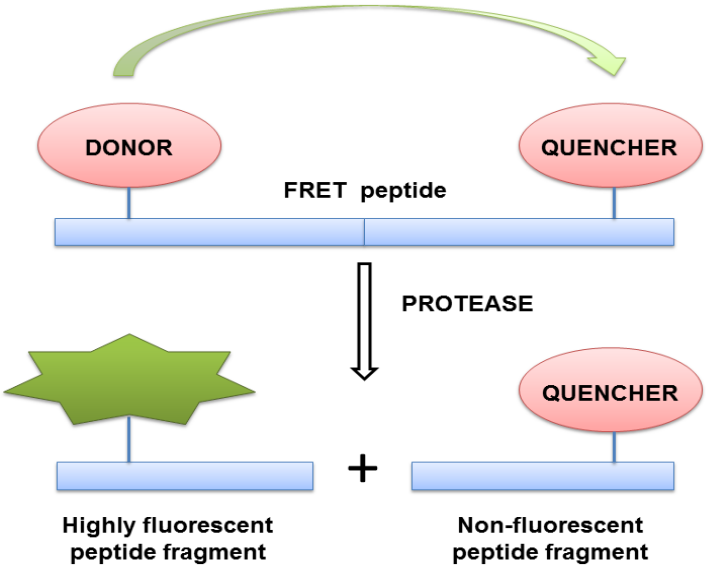

Figure 17. Active TACE cleaves FRET substrate into two separate fragments resulting in an increase of 5-FAM fluorescence

TACE activity was measured in 50 µl of cell lysates and in undiluted cell-free supernatants from 2 x 10^6 macrophages. As positive control wells in the kinetic measurement, 0.5 µM and 1µM of 5-FAM were used diluted in the kit supplied assay buffer. Right before the reading 50 µl of TACE substrate solution at 1 µM in assay buffer was added to each well. The activity was measured

using a black clear bottom 96 well plate (Costar Corning Life Sciences) in the Synergy Mx plate reader at λ excitation = 490 ± 6.75 nm, and λ emission = 520 ± 6.75 nm every 5 min during 40 min at 30 ºC. The values used were the fluorescence intensity at 20 min and values shown in results were expressed as relative fluorescence units (rfu).

15. Cathepsin B activity determination

Cathepsin B activity was measured from cell-free supernatants using a fluorescence-based assay that utilizes the preferred cathepsin-B peptide substrate sequence Arg-Arg labeled with amino-4-methylcoumarin (Z-RR-AMC, from AnaSpec). Upon cleavage with cathepsin B the AMC fluorophore is generated emitting bright blue fluorescence, being the amount of AMC fluorescence proportional to the cathepsin B activity. Supernatants were diluted 1:4 in black clear bottom 96 well plates with cathepsin assay buffer: 0.2 M sodium acetate (Sigma-Aldrich), 4 mM DTT (Sigma-Aldrich), 4 mM EDTA and 1 mM Z-RR-AMC. Samples were measured using black clear bottom 96 well plates for 90 min at 30 ºC in a Synergy Mx plate reader reading fluorescence every 5 min at λ exitation = 335 ± 60 nm and λ emmission = 460 ± 35 nm to record a kinetic of enzimatic activity and avoid possible saturation of certain samples. In the results cathepsin B activity represented was the measure obtained at 40 min of triplicated wells in each experiment and was normalized to resting or control conditions.

16. Intracellular calcium measuraments

The intracellular calcium was measured using Fura 2-acetoxymethyl (AM), that is a high affinity intracellular calcium indicator that is ratiometric and UV light excitable. The AM ester form is useful for non-invasive intracellular loading. BMDMs plated in 96-well black plates with clear bottoms were incubated at 37 °C and 5 % CO_2 for 40 min in Fura 2-AM loading buffer with 4 µM Fura 2-AM (Life Technologies) and 0.02 % pluronic acid (Merck). At the end

of this incubation, Fura 2-AM was removed and replaced with Et buffer. Fluorescence was recorded in a Synergy Mx plate reader for 200 s at 4 s intervals at a dual λ excitation coupled = 340/380 ± 6.75 nm, and λ emission = 510 ± 6.75 nm. The different nucleotides or nigericin were automatically injected into the wells at the designated time points (indicated by arrows in the Figures 32D,E, 33B y 34B). Intracellular calcium level was expressed as the ratio of the emission intensities excited at 340 and 380 nm, and the value was normalized to the fluorescence at time 0 (F/F0). The data was fitted using KaleidaGraph 4.0 for Mac (Synergy Software) by applying a Stineman function and the output was then a geometrically weighted to the current point and ± 10 % of the data range to achieve a smoothed curve, which is the one presented in the results.

17. Statistical analysis

All data are shown as mean values and error bars represent standard error (s.e.m.) from the number of independent assays indicated in the figure legends (experiments were performed 3 or more independent times to ensure reproducibility). For two-group statistical comparisons, a two-tailed unpaired Student's t-test was used, comparisons of multiple groups were analysed by one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison post-test using Prism software (Graph-Pad Software, Inc.). The "p" values are indicated as ***p <0.001; **p >0.001 <0.01; *p >0.01 <0.05; p >0.05 not significant (*ns*).

Results

1. Purinergic signaling during macrophage differentiation: activation of alternative M2 macrophages

1.1. Extracellular ATP impairs growth but not differentiation of bone marrow precursors into macrophages

We first explored the role of purinergic signaling during the differentiation of bone marrow precursors into macrophages by supplementing macrophage differentiation media with different concentrations of ATP (0.05, 0.1 and 1 mM). After plating bone marrow precursors (day 0) we monitored cell growth at different days and found that macrophages were bursting growth after day 5 of differentiation (Figure 18A). Repeated ATP application (0.1 and 1 mM) at days 0, 2, 5 and 7 resulted in a statistically significant decrease of macrophage growth (Figure 18A). Similar result was found when the number of macrophages was determined at day 8 of differentiation and a single ATP application was added at day 0, but in this case the reduction of cells only appeared statistically significant when 1 mM of ATP was used (Figure 18B). We then analyzed if bone marrow precursors differentiated for 8 days in the presence of 1 mM ATP were mature macrophages. We found similar expression of the surface mouse mature macrophage antigen F4/80 by flow cytometry in bone marrow cells differentiated during 8 days in the absence or presence of ATP (Figure 18C,D). Approximately 80% of the cells at day 8 of differentiation were mature macrophages independently of ATP treatment

(Figure 18D), suggesting that extracellular ATP do not affect macrophage differentiation, but impairs macrophage growth.

High extracellular ATP concentrations activate P2X7R and, in myeloid cells, prolonged activation of this receptor leads to cell death (Di Virgilio et al., 1998; MacKenzie et al., 2005). We then tested the hypothesis if the inhibition of cell growth induced by extracellular ATP during macrophage differentiation could be due to P2X7R activation and cell death. We analyzed *P2rx7* mRNA expression by qPCR through the differentiation of bone marrow precursors into mature macrophages and found that *P2rx7* expression significantly increased from day 6 of differentiation (Figure 19A). Treatment with the specific P2X7R antagonist A438079 prior ATP application did not recover macrophage growth (Figure 19B). Single (at day 0) or multiple (at days 0, 2, 5, 7) ATP treatments of bone marrow cultures inhibited growth of both WT and *P2rx7*$^{-/-}$ macrophages (Figure 19C,D). We next assess cell death and we found that ATP stimulation was not inducing death of cells during macrophage differentiation (Figure 19E). Furthermore, ATP was not arresting macrophage cell cycle (Figure 20). We therefore ruled out the involvement of P2X7R and cell death on macrophage growth inhibition upon ATP treatment.

1.2. Adenosine nucleoside reduce macrophage growth during differentiation

After these results we next explored the action of different nucleotides and nuecleosides on macrophage growth, and found that ADP and adenosine were also able to inhibit macrophage growth, resulting adenosine treatment with a similar effect than extracellular ATP application (Figure 21A). We then analyzed the expression of different purine receptors during macrophage differentiation (Figure 21B) and, from the expressed receptors, purine P2X and P2Y receptors increased over the time of differentiation (Figure 21B,C). A_{2B} adenosine receptor expression slightly decreased during macrophage differentiation (Figure 21B,C). We found very little or no expression for A_1, A_{2A} and A_3 adenosine receptors, as well as for $P2Y_1$ receptors at any time point of

macrophage differentiation examined. Bone marrow precursors and mature macrophages were able to quickly degrade extracellular ATP (Figure 21D), suggesting that ATP addition during macrophage differentiation could be primarily mediating its effect *via* its degradation into adenosine. This result is in accordance with previous publication where adenosine was found to inhibit macrophage growth *via* the A_{2B} adenosine receptor (Xaus et al., 1999). Here we confirmed this study, and found that the highly potent and general adenosine receptor agonist NECA was also able to arrest macrophage growth during differentiation (Figure 22A). F4/80 staining of macrophages differentiated in the presence of adenosine revealed that they were mature macrophages (Figure 22B,C). We next found that adenosine stimulation was not increasing cell death or altering cell cycle during macrophage differentiation (Figure 22D and Figure 20).

1.3. Macrophage differentiated in the presence of adenosine results in M2-like phenotype

Recently, it has been demonstrated that adenosine was able to enhance macrophage polarization towards M2 when incubated with IL-4 or IL-13 (Csóka et al., 2012). We found that macrophages differentiated in the presence of adenosine (Figure 23A) presented an increased expression of the M2-related genes *Chi3l3* (which encodes for Ym1), *Arg1* and *Mrc1*, but not for *Retnla* (which encodes for FIZZ1) (Figure 23B). Expression of proinflammatory genes associated with M1 type of macrophages (*Il1b*, *Tnfa* and *Il6*) was not affected when macrophages were differentiated in the presence of adenosine, except for *Nos2* (the gene encoding for the inducible form of nitric oxide synthase, iNOS), which expression trend to increase when macrophages were differentiated with adenosine (Figure 23B).

The use of different house keeping genes to normalize the data from the qPCR did not modify this result (Figure 24). The expression of four housekeeping genes was evaluated: *Gapdh*, *Ywhaz*, *Hprt1*, and *Sdha*. No significant differences were observed in the normalized data to each of the four

different housekeeping genes. The adenosine effect on different house keeping genes expression was analyzed by qPCR after macrophage differentiation with or without a single application of adenosine at day 0 as indicated (Figure 24A). Then *Chi3l3* gene expression was measured by qPCR after macrophage differentiation with or without a single application of adenosine at day 0, and normalized to different house keeping genes as indicated. We observed a significant increase of *Chi3l3* gene expression in the macrophage trated with adenosine when normalizing to each of the four different house keeping genes (Figure 24B).

Adenosine treatment during differentiation did not change expression of adenosine receptors A_{2A} (*Adora2a*) or A_{2B} (*Adora2b*) (Figure 23C). From all genes examined, we found that the expression of the M2 marker gene *Chi3l3* quickly decreased during macrophage growth (Figure 23D) and its expression levels were inversely correlated to the number of cells during differentiation (Figure 23D,E).

Macrophages differentiated in the presence of adenosine (Figure 25A), were able to further increase the expression of M2 related gene markers after IL-4 treatment, and presented higher expression when compared with macrophages differentiated without adenosine (Figure 25B). On the contrary, macrophages differentiated in the presence of adenosine presented a decreased expression of proinflammatory genes *Il1b*, *Il6* and *Nos2* when polarized to M1 with LPS and IFNγ. Suggesting that macrophages differentiated in the presence of adenosine present preference to polarize to M2 rather than to M1 phenotypes.

Recently, it was described that adenosine was able to increase arginase activity during M2 macrophage polarization by IL-4 and IL-13 (Csóka et al., 2012). Here we confirmed that adenosine increased *Arg1* expression induced by IL-4, and further validate that adenosine and IL-4 treatment on mature macrophages was able to increase expression of M2 related genes *Chi3l3* and *Mrc1* (Figure 26A,B). However adenosine did not change expression of the M2 gene *Retnla* after IL-4 treatment (Figure 26B). On the contrary, adenosine

decreased expression of proinflammatory genes *Tnfa*, *Il6* and *Nos2* on macrophages polarized to M1 by LPS and IFNγ stimulation (Figure 26B), but increased expression of *Il1b* gene (Figure 26B). These data suggests that adenosine affected the expression of a specific set of M1 and M2 related genes when the macrophage polarizes.

We then asked whether adenosine was able to change the fate of macrophages after differentiation (Figure 27A). The amount of mature macrophages significantly decreased upon 5 days after differentiation and adenosine did not rescued macrophage death after differentiation (Figure 27B). We then found that during macrophage senescence the expression of proinflammatory genes decreased (Figure 27C), but expression of *Chi3l3* and *Mrc1* M2 markers increased (Figure 27C). However, adenosine was not able to modify the gene expression pattern during macrophage senescence (Figure 27C). This data also support the notion that *Chi3l3* expression upregulate when the number of macrophage decreases.

1.4. LPS arrest macrophage growth and induce the expression of Chi3l3

Finally, we aimed to compare macrophage growth inhibition induced with LPS (Vadiveloo et al., 1996; Vairo et al., 1992), a classical M1 polarizing PAMP, with the effect of adenosine, a M2 promoting molecule. We found that LPS arrested macrophage growth to a lesser extent than adenosine (Figure 28A), and combination of adenosine together with LPS was not able to further decrease the number of macrophages below LPS treatment alone (Figure 28A). As expected, macrophages differentiated in the presence of LPS presented an increase in the expression of proinflammatory genes *Il1b*, *Tnfa*, *Il6* and *Nos2* (Figure 28B). Surprisingly, it also increased the expression of M2 related genes *Chi3l3* and *Arg1*, but expression of *Mrc1* and *Retnla* was decreased (Figure 28B). LPS delayed the decrease of *Chi3l3* expression found during macrophage differentiation (Figure 28C). We then found that the effect of adenosine was synergistic with LPS in increasing *Chi3l3* expression, but did not affected the

expression of A_{2B} adenosine receptor (*Adora2b*) (Figure 28D) or any of the other M1 or M2 marker genes analyzed. However, differentiation of bone marrow precursor in the presence of LPS resulted in a decrease of mature macrophages after 8 days of differentiation (Figure 28E,F).

Altogether, we found the expression of the M2 related gene *Chi3l3* (that encodes for Ym1) decreasing during macrophage differentiation from bone marrow precursors. When macrophage growth was inhibited during differentiation by adenosine (toward M2 polarization) or LPS (toward M1 polarization) the expression of *Chi3l3* expression was increased. Therefore, Ym1 emerges as a novel marker for macrophage growth inhibition, as well as for the M2 polarization phenotype of mouse macrophages.

1.5. Figures for the Results section: Purinergic signaling during macrophage differentiation leads to alternative M2 macrophages

Figure 18

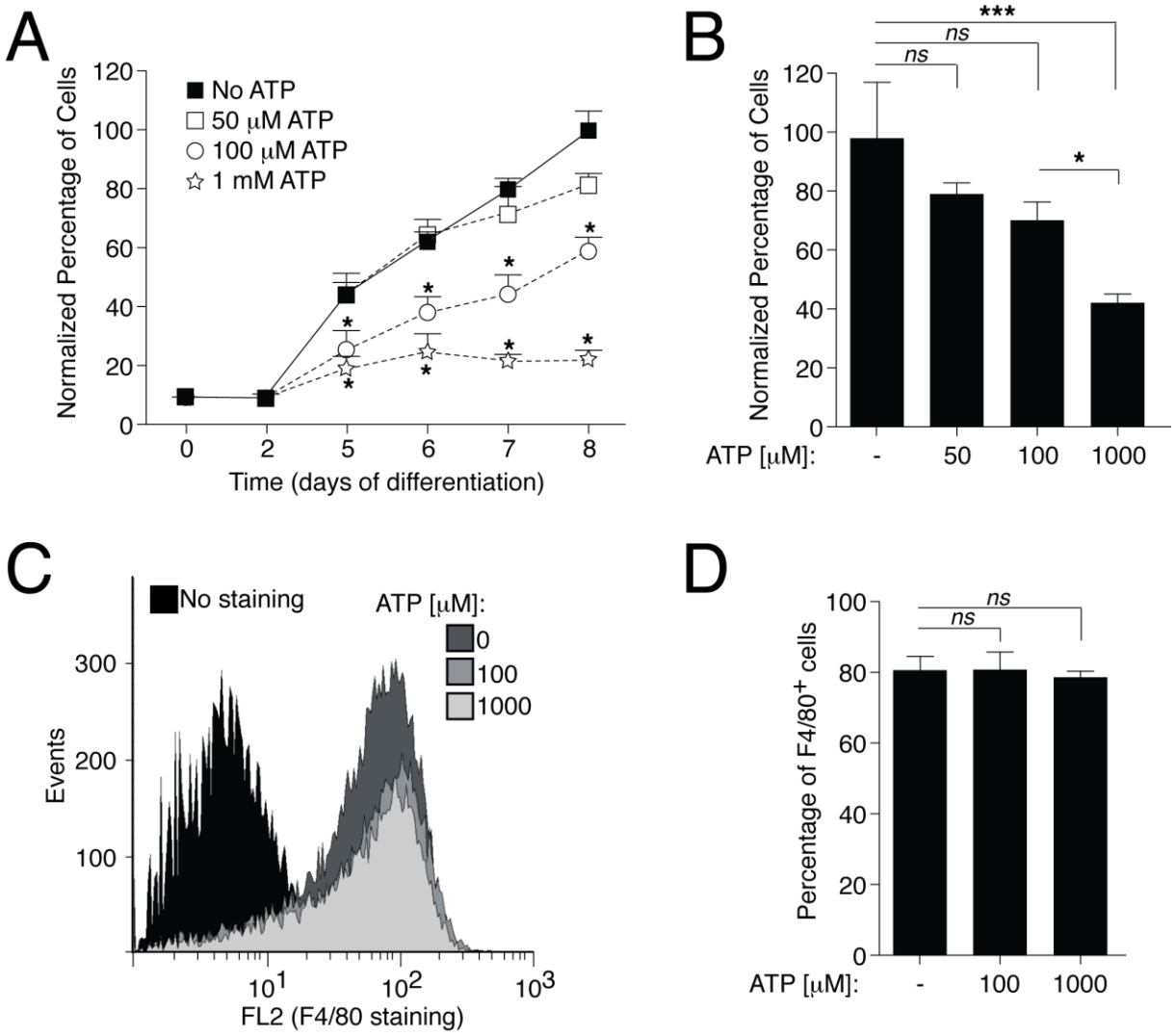
A
Normalized Percentage of Cells
No ATP
50 μM ATP
100 μM ATP
1 mM ATP
Time (days of differentiation)
B
Normalized Percentage of Cells
ns
ns
***
*
ATP [μM]:
C
No staining
ATP [μM]:
0
100
1000
Events
FL2 (F4/80 staining)
D
Percentage of F4/80+ cells
ns
ns
ATP [μM]:

Figure 18. Effect of extracellular ATP during macrophage growth and differentiation. (A) Growth of bone marrow precursors measured by MTT assay during 8 days of differentiation in the presence of different concentrations of ATP as indicated. Day 0 correspond to the day bone marrow is isolated from mice and plated. ATP was added at day 0 and then supplemented at days 2, 5 and 7; data presented as average ± s.m.e. of 8 independent experiments. (B) Growth of bone marrow precursors measured by MTT assay after 8 days of differentiation with different concentrations of ATP as indicated. A single ATP application was added at day 0; data presented as average ± s.m.e. of 7 independent experiments. (C) Representative detection of F4/80 mature macrophage antigen by flow cytometry on macrophages after 8 days of differentiation in the presence of different concentrations of ATP as indicated. A single ATP application was added at day 0. (D) Average ± s.m.e. of the percentage of F4/80 positive macrophages from 3 independent experiments as the one shown in panel C.

Figure 19

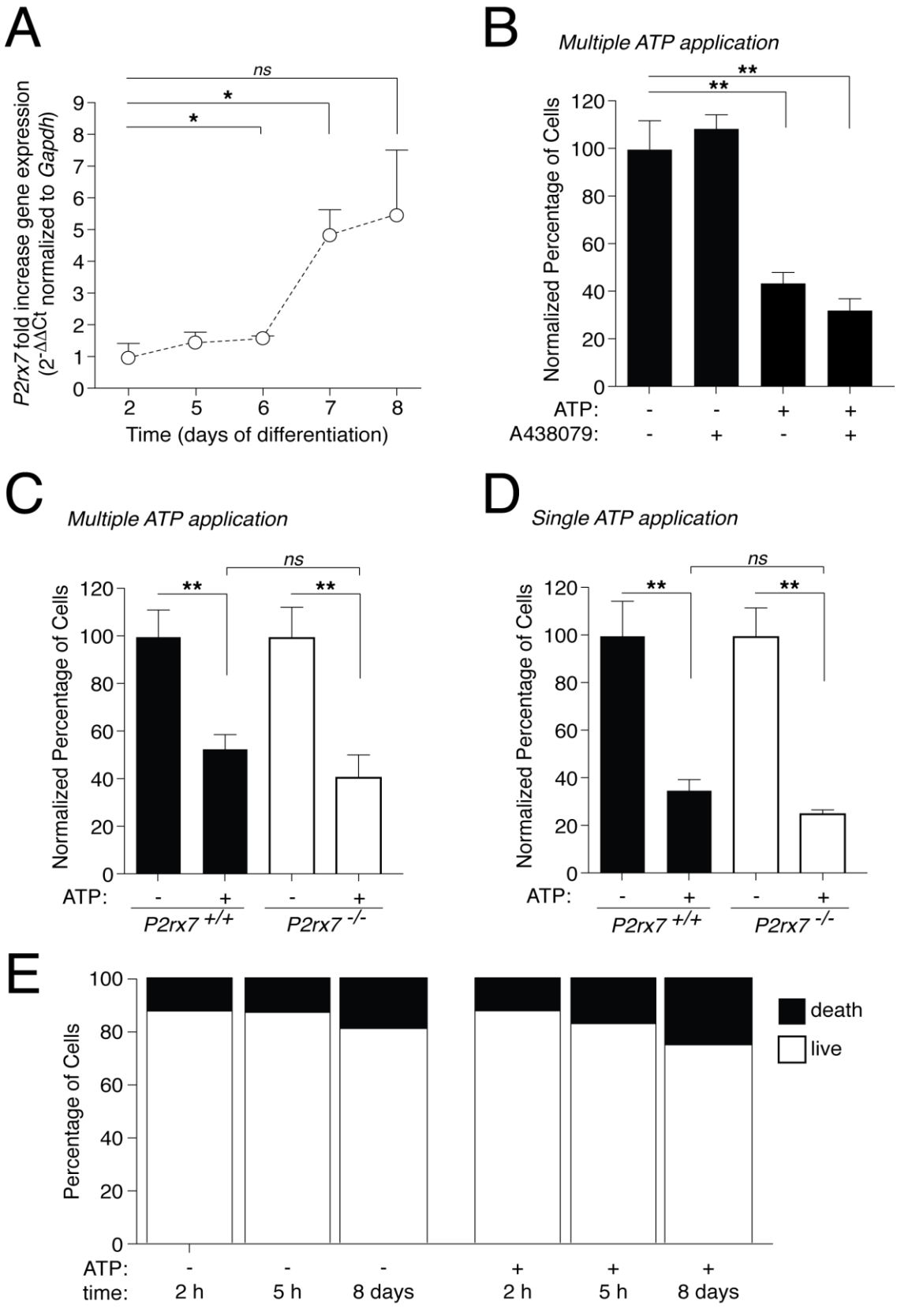
A
P2rx7 fold increase gene expression (2-ΔΔCt normalized to Gapdh)
ns
*
*
Time (days of differentiation)
B
Multiple ATP application
Normalized Percentage of Cells
**
**
ATP:
A438079:
C
Multiple ATP application
Normalized Percentage of Cells
ns
**
**
ATP:
P2rx7 +/+
P2rx7 -/-
D
Single ATP application
Normalized Percentage of Cells
ns
**
**
ATP:
P2rx7 +/+
P2rx7 -/-
E
Percentage of Cells
death
live
ATP:
time:
2 h
5 h
8 days
2 h
5 h
8 days

Figure 19. Macrophage growth inhibition by extracellular ATP is not dependent on P2X7R. (A) Expression of *P2rx7* gene analyzed by qPCR during macrophage differentiation; data presented as average ± s.m.e. of 3 independent experiments. (B) Growth of bone marrow precursors measured by MTT assay after 8 days of differentiation with ATP (1 mM) in the presence or absence of A438079 (10 μM). ATP and A438079 were supplemented at days 0, 2, 5 and 7; data presented as average ± s.m.e. of 4 independent experiments. (C,D) Growth of wild type (*P2rx7*$^{+/+}$) or *P2rx7*$^{-/-}$ bone marrow precursors measured by MTT assay after 8 days of differentiation with multiple applications of ATP (1 mM) at days 0, 2, 5 and 7 (C) or a single ATP (1 mM) application at day 0 (D); data presented as average ± s.m.e. of 3 independent experiments. (E) Percentage of viable and dead cells measured by flow cytometry as described in material and methods section 13.2 *"Cell death measurement"* at different times of differentiation with ATP (1 mM) as indicated; data presented as average of 4 experiments.

Figure 20

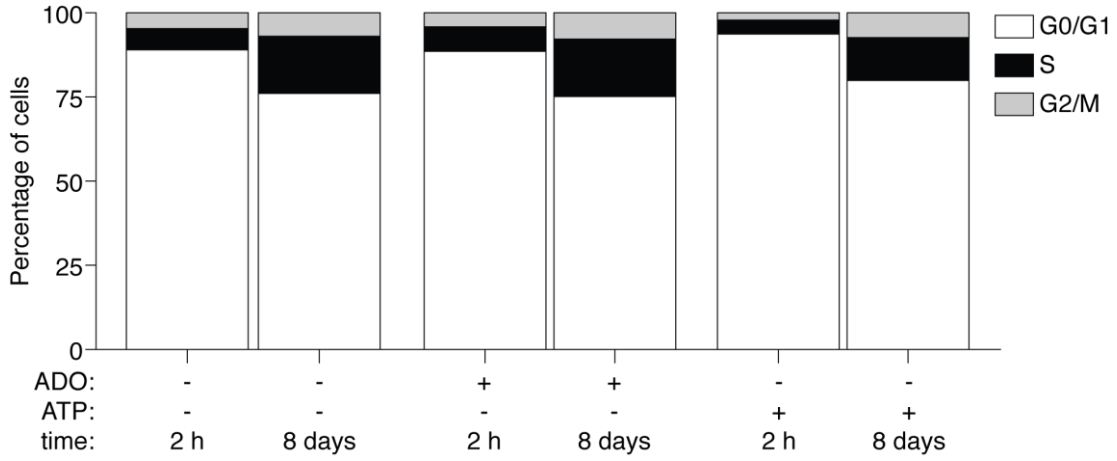
100
75
50
25
0
Percentage of cells
G0/G1
S
G2/M
ADO: - - + + - -
ATP: - - - - + +
time: 2 h 8 days 2 h 8 days 2 h 8 days

Figure 20. Effect of adenosine and ATP on macrophage cell cycle. Cell cycle analyzed by flow cytometry of bone marrow precursors after 2 h or 8 days of differentiation with ATP (1 mM) or adenosine (ADO, 1 mM) as indicated. A single dose of ATP or adenosine was added at day 0.

Figure 21

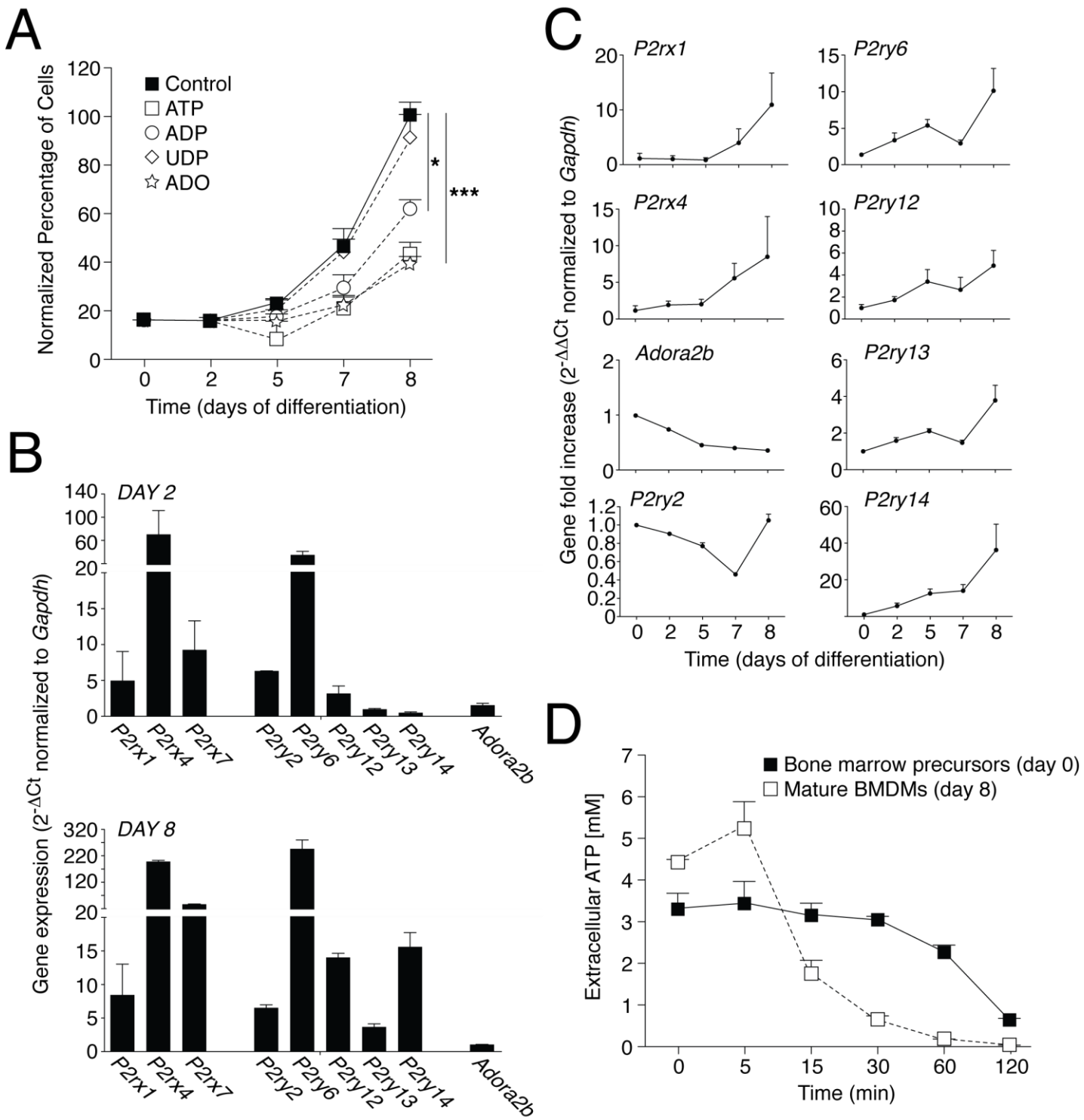
A
Normalized Percentage of Cells
Control
ATP
ADP
UDP
ADO
*
***
Time (days of differentiation)
B
DAY 2
DAY 8
Gene expression (2-ΔCt normalized to Gapdh)
P2rx1
P2rx4
P2rx7
P2ry2
P2ry6
P2ry12
P2ry13
P2ry14
Adora2b
C
P2rx1
P2ry6
P2rx4
P2ry12
Adora2b
P2ry13
P2ry2
P2ry14
Gene fold increase (2-ΔΔCt normalized to Gapdh)
Time (days of differentiation)
D
Bone marrow precursors (day 0)
Mature BMDMs (day 8)
Extracellular ATP [mM]
Time (min)

Figure 21. Effect of adenosine on macrophage growth during differentiation. (A) Growth of bone marrow precursors measured by MTT assay during 8 days of differentiation with different extracellular nucleotides as indicated (all at 0.1 mM). Day 0 correspond to the day bone marrow is isolated from mice and plated. Nucleotides were added at day 0 and then supplemented at days 2, 5 and 7; data presented as average ± s.m.e. of 3 independent experiments. ADO: adenosine. (B,C) qPCR expression of different purine receptors genes during macrophage differentiation; data presented as average ± s.m.e. of 3 independent experiments. (D) Degradation of extracellular ATP by bone marrow precursors (day 0) and mature BMDMs (day 8); data presented as average ± s.m.e. of 3 independent experiments.

Figure 22

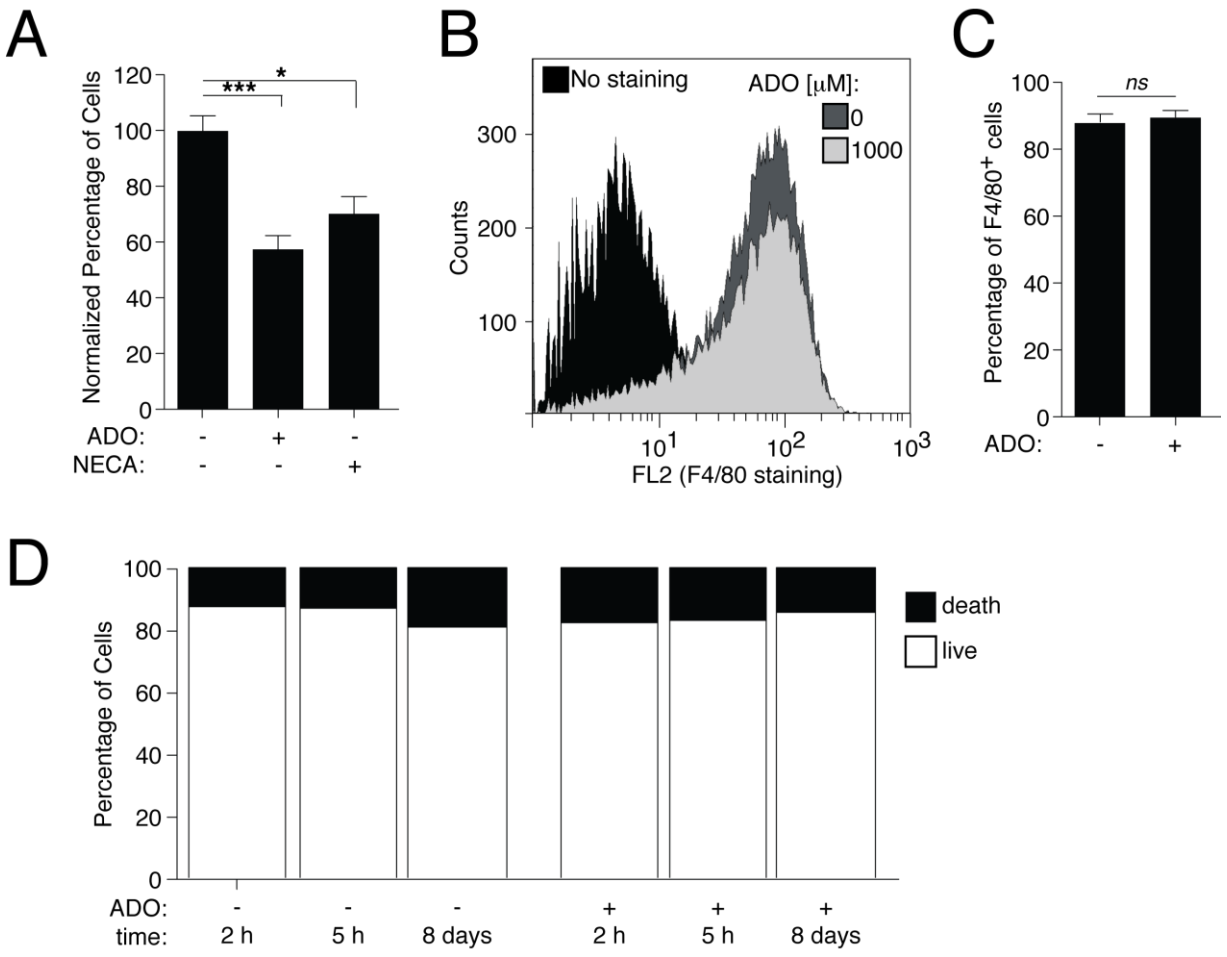
A
Normalized Percentage of Cells
120
100
80
60
40
20
0
***
*
ADO: - + -
NECA: - - +
B
No staining
ADO [μM]:
0
1000
Counts
300
200
100
10^1
10^2
10^3
FL2 (F4/80 staining)
C
Percentage of F4/80+ cells
ns
100
80
60
40
20
0
ADO: - +
D
Percentage of Cells
100
80
60
40
20
0
death
live
ADO: - - - + + +
time: 2 h 5 h 8 days 2 h 5 h 8 days

Figure 22. Macrophage growth inhibition by adenosine depends on adenosine receptors. (A) Growth of bone marrow precursors measured by MTT assay after 8 days of differentiation with a single application at day 0 of adenosine (ADO, 0.1 mM), NECA (0.1 mM); data presented as average ± s.m.e. of 8 independent experiments. (B) Representative detection of F4/80 mature macrophage antigen by flow cytometry on macrophages after 8 days of differentiation in the presence of a single application at day 0 of adenosine (ADO, 1 mM) as indicated. (C) Average ± s.m.e. of the percentage of F4/80 positive macrophages of 5 independent experiments as shown in panel B. (D) Percentage of viable and dead cells measured by flow cytometry as described in material and methods section 13.2 *"Cell death measurement"* at different times of differentiation with a single application of adenosine (ADO, 1 mM) at day 0; data presented as average of 5 experiments.

Figure 23

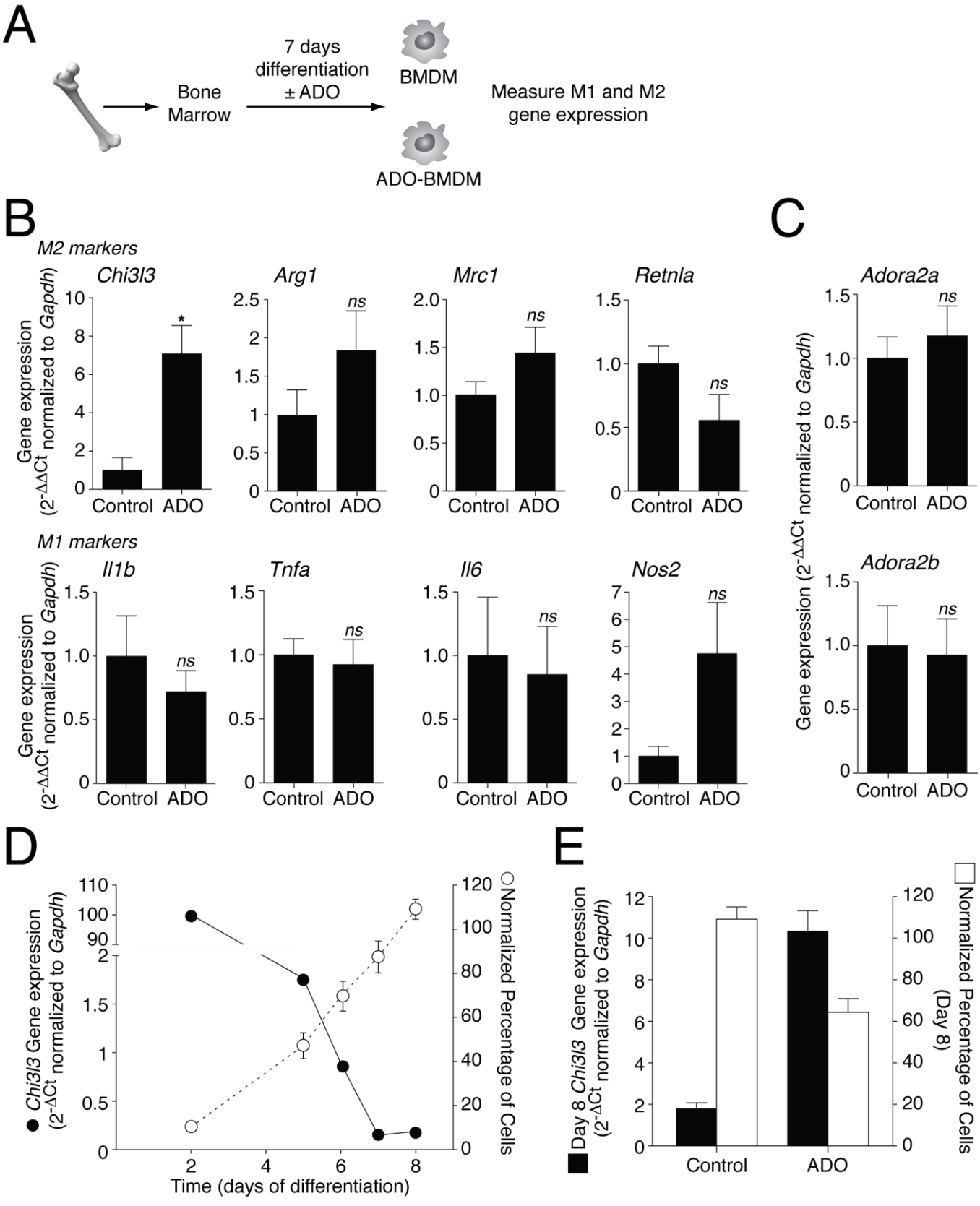
A
Bone Marrow
7 days differentiation ± ADO
BMDM
ADO-BMDM
Measure M1 and M2 gene expression
B
M2 markers
Chi3l3
Arg1
Mrc1
Retnla
M1 markers
Il1b
Tnfa
Il6
Nos2
Gene expression (2-ΔΔCt normalized to Gapdh)
Control
ADO
ns
*
C
Adora2a
Adora2b
D
Chi3l3 Gene expression (2-ΔCt normalized to Gapdh)
Normalized Percentage of Cells
Time (days of differentiation)
E
Day 8 Chi3l3 Gene expression (2-ΔCt normalized to Gapdh)
Normalized Percentage of Cells (Day 8)

Figure 23. Macrophages differentiated with adenosine result in M2-like polarization. (A) Experimental approach diagram. (B,C) M2 and M1 markers (B), and adenosine receptors (C) gene expression analyzed by qPCR after macrophage differentiation with adenosine (ADO, 0.1 mM) as indicated; data presented as average ± s.m.e. of 3 independent experiments. (D) *Chi3l3* gene expression measured by qPCR and percentage of cells determined by MTT assay during macrophage differentiation; data presented as average ± s.m.e. of 3 independent experiments. (E) *Chi3l3* gene expression analyzed by qPCR and percentage of cells measured by MTT assay after macrophage differentiation with a single application of adenosine (ADO, 0.1 mM) at day 0 as indicated; data presented as average ± s.m.e. of 3 independent experiments.

Figure 24

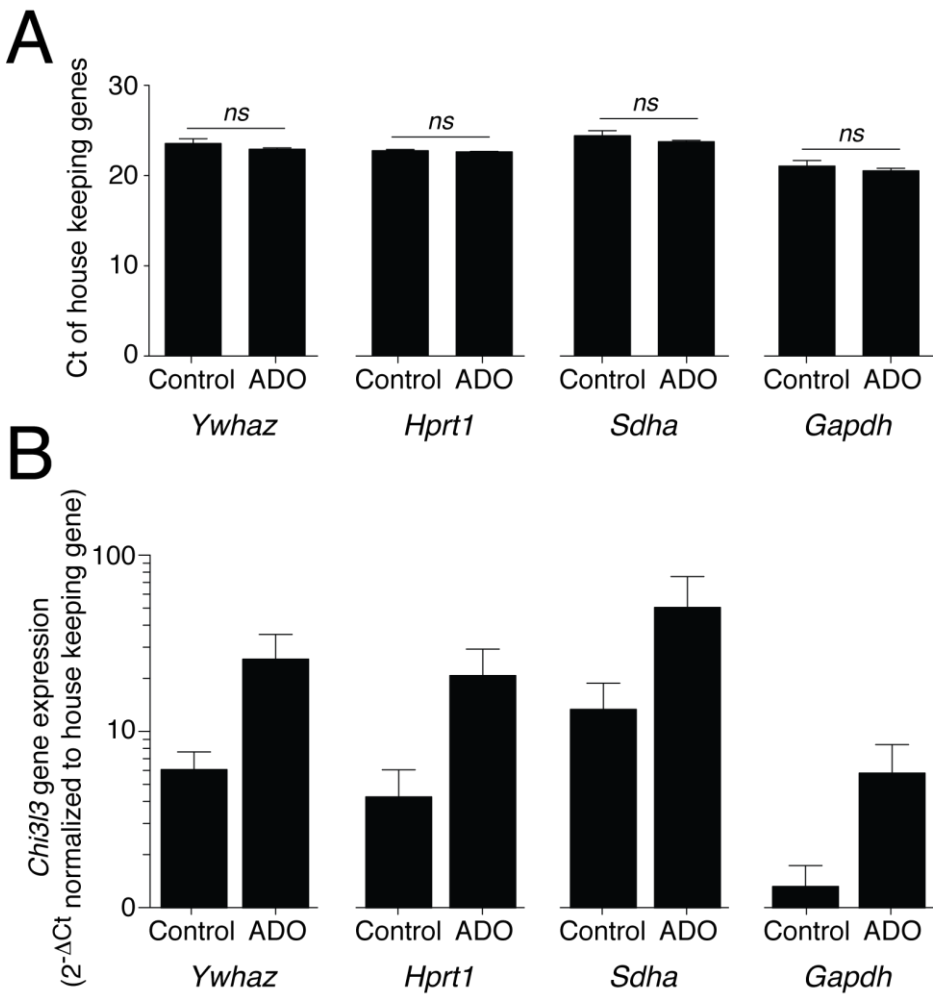
A
Ct of house keeping genes
30
20
10
0
ns
ns
ns
ns
Control ADO
Control ADO
Control ADO
Control ADO
Ywhaz
Hprt1
Sdha
Gapdh
B
Chi3l3 gene expression
(2-ΔCt normalized to house keeping gene)
100
10
0
Control ADO
Control ADO
Control ADO
Control ADO
Ywhaz
Hprt1
Sdha
Gapdh

Figure 24. Adenosine effect on house keeping gene expression. (A) Ct for different house keeping genes analyzed by qPCR after macrophage differentiation with a single application of adenosine (ADO, 0.1 mM) at day 0 as indicated; data presented as average ± s.m.e. of 5 independent experiments. (B) *Chi3l3* gene expression measured by qPCR after macrophage differentiation with a single application of adenosine (ADO, 0.1 mM) at day 0 and normalized to different house keeping genes as indicated; data presented as average ± s.m.e. of 4 independent experiments.

Figure 25

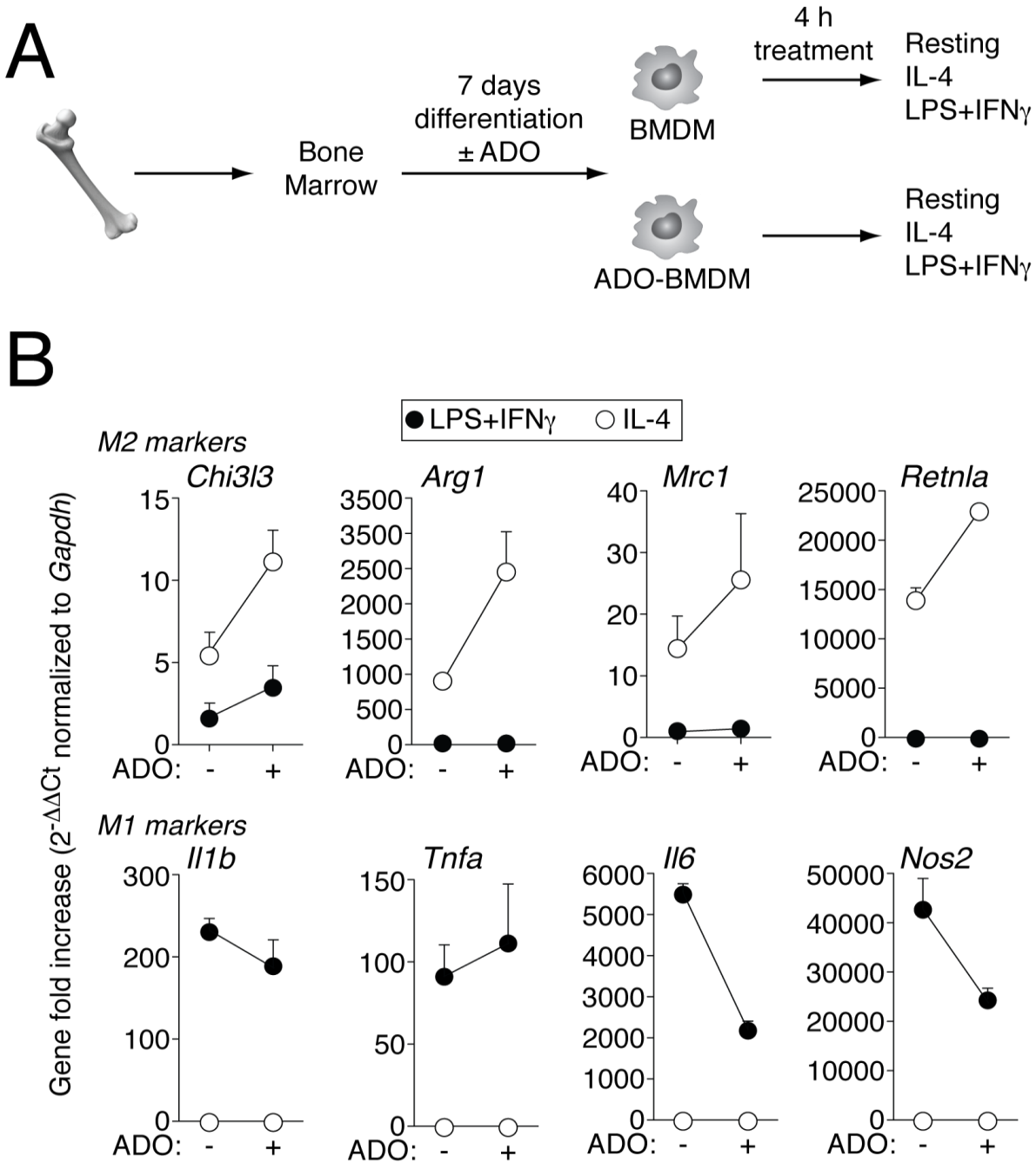
A
Bone Marrow
7 days differentiation ± ADO
BMDM
ADO-BMDM
4 h treatment
Resting
IL-4
LPS+IFNγ
Resting
IL-4
LPS+IFNγ
B
LPS+IFNγ
IL-4
M2 markers
Chi3l3
Arg1
Mrc1
Retnla
M1 markers
Il1b
Tnfa
Il6
Nos2
Gene fold increase (2-ΔΔCt normalized to Gapdh)
ADO: - +

Figure 25. Macrophages differentiated with adenosine increase M2 polarization after IL-4 treatment. (A) Experimental approach diagram. (B) Gene expression analyzed by qPCR for different M2 and M1 markers after macrophage differentiation with M-CSF and a single application of adenosine (ADO, 0.1 mM) at day 0 as indicated and subsequent polarized to M1 by LPS+IFNγ (black circles) or to M2 by IL-4 (white circles); data presented as average ± s.m.e. of 3 independent experiments.

Figure 26

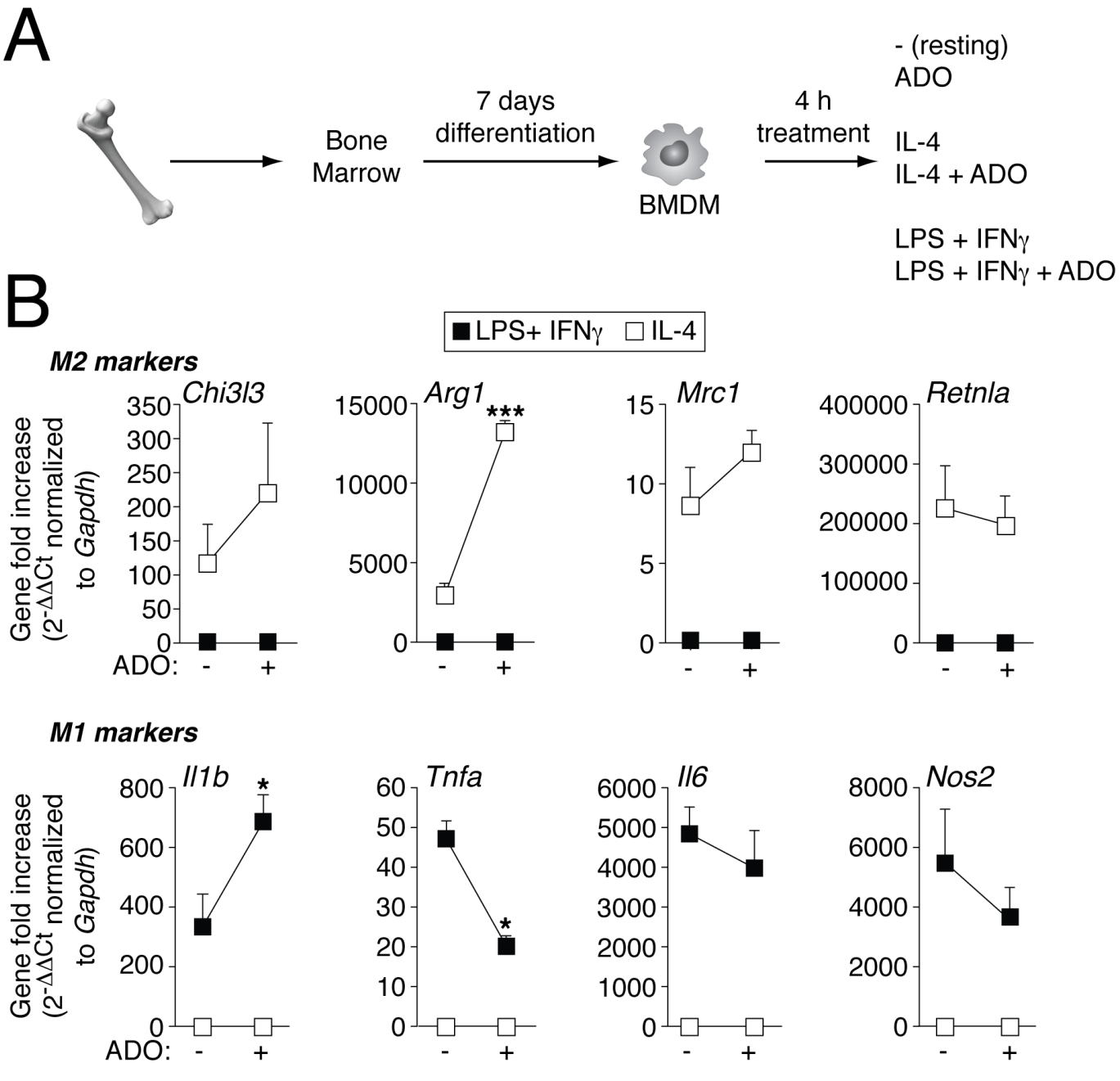
A
Bone Marrow
7 days differentiation
BMDM
4 h treatment
- (resting)
ADO
IL-4
IL-4 + ADO
LPS + IFNγ
LPS + IFNγ + ADO
B
LPS+ IFNγ
IL-4
M2 markers
Chi3l3
Arg1
Mrc1
Retnla
Gene fold increase (2-ΔΔCt normalized to Gapdh)
ADO: - +
M1 markers
Il1b
Tnfa
Il6
Nos2

Figure 26. M1 and M2 macrophage polarization is affected by adenosine. (A) Experimental approach diagram. (B) Mature BMDMs were polarized to M1 by LPS + IFNγ (black squares) or to M2 by IL-4 (white squares) in the presence of adenosine (ADO, 0.1 mM) as indicated. Gene expression for different M2 and M1 markers after polarization was analyzed by qPCR; data presented as average ± s.m.e. of 2 independent experiments.

Figure 27

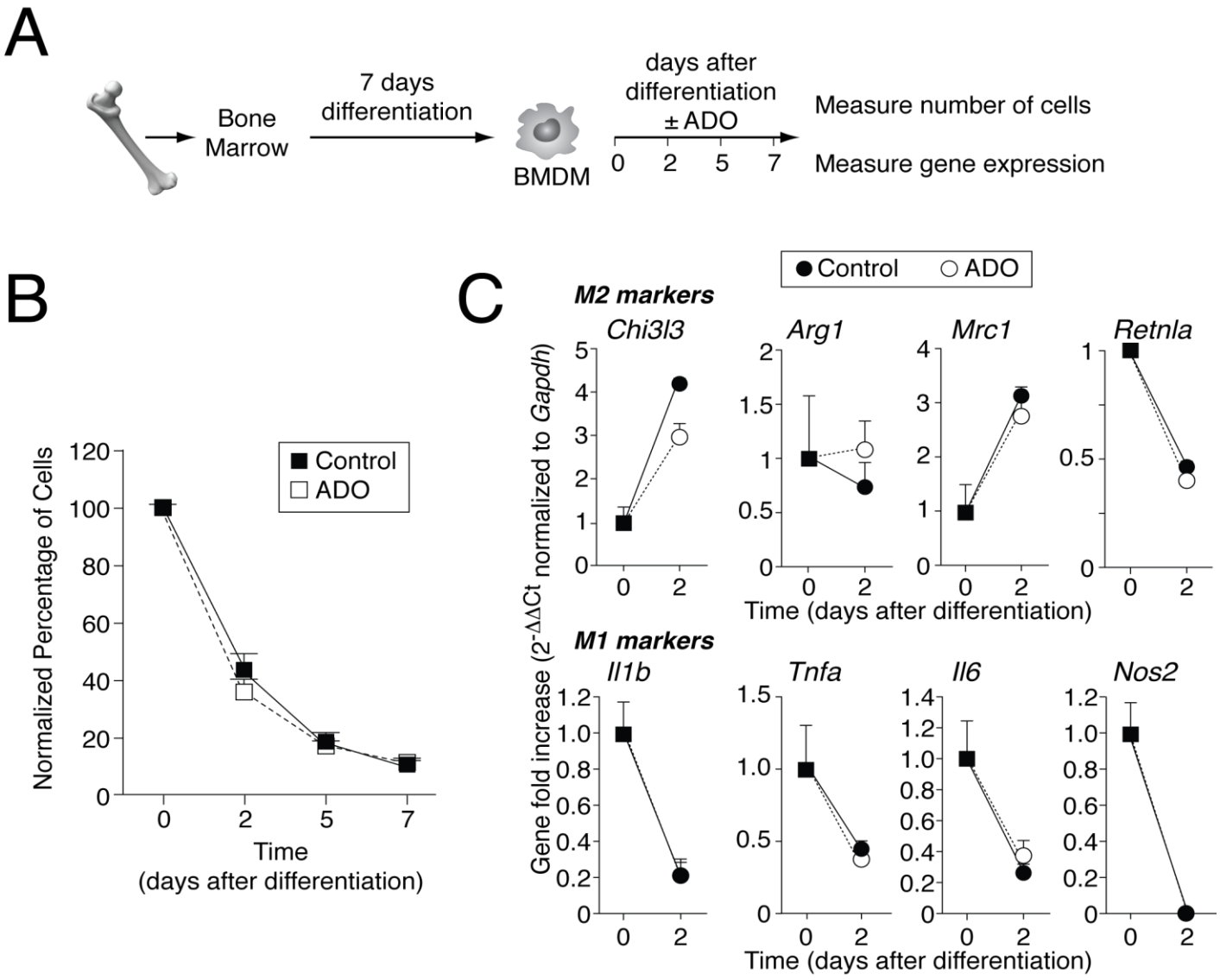
A
Bone Marrow
7 days differentiation
BMDM
days after differentiation ± ADO
0 2 5 7
Measure number of cells
Measure gene expression
B
Normalized Percentage of Cells
Control
ADO
Time (days after differentiation)
C
Control
ADO
M2 markers
Chi3l3
Arg1
Mrc1
Retnla
M1 markers
Il1b
Tnfa
Il6
Nos2
Gene fold increase (2-ΔΔCt normalized to Gapdh)
Time (days after differentiation)

Figure 27. Effect of adenosine on macrophage senescence. (A) Experimental approach diagram. (B) Mature BMDMs were cultured for 7 days with a single dose of adenosine (ADO, 0.1 mM) as indicated and viability was monitored by MTT assay at indicated times. Day 0 denotes the day macrophages were mature (and correspond to the 8th day after differentiation); data presented as average ± s.m.e. of 3 independent experiments. (C) Gene expression analyzed by qPCR for different M2 and M1 markers after 2 days of mature macrophage culture with adenosine (ADO, 0.1 mM) as indicated; data presented as average ± s.m.e. of 2 independent experiments.

Figure 28

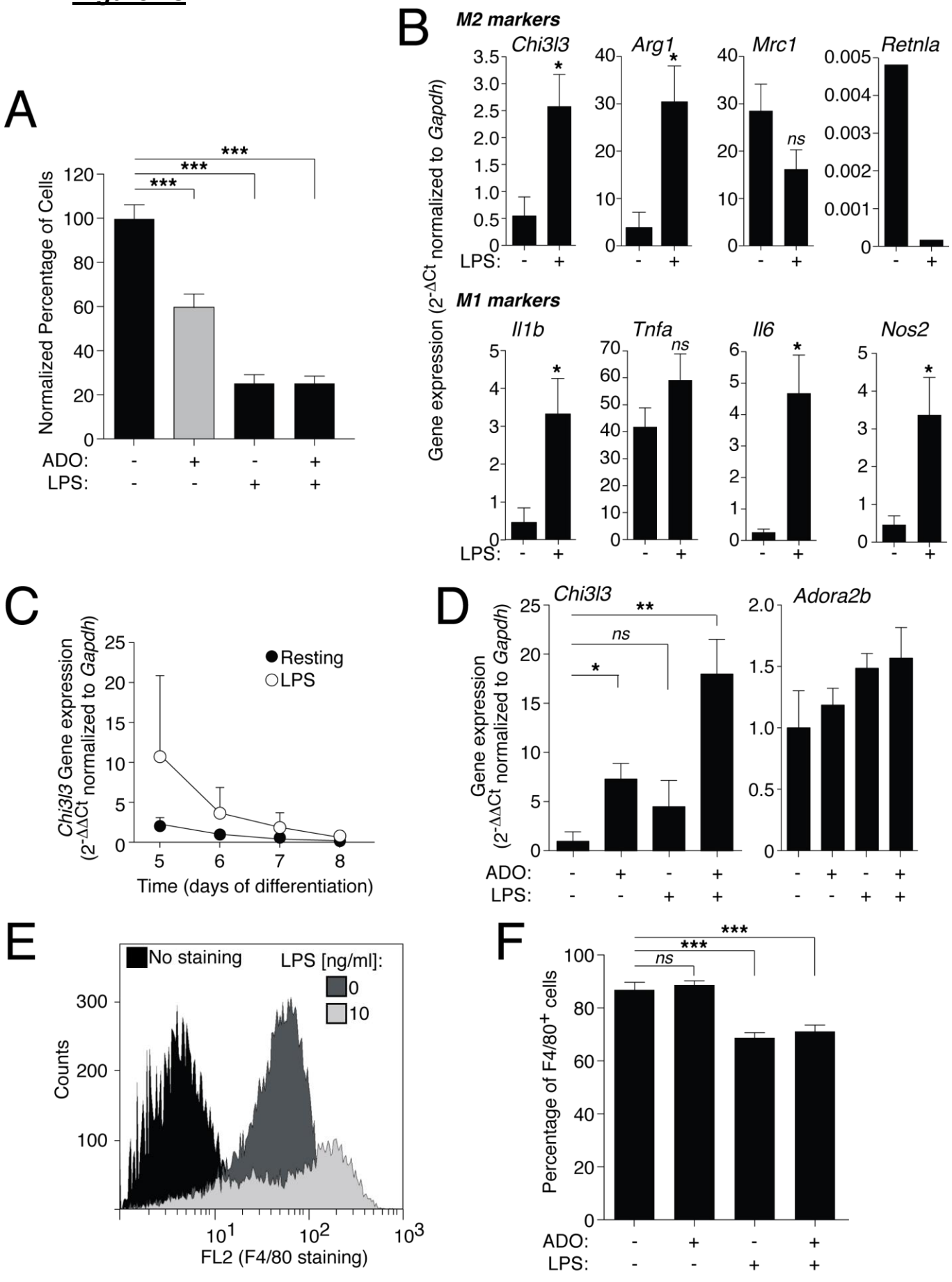
A
Normalized Percentage of Cells
ADO:
LPS:
B
M2 markers
Chi3l3
Arg1
Mrc1
Retnla
M1 markers
Il1b
Tnfa
Il6
Nos2
Gene expression (2-ΔCt normalized to Gapdh)
LPS:
ns
C
Chi3l3 Gene expression (2-ΔΔCt normalized to Gapdh)
Resting
LPS
Time (days of differentiation)
D
Chi3l3
Adora2b
Gene expression (2-ΔΔCt normalized to Gapdh)
ADO:
LPS:
E
No staining
LPS [ng/ml]:
0
10
Counts
FL2 (F4/80 staining)
F
Percentage of F4/80+ cells
ADO:
LPS:

Figure 28. Effect of LPS on macrophage growth and differentiation. (A) Growth of bone marrow precursors measured by MTT assay after 8 days of differentiation with a single dose at day 0 of adenosine (ADO, 0.1 mM) or LPS (10 ng/ml) as indicated; data is presented as average ± s.m.e. of 3 independent experiments. Please note that graph bar for adenosine treatment is the same as presented in Figure 22A and is presented here for comparison. (B) M2 and M1 gene expression analyzed by qPCR after macrophage differentiation with a single dose of LPS (10 ng/ml) at day 0 as indicated; data presented as average ± s.m.e. of 3 independent experiments. (C) *Chi3l3* gene expression measured by qPCR at days 5, 6, 7 and 8 of macrophage differentiation with a single dose of LPS (10 ng/ml) at day 0 as indicated; data presented as average ± s.m.e. of 3 independent experiments. (D) *Chi3l3* and *Adora2b* gene expression analyzed by qPCR after macrophage differentiation with a single dose of adenosine (ADO, 0.1 mM) or LPS (10 ng/ml) at day 0 as indicated; data presented as average ± s.m.e. of 3 independent experiments. (E) Representative detection of F4/80 mature macrophage antigen by flow cytometry on macrophages after 8 days of differentiation in the presence of a single LPS (10 ng/ml) application at day 0 as indicated. (F) Average ± s.m.e. of the percentage of F4/80 positive cells of 5 independent experiments as shown in panel E.

2. P2X7 receptor activation in macrophages causes fever *via* PGE2 and IL-1β release

2.1. P2X7R activation induces PGE2 release

After assessing the role of purines on macrophage differentiation, we next aimed to assess the function of ATP stimulation on mature macrophages polarized to M1 phenotype. Specially, we focused on the production of lipid mediators as important immune regulators. The application of short treatment of 30 min with 3 mM of ATP increased the levels of PGE2 in the supernatants of LPS-primed mouse macrophages by 4- to 8-fold (Figure 29A). We found a similar increase when ATP was applied over cells without washing with LPS-stimulation medium (Figure 30A). The release of PGE2 in response to ATP occurred at the same time as the release of IL-1β (Figure 29C), a well-characterized response due to P2X7R-dependent NLRP3 inflammasome and caspase-1 activation (Ferrari et al., 2006). ATP was also a stimulus for release of PGE2 and IL-1β from primary human peritoneal macrophages or blood monocytes (Figure 29E,F). Treatment with an irreversible caspase-1 inhibitor (Ac-YVAD-AOM), prior to ATP stimulation blocked IL-1β but not PGE2 release (Figure 29A,C), suggesting separate signaling pathways downstream of P2X7R for inflammasome-dependent IL-1β release and PGE2 production, respectively. Unprimed macrophages failed to release PGE2 or IL-1β in response to extracellular ATP (Figure 29A,C). The use of BzATP, a specific P2X7R agonist, also induced PGE2 and IL-1β release from LPS-primed macrophage (Figure

29B,D). Furthermore, we found that the use of the selective P2X7R antagonist A438079 reduced release of both PGE2 and IL-1β in response to ATP or BzATP (Figure 29A–D). Dose-response curves revealed an ATP EC_{50} (half maximal effective concentration) for PGE2 and IL-1β release of 1.58 mM (Figure 31A) and 1.23 mM (Figure 31B), respectively. Both EC_{50} values are in line with the ATP EC_{50} for the activation of ion currents through P2X7R (Roger et al., 2010). The P2X7R antagonist A438079 potently inhibited ATP-mediated PGE2 release at all ATP concentrations used (Figure 31A). Because prolonged P2X7R stimulation is known to cause cell death (MacKenzie et al., 2005), LDH was also measured in cell supernatants during PGE2 release experiments. Under no conditions we measured that LDH levels increase above 20 % of total cellular LDH content (Figure 31C), suggesting that PGE2 release was not a consequence of cell death.

2.2. P2X7R and P2X4R differentially regulate PGE2 release from macrophages

We then found that ATP was unable to trigger PGE2 release from macrophages derived from *P2rx7*$^{-/-}$ mice, while on the contrary, ATP-stimulated PGE2 release was unaffected by lack of P2X4R (Figure 32A), thus confirming that P2X7R is the main purinergic receptor coupled to a robust PGE2 release in macrophages. Furthermore, A438079 decreased ATP-induced PGE2 release in both WT and *P2rx4*$^{-/-}$ macrophages, again suggesting a negligible role for P2X4R in PGE2 release in response to high ATP concentrations. On the other hand, as previously shown by Rassendren and coworkers (Ulmann et al., 2010), we determinate that a low ATP dose (100 µM), subthreshold for P2X7 activation, could trigger a modest PGE2 release but to a level 3-fold lower than that caused by an ATP dose fully stimulatory for P2X7R (Figure 32B). To further characterize the involvement of P2X4R in our experiments, we used ivermectin, a drug that specifically potentiates P2X4R responses (North, 2002). Treatment of macrophages with 100 µM ATP plus ivermectin caused a 2-fold increase in PGE2 release compared to 100 µM ATP alone (Figure 32B), confirming a role for P2X4R in PGE2 release in response to low ATP concentrations. Ivermectin

treatment did not enhance PGE2 release in response to 3 mM of ATP (141.83 ± 10.17 pg/ml for 3 mM ATP, 48.17 ± 4.81 for 3 mM ATP + 3 μM ivermectin, $p > 0.05$, $n = 3$). As already reported (Ulmann et al., 2010), treatment with 100 μM of ATP plus ivermectin was ineffective to increase PGE2 release from *P2rx4*$^{-/-}$ macrophages (Figure 32B). As a control, IL-1β release was monitored in parallel and found to occur to the same level in response to ATP stimulation in WT and *P2rx4*$^{-/-}$ macrophages, being in both cases sensitive to A438079 (Figure 32C). On the contrary, ATP could not trigger IL-1β release from *P2rx7*$^{-/-}$ macrophages at any concentration tested (Figure 32C) and when used at low concentration (100 μM) from WT or *P2rx4*$^{-/-}$ macrophages, whether alone or in the presence of ivermectin (Figure 30C). P2X4R and P2X7R functional responses were validated further by checking intracellular calcium changes in response to different ATP concentrations in WT and genetically deleted mice (Figure 32D,E). At variance with mouse macrophages, human peritoneal macrophages and blood monocytes released negligible PGE2 levels in response to 100 μM ATP, and this response was not potentiated by ivermectin (Table 5), although in both primary human macrophages and monocytes, expression of P2X4R was significantly higher than that of P2X7R (Figure 32F). Adenosine (physiological agonist of P1 receptors) and various nucleotides selective for P2YR, all at 100 μM, lacked PGE2-releasing activity and did not increase intracellular calcium (Figure 33A,B), ruling out the involvement of adenosine or P2YR or receptors in ATP-induced PGE2 release from macrophages.

PGE2 release was measured using specific metabolite ELISA competition system from three different manufacturers: Cayman Chemical Company, Assay designs and Arbor Assays, all according to the manufacturer's instructions. Due to the use of three manufacturers, we ensure reproducibility of the results by comparing the different brands (Figure 30B).

2.3. Sustained intracellular calcium mediates PGE2 release after ATP stimulation

Levels of PGE2 release in response to different extracellular ATP concentrations correlated with the P2X7-dependent sustained rise in intracellular calcium, as also shown by the selective effect of the P2X7R antagonist A438079 on the late, sustained phase of calcium increase (Figure 32D). A sustained increase in intracellular calcium is also likely to play a role in response to P2X4R stimulation, as ivermectin potentiated and prolonged the increase in intracellular calcium to low ATP concentrations (Figure 32E) and the initial transient calcium rise occurring in the presence of P2X7R blockage is probably due to activation of phospholipase C *via* activation of G-protein-coupled P2YR (von Kügelgen, 2006), as it was abolished by the phospholipase C inhibitor U73122 (Figure 33B). ATP treatment in Et-EGTA buffer significantly decreased PGE2 release (Figure 34A), accordingly, the P2X7R-dependent sustained increase in intracellular calcium was also obliterated (Figure 34B). To confirm that P2Y receptors were not involved in ATP-dependent PGE2 release, we didn't observed significant differences in the presence of the phospholipase C inhibitor U73122 (Figure 34A), which fully blocked P2YR dependent release of calcium from intracellular stores but did not affect the sustained increase in intracellular calcium (Figure 34B). These data strongly suggest that calcium influx through P2X7R is the signal responsible for PGE2 release.

2.4. MAPK and COX-2 meditate PGE2 release after P2X7R stimulation

Then we desmostrated that PGE2 release in response to P2X7R stimulation was blocked by a selective COX-2 inhibitor (SC-791) but was unaffected by FR122047, a COX-1 selective inhibitor (Figure 34C). ETYA, a nonmetabolizable analog of arachidonic acid that blocks generation of all arachidonic acid metabolites, also reduced PGE2 release in response to P2X7R stimulation (Figure 34C), suggesting that the site of action of P2X7R is upstream to arachidonic acid, probably at the level of calcium-dependent cytosolic phospholipase A2 (cPLA2).

As is described MAPK are largely involved in PGE2 release by modulating arachidonic acid release and by increasing the expression levels of COX-2 (Hunot et al., 2004; Nieminen et al., 2006). It is known that P2X7R activates MAPK signaling, and participation of this pathway in P2X7R-dependent PGE2 release was confirmed by using specific JNK (SP600125) or ERK (U0126) inhibitors (Figure 34D) . On the contrary, a p38-selective antagonist (SB202190) had no effect on ATP-stimulated PGE2 release (Figure 34D). JNK mediates the synthesis of COX-2 both *in vitro* and *in vivo* (Hunot et al., 2004; Nieminen et al., 2006); thus, it might be possible that ATP effects on PGE2 release were at least in part due to induction of COX-2 synthesis. However, ATP stimulation of unprimed or primed macrophages did not change expression levels of COX-2 or PGE synthase (Figure 34E,F).

2.5. P2X7R is important for the development of fever

It has been caracterizated that IL-1β and PGE2 are key mediators of the febrile response, and as such are also known as endogenous pyrogens (Dinarello et al., 1986; Li et al., 2001; Portanova et al., 1996). Since P2X7R is a key trigger for release of both pyrogens, we investigated its involvement in the pathogenesis of the febrile response. *P2rx7*$^{-/-}$ mice presented the same temperature profile as their WT counterparts at 3 different times of the day (Table 6). Then we used these time points to measure the temperature before and after LPS injection. The temperature of both WT and *P2rx7*$^{-/-}$ mice intraperitoneal (i.p.) injected with sterile vehicle saline solution significantly decreased during the day (Table 6). After injecting LPS i.p., *P2rx7*$^{-/-}$ mice developed an attenuated febrile response compared to their WT counterparts (Figure 35A). Treatment with A438079 in WT mice before LPS injection impaired the increase of temperature associated with LPS inoculation (Figure 35B).

We next analyzed by *in vivo* bioluminescence imaging whether ATP was released in the peritoneum of mice after injection of LPS. Intraperitoneal LPS administration triggered a larger ATP release compared to saline-injected mice,

as detected by bioluminescence emission from HEK293-pmeLUC cells (Figure 35C). These data show that injection of the exogenous pyrogen LPS causes release of the endogenous danger signal ATP, which in turn activates P2X7R to cause fever.

After that we explored the role of PGE2 and IL-1β in the reduced febrile response in *P2rx7*$^{-/-}$ mice. We failed to detect an increase of PGE2 in serum or peritoneal lavage fluid of mice injected with LPS compared to mice injected with saline (data not shown). On the contrary, a robust increase in IL-1β was easily and reproducibly detected in both serum and peritoneal lavage fluid of LPS-injected mice (Figure 35D). IL-1β levels were substantially reduced in LPS-challenged *P2rx7*$^{-/-}$ mice (Figure 35D), suggesting that the lack of IL-1β release in *P2rx7*$^{-/-}$ mice could explain the reduced febrile response.

To assess whether P2X7R-derived PGE2 release was an important mediator for the development of the febrile response *in vivo*, we challenged mice with recombinant IL-1β and monitored body temperature at different time points. In contrast to LPS-induced fever, IL-1β was a powerful pyrogen in both WT and *P2rx7*$^{-/-}$ mice (Figure 35E). However, the increase in body temperature in *P2rx7*$^{-/-}$ mice was significantly shorter that in the WT animals (Figure 35E,F).

2.6. P2X7R activation induces TX and LT release

Since the effect of P2X7R stimulation on PGE2 release was mainly due to an increase of free arachidonic acid, we asked whether P2X7R could also couple to the production of other arachidonic acid metabolites. Treatment of macrophages with 3 mM ATP caused accumulation of the COX-2-dependent TXB2 and to a lesser extent the amount of COX-2-independent LTB4 (Figure 36C,D). Primary human peritoneal macrophages and blood monocytes also released TXB2 and LTB4 after ATP application (Figure 36E,F). The P2X7R antagonist A438079 powerfully blocked macrophage release of TXB2, LTB4, PGE2, and IL-1β (Figure 36A–D). The selective COX-2 inhibitor (SC-791) blocked TXB2 and PGE2, but not LTB4 or IL-1β release (Figure 36A–D),

indicating that P2X7R antagonism offers a wider anti-inflammatory range than the blocking spectra of the currently used NSAID.

2.7. Figures for the Results section: P2X7 receptor activation in macrophages causes fever via PGE2 and IL-1 β release

Figure 29

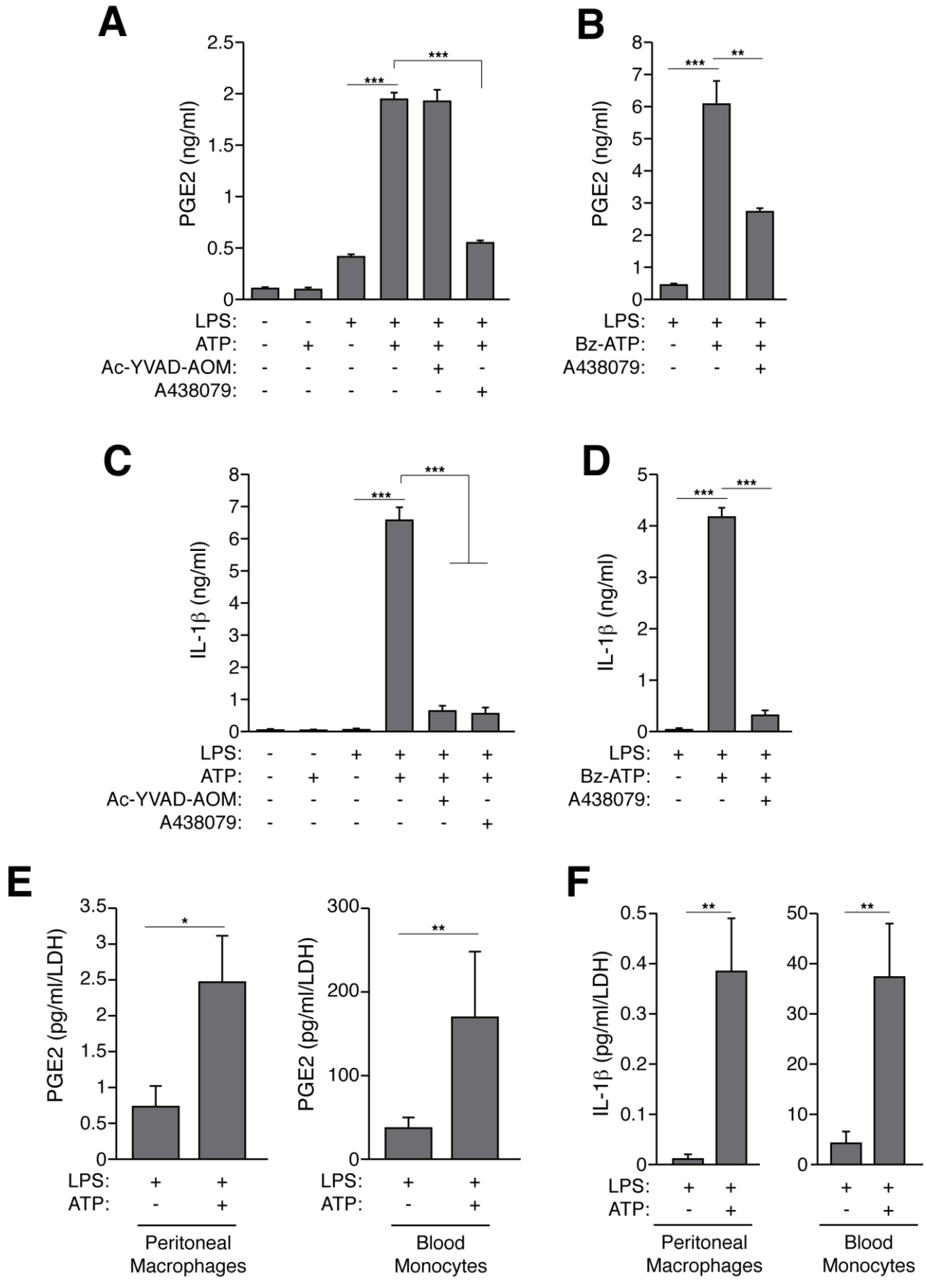
A
PGE2 (ng/ml)
LPS:
ATP:
Ac-YVAD-AOM:
A438079:
B
PGE2 (ng/ml)
LPS:
Bz-ATP:
A438079:
C
IL-1β (ng/ml)
LPS:
ATP:
Ac-YVAD-AOM:
A438079:
D
IL-1β (ng/ml)
LPS:
Bz-ATP:
A438079:
E
PGE2 (pg/ml/LDH)
LPS:
ATP:
Peritoneal Macrophages
PGE2 (pg/ml/LDH)
LPS:
ATP:
Blood Monocytes
F
IL-1β (pg/ml/LDH)
LPS:
ATP:
Peritoneal Macrophages
Blood Monocytes

Figure 29. ATP and BzATP induce release of PGE2 and IL-1β from human and murine macrophage and monocyte. A, B) ELISA for released PGE2 from mouse BMDMs primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP (A) or 300 µM of BzATP (B). The caspase-1 inhibitor (Ac-YVAD-AOM, 100 µM) or the P2X7R selective antagonist (A438079, 10 µM) was applied 10 min before and during ATP or BzATP treatment (*n* = 4–8 independent experiments). C, D) ELISA for released IL-1β from mouse BMDMs was treated with ATP (C) or BzATP (D) as in A and B (*n* = 4–8 independent experiments). E, F) ELISA for released PGE2 (E) or IL-1β (F) from primary human peritoneal macrophages or blood monocytes primed with LPS and subsequently stimulated with 3 mM of ATP (*n* = 4–7 different individuals). *ns*, not significant ($p > 0.05$). *$p > 0.01 < 0.05$; **$p > 0.001 < 0.01$; ***$p < 0.001$.

Figure 30

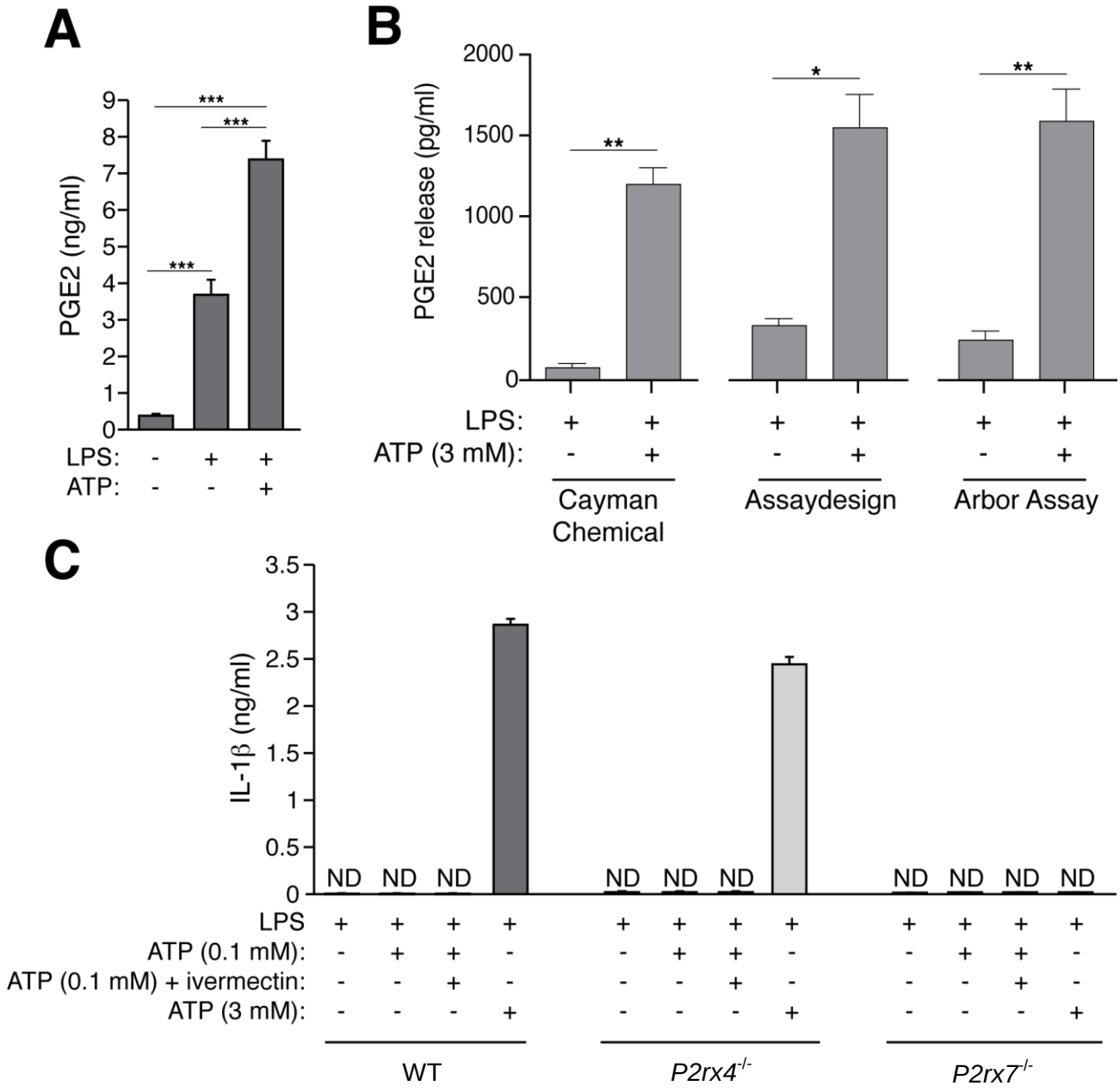
A
PGE2 (ng/ml)
***
***
***
LPS: - + +
ATP: - - +
B
PGE2 release (pg/ml)
**
*
**
LPS: + + + + + +
ATP (3 mM): - + - + - +
Cayman Chemical
Assaydesign
Arbor Assay
C
IL-1β (ng/ml)
ND ND ND
LPS + + + + + + + + + + + +
ATP (0.1 mM): - + + - - + + - - + + -
ATP (0.1 mM) + ivermectin: - - + - - - + - - - + -
ATP (3 mM): - - - + - - - + - - - +
WT
P2rx4-/-
P2rx7-/-

Figure 30 A) ELISA for released PGE2 from mouse BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP on the top of LPS (without washing LPS stimulation medium). B) ELISA for PGE2 release from BMDM primed with LPS and subsequently washed with Et-buffer and stimulated for 30 min with 3 mM of ATP. The PGE2 determination was performed using three different ELISA kit brands; n = 4 independent experiments for each ELISA kit (samples were not the same among the different ELISA kit). C) ELISA for IL-1β release from BMDM WT, *P2rx4*$^{-/-}$ or *P2rx7*$^{-/-}$ mice primed with LPS and subsequently stimulated for 30 min with 0.1 mM ATP or 3 mM of ATP in the presence or absence of ivermectin (3 μM) 10 min before and during ATP stimulation (n = 4 independent experiments for each genotype); ND: not detected.

Figure 31

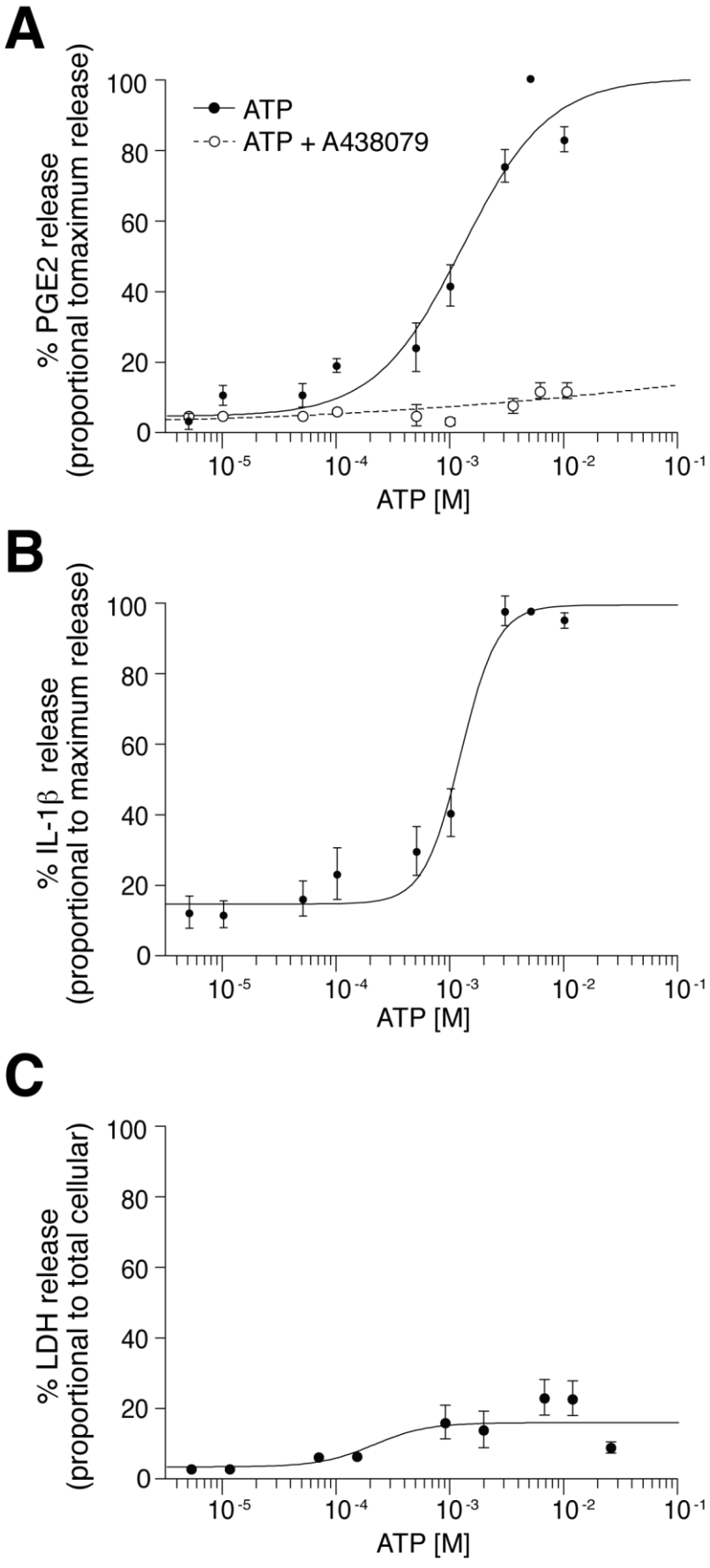
A
% PGE2 release
(proportional tomaximum release)
ATP
ATP + A438079
ATP [M]
B
% IL-1β release
(proportional to maximum release)
ATP [M]
C
% LDH release
(proportional to total cellular)
ATP [M]

Figure 31. ATP dose-response relations for PGE2, IL-1β and LDH release. A) ELISA for released PGE2 from mouse BMDMs primed with LPS and subsequently stimulated for 30 min with different concentrations of ATP in the presence or absence of the P2X7R selective antagonist (A438079, 10 μM) 10 min before and during ATP stimulation (*n* = 5–8 independent experiments for ATP; *n* = 3–6 independent experiments for ATP+A438079). ATP EC_{50} for PGE2 release was 1.58 mM; maximum concentration of PGE2 released was 2.05 ± 0.4 ng/ml (*n* = 7). B) ELISA for released IL-1β from mouse BMDMs treated as in A (*n* = 4 independent experiments for each ATP concentration). ATP EC_{50} for IL-1β release was 1.23 mM; maximum concentration of IL-1β released was 5.17 ± 0.8 ng/ml (*n* = 4). C) Presence of LDH in the supernatants of mouse BMDMs treated as in A as a test for cell death. In any conditions, the levels of LDH were >20% of the total cellular LDH content (n = 6 independent experiments for each ATP concentration).

Figure 32

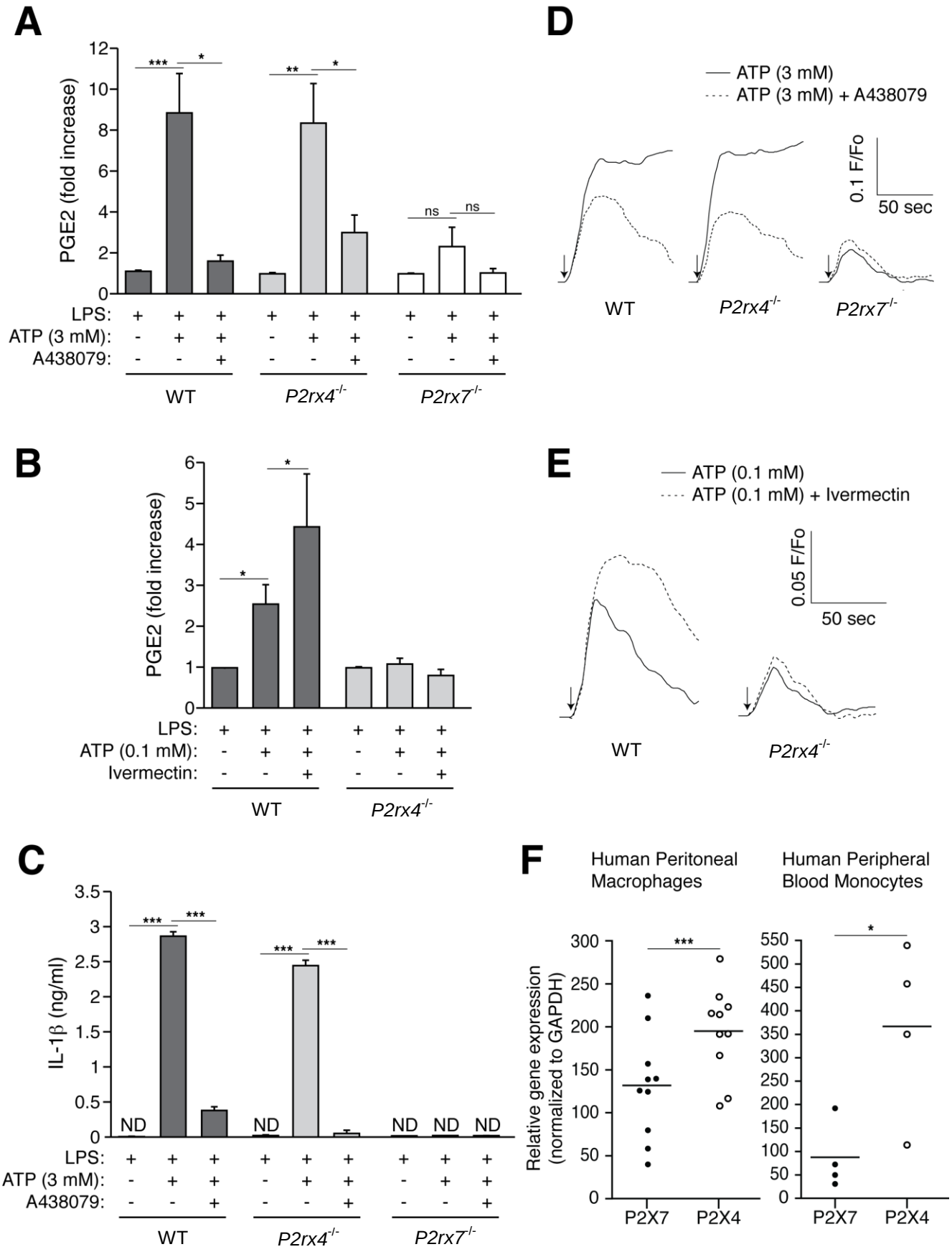
A
PGE2 (fold increase)
LPS:
ATP (3 mM):
A438079:
WT
P2rx4-/-
P2rx7-/-
ns
B
PGE2 (fold increase)
LPS:
ATP (0.1 mM):
Ivermectin:
WT
P2rx4-/-
C
IL-1β (ng/ml)
ND
LPS:
ATP (3 mM):
A438079:
WT
P2rx4-/-
P2rx7-/-
D
ATP (3 mM)
ATP (3 mM) + A438079
0.1 F/Fo
50 sec
WT
P2rx4-/-
P2rx7-/-
E
ATP (0.1 mM)
ATP (0.1 mM) + Ivermectin
0.05 F/Fo
50 sec
WT
P2rx4-/-
F
Human Peritoneal Macrophages
Human Peripheral Blood Monocytes
Relative gene expression (normalized to GAPDH)
P2X7
P2X4
P2X7
P2X4

Figure 32. P2X7R and P2X4R differentially regulate PGE2 release from macrophages in response to extracellular ATP. A) ELISA for released PGE2 from BMDM WT, *P2rx4*$^{-/-}$, or *P2rx7*$^{-/-}$mice primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of the P2X7R selective antagonist (A438079, 10 µM) 10 min before and during ATP stimulation (*n* = 6–12 for WT; *n* = 4 for *P2rx4*$^{-/-}$and *P2rx7*$^{-/-}$independent experiments). Basal PGE2 releases from the different genotypes (n = 4 independent experiments) used to calculate the fold increase showed in the figure were (pg/ml): 99.0 ± 15.9 for WT, 79.2 ± 17.3 for *P2rx4*$^{-/-}$, and 80.5 ± 16.2 for *P2rx7*$^{-/-}$. B) ELISA for released PGE2 from BMDMs from WT or *P2rx4*$^{-/-}$ mice primed as in A and stimulated for 30 min with 0.1 mM of ATP in the presence or absence of 3 µM of ivermectin (n4 independent experiments). C) IL-1β release was monitored in parallel to PGE2 release as explained in A (*n* = 4–6 independent experiments). D, E) Representative trace of 4–11 independent experiments for intracellular calcium rise in BMDMs derived from WT, *P2rx4*$^{-/-}$, or *P2rx7*$^{-/-}$ mice stimulated as in A and B with A438079 (C) or ivermectin (D). F) Relative gene expression for P2X4R and P2X7R, determined by quantitative RT-PCR in different individual samples of primary human macrophages (*n* = 10) and blood monocytes (*n* = 4). ND, not detected; *ns*, not significant (p >0.05). *p >0.01 <0.05; **p >0.001 <0.01; ***p <0.001.

Figure 33

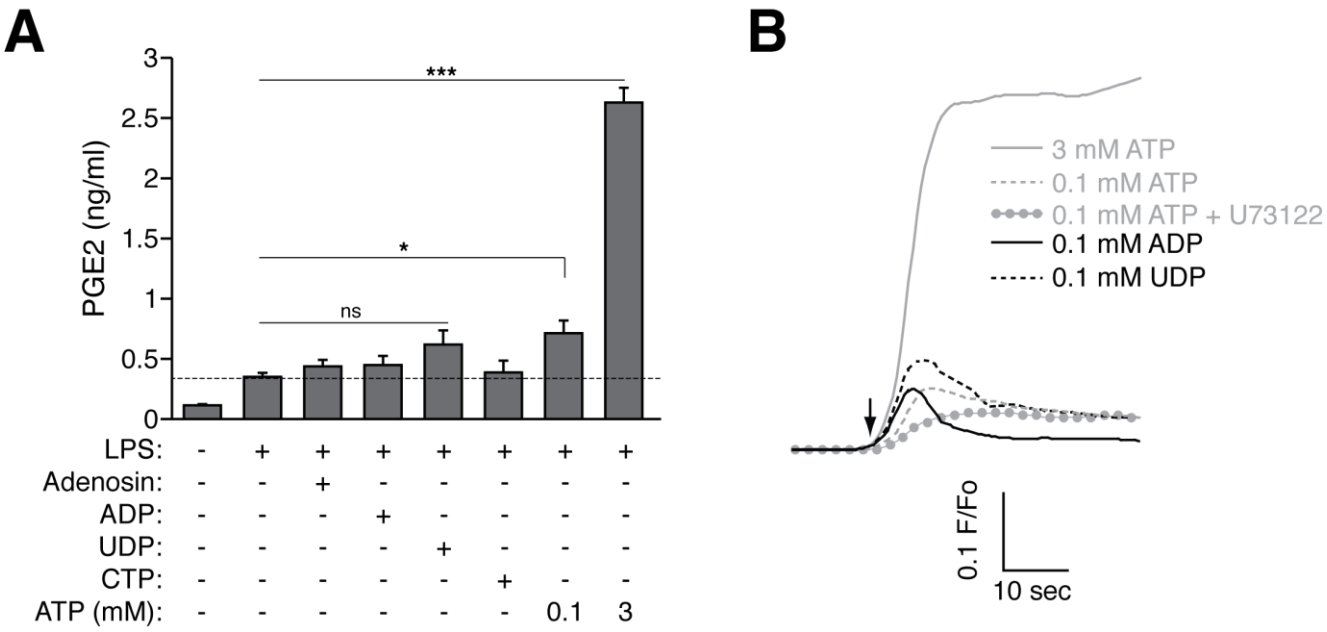
A
PGE2 (ng/ml)
3
2.5
2
1.5
1
0.5
0
***
*
ns
LPS: - + + + + + + +
Adenosin: - - + - - - - -
ADP: - - - + - - - -
UDP: - - - - + - - -
CTP: - - - - - + - -
ATP (mM): - - - - - - 0.1 3
B
3 mM ATP
0.1 mM ATP
0.1 mM ATP + U73122
0.1 mM ADP
0.1 mM UDP
0.1 F/Fo
10 sec

Figure 33. A) ELISA for PGE2 release from BMDM primed with LPS and subsequently stimulated for 30 min with 0.1 mM of different nucleotides or 3 mM of ATP. B) Representative trace out of 3 independent experiments for intracellular calcium rise in BMDM stimulated as in A in the presence or absence of the PLC inhibitor U73122 (10 μM). Asterisks represent significance compared with LPS treated group. *ns*, not significant ($p > 0.05$); *$p > 0.01 < 0.05$; ***$p < 0.001$.

Figure 34

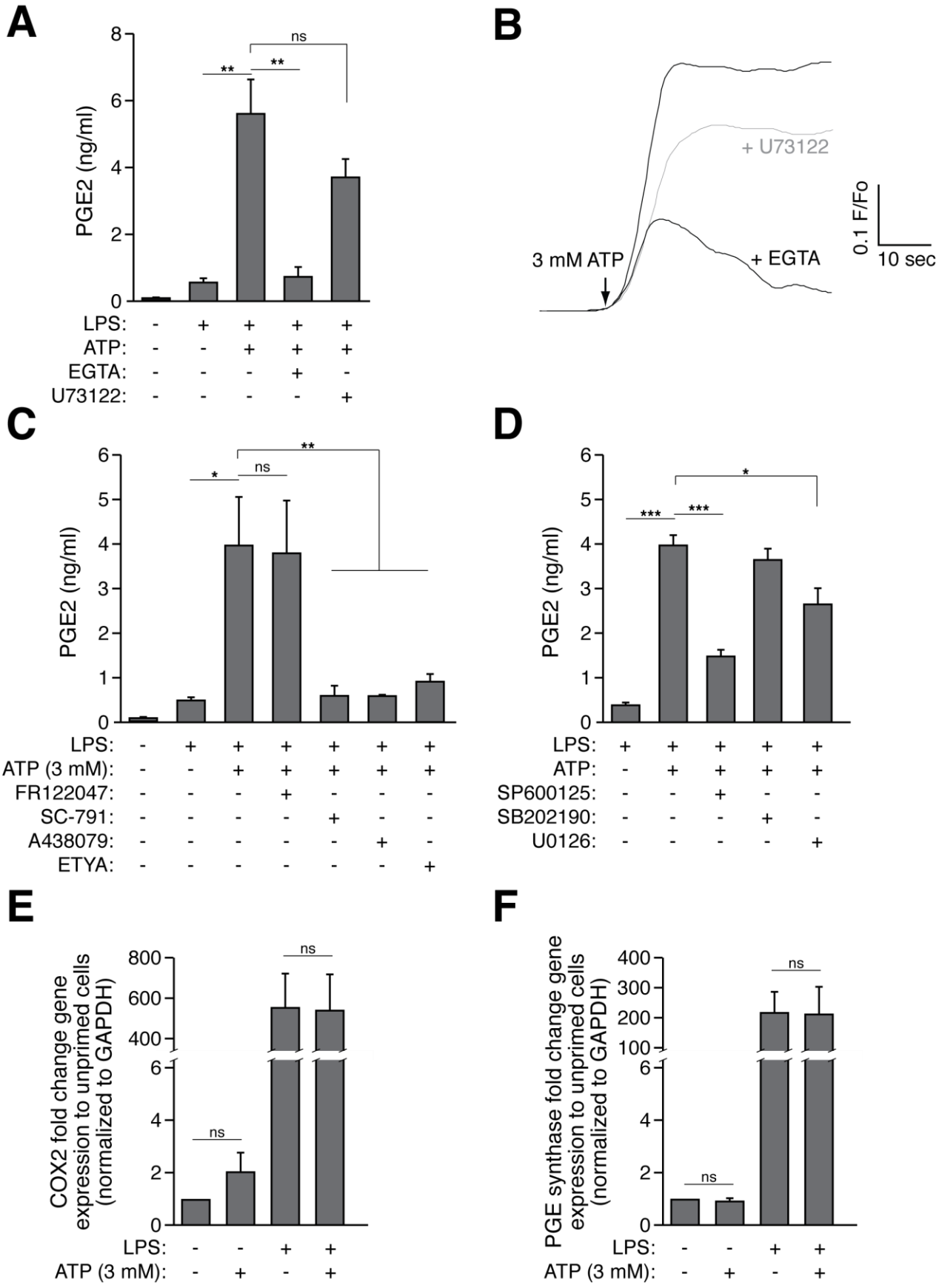
A
PGE2 (ng/ml)
ns
**
**
LPS:
ATP:
EGTA:
U73122:
B
+ U73122
3 mM ATP
+ EGTA
0.1 F/Fo
10 sec
C
PGE2 (ng/ml)
*
ns
**
LPS:
ATP (3 mM):
FR122047:
SC-791:
A438079:
ETYA:
D
PGE2 (ng/ml)
***
***
*
LPS:
ATP:
SP600125:
SB202190:
U0126:
E
COX2 fold change gene expression to unprimed cells (normalized to GAPDH)
ns
ns
LPS:
ATP (3 mM):
F
PGE synthase fold change gene expression to unprimed cells (normalized to GAPDH)
ns
ns
LPS:
ATP (3 mM):

Figure 34. MAPK and COX-2 meditate PGE2 release after P2X7R stimulation. A) ELISA for released PGE2 from BMDMs primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of extracellular calcium (EGTA) or PLC inhibitor U73122 (10 µM) (*n* = 4–7 independent experiments). B) Representative trace of 2–5 independent experiments for intracellular calcium rise in BMDMs stimulated as in A. C, D) ELISA for released PGE2 from BMDMs stimulated as in A in the presence or absence of COX1 selective inhibitor (FR122047, 2 µM), selective COX2 inhibitor (SC-791, 2 µM), P2X7R selective antagonist (A438079, 10 µM), and a non-metabolizable analog of arachidonic acid (ETYA, 10 µM) (C) or a specific JNK inhibitor (SP600125, 10 µM), specific ERK inhibitor (U0126, 10 µM), or p38 selective inhibitor (SB202190, 10 µM) (D) 10 min before and during ATP stimulation (*n* = 4–7 independent experiments). E, F) Relative gene expression for COX2 (E) and PGE synthase (F) determined by quantitative RT-PCR in unprimed- or LPS primed-BMDMs and subsequently stimulated with ATP for 30 min (*n* = 3 independent experiments). *ns*, not significant ($p > 0.05$). *$p > 0.01 < 0.05$; **$p > 0.001 < 0.01$; ***$p < 0.001$.

Figure 35

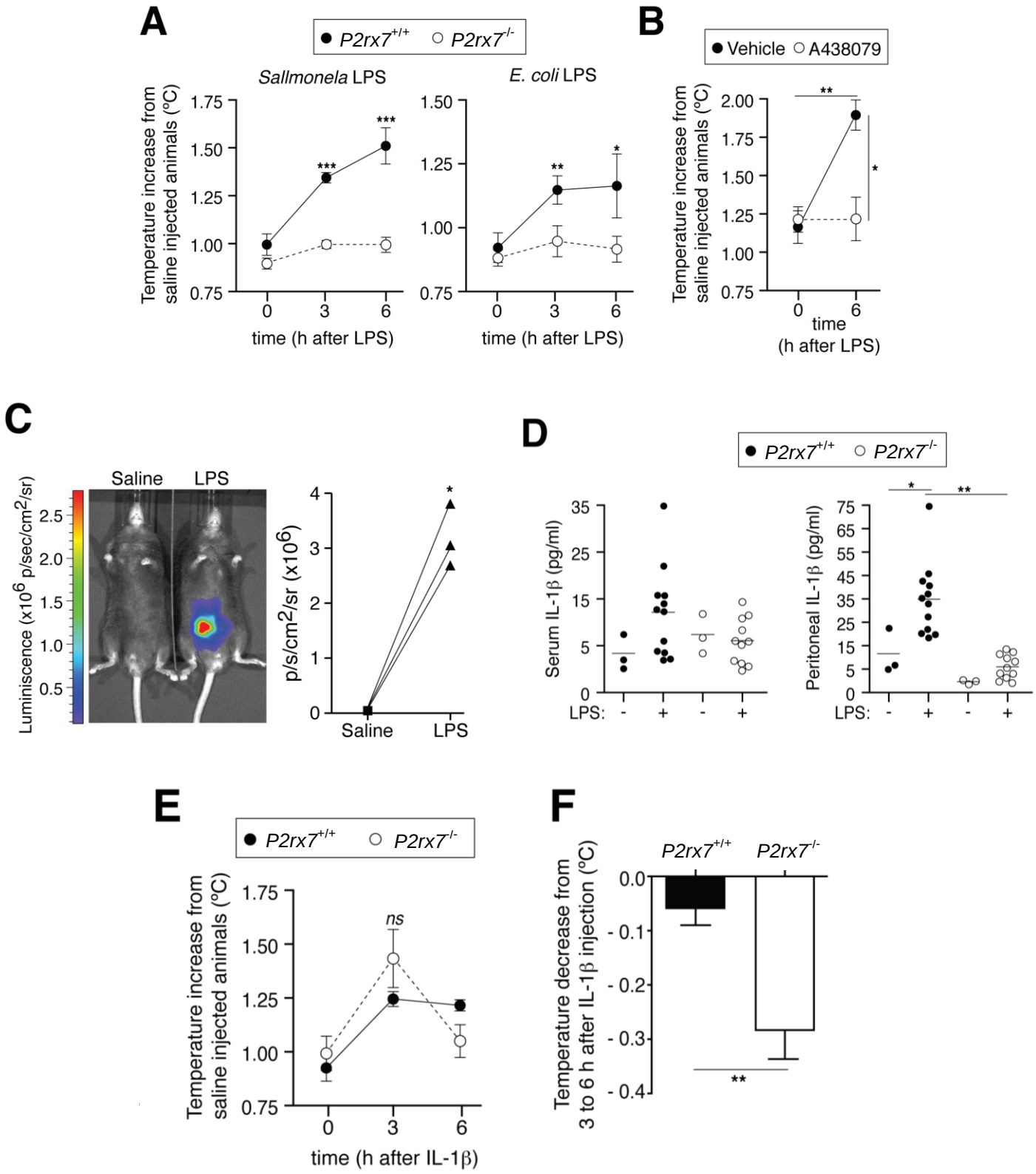
A
P2rx7+/+ P2rx7-/-
Sallmonela LPS
E. coli LPS
Temperature increase from saline injected animals (ºC)
time (h after LPS)
B
Vehicle A438079
time (h after LPS)
C
Saline LPS
Luminiscence (x10^6 p/sec/cm2/sr)
p/s/cm2/sr (x10^6)
D
Serum IL-1β (pg/ml)
Peritoneal IL-1β (pg/ml)
LPS:
E
ns
time (h after IL-1β)
F
Temperature decrease from 3 to 6 h after IL-1β injection (ºC)

Figure 35. P2X7R is important for the development of fever. A) C57 BL/6 (WT; *P2rx7*$^{+/+}$) and *P2rx7*$^{-/-}$ mice were i.p. injected with *Salmonella* or *E. coli* LPS (*n* = 6–10 mice/group). Rectal temperature of mice was measured with an electronic thermometer immediately before injections and after 3 and 6 h postinjection; asterisks mark significant differences between WT and *P2rx7*$^{-/-}$ group. B) WT mice were injected i.p. with A438079 or vehicle, and 1 h later with *Salmonella* LPS (*n* = 4 mice/group). Rectal temperature was measured as in A. C) ATP release in the peritoneum of mice after 4 h injection with saline or *Salmonella* LPS. Mice received ATP-dependent luciferase-expressing HEK293 cells into the peritoneum after 2 h saline or LPS injection, and luciferin was injected i.p. before 10 min of imaging. Left panel: one representative experiment. Right panel: quantification of bioluminescence as average radiance (p/s/cm2/sr) from 3 different experiments; asterisk marks significant differences between saline and LPS group (n = 3 mice/group). D) ELISA for IL-1β in the serum or peritoneal lavage of WT or *P2rx7*$^{-/-}$ mice after 4 h injection i.p. with saline or *Salmonella* LPS (*n* = 3–12 mice/group). E) WT or *P2rx7*$^{-/-}$ mice were injected i.p. with recombinant IL-1β, and rectal temperature was monitored as in A (*n* = 6 mice). F) Temperature decrease in WT and *P2rx7*$^{-/-}$ mice from 3 to 6 h after IL-1β injection (*n* = 6 mice/group). *ns*, not significant ($p > 0.05$). *$p > 0.01 < 0.05$; **$p > 0.001 < 0.01$; ***$p < 0.001$.

Figure 36

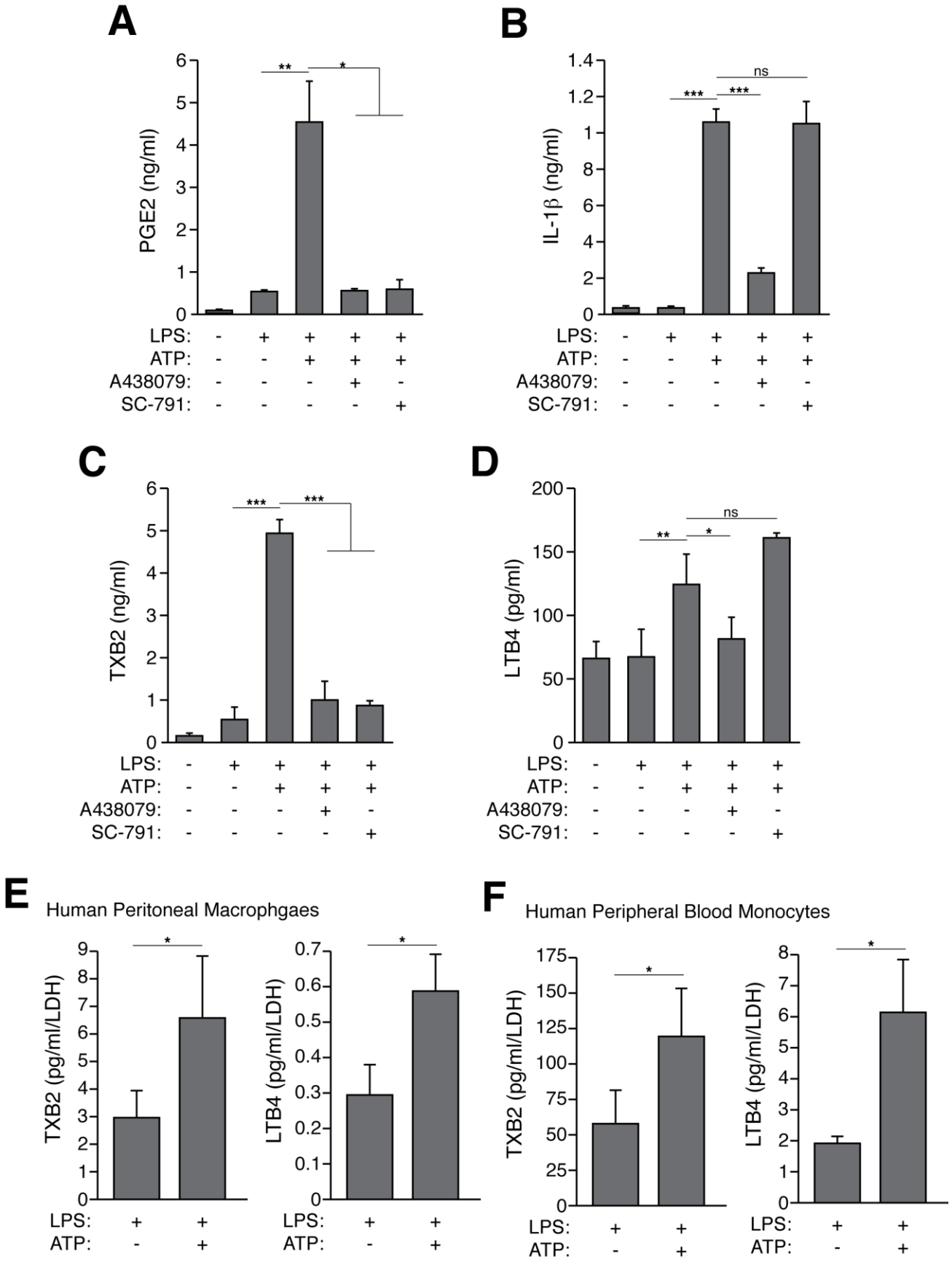
A
PGE2 (ng/ml)
LPS:
ATP:
A438079:
SC-791:
B
IL-1β (ng/ml)
ns
C
TXB2 (ng/ml)
D
LTB4 (pg/ml)
E
Human Peritoneal Macrophgaes
TXB2 (pg/ml/LDH)
LTB4 (pg/ml/LDH)
F
Human Peripheral Blood Monocytes

Figure 36. P2X7R activation couple to TX and LT release. A–D) ELISA for PGE2 (A), IL-1β (B), TXB2 (C), and LTB4 (D) release from BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of P2X7R selective antagonist (A438079, 10 μM) or the selective COX-2 inhibitor (SC-791, 1 μM) 10 min before and during ATP stimulation (*n* = 3–5 independent experiments). E,F) ELISA for TXB2 and LTB4 from primary human peritoneal macrophages (E; *n* = 3 different individuals) and blood monocytes (F; *n* = 4 different individuals) treated as in A. *ns*, not significant (p >0.05). *p >0.01 <0.05; **p >0.001 <0.01; ***p <0.001.

2.8. Tables for the Results section: P2X7 receptor activation in macrophages causes fever via PGE2 and IL-1 β release

Table 5

	Stimulation			
	LPS	**$ATP_{100\mu M}$**	**$ATP_{100\mu M}$ + IVM**	**ATP_{3mM}**
	PGE2 release [pg/ml (FI)]			
PMs				
01	3.05	4.73 (1.55)	3.62 (1.19)	5.64 (1.85)
02	1.06	0.65 (0.61)	0.78 (0.73)	1.79 (1.68)
03	0.36	0.73 (2.00)	0.84 (2.32)	1.17 (3.21)
04	0.54	0.97 (1.82)	0.63 (1.18)	1.07 (1.99)
FI_{AV}	-	1.49 ± 0.31	1.35 ± 0.34	2.18 ± 0.35
P		0.21	0.37	0.04*
HBMs				
05	43.38	65.24 (1.50)	90.34 (1.39)	85.17 (1.96)
06	75.75	221.01 (2.92)	313.48 (1.42)	295.34 (3.90)
07	3.50	3.94 (1.12)	11.94 (3.03)	10.56 (3.01)
08	38.28	30.76 (0.80)	48.64 (1.58)	187.22 (4.89)
FI_{AV}	-	1.58 ± 0.46	1.85 ± 0.39	3.44 ± 0.62
P		0.26	0.12	0.02*

Table 5. PGE2 release from primary human peritoneal macrophages and blood monocytes stimulated with ATP from 4 different individuals. PGE2 release values are normalized to the total amount of cellular LDH content, as detailed in Materials and Methods. Macrophages and monocytes were stimulated with LPS (1 µg/ml) for 4 h, and then 30 min of ATP (100 µM or 3 mM) in the presence or absence of ivermectin (IVM, 3 µM). PM, peritoneal macrophage; HBM, human blood monocyte; FI, fold increase *vs*. LPS; FI_{AV}, average fold increase *vs*. LPS. *p >0.01 <0.05.

Table 6

	9:30 AM		1:30 PM		4:30 PM	
Variable	**WT**	**KO**	**WT**	**KO**	**WT**	**KO**
T (ºC)	37.08 ± 0.04	36.96 ± 0.06	36.54 ± 0.12***	36.47 ± 0.15**	36.42 ± 0.12***	36.54 ± 0.12
n	146	89	18	12	18	12

Table 6. Rectal temperature of C57 BL/6 (WT, *P2rx7*$^{+/+}$) and P2X7R-deficient (KO, *P2rx7*$^{-/-}$) mice at different day times. Values are means ± s.e.m. No differences were found in basal temperatures between *P2rx7*$^{+/+}$ (WT) and *P2rx7*$^{-/-}$ (KO, knockout) genotypes at any time point measured. **p >0.001 <0.01, ***p <0.001 *vs*. corresponding 9:30 AM group.

3. P2X7 receptor controls TNF-α release

3.1. P2X7R controls TNF-α release in macrophages uncoupled from gene expression

Previous reports suggest that P2X7R could be associated with the release of different proinflammatory cytokines, such as the release of TNF-α by microglia (Shieh, et al., P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-alpha in cultured mouse microglia (Shieh et al., 2014a). We therefore aimed to analyze the release of TNF-α induced by P2X7R activation in macrophages as a protein following the classical release endoplasmic reticulum-Golgi pathway and with a release independent of the inflammasome. TNF-α expression and release is inducible in macrophages by LPS treatment, and as expected we found that LPS induced the accumulation of TNF-α on macrophage supernatants with an exponential increase from 90 to 120 min of stimulation, and then reaching a plateau (Figure 37A), suggesting a decrease on TNF-α release after 120 min of LPS stimulation. This decline on TNF-α release was evident when TNF-α was detected in “pulse-chase” experiments: macrophages were pulsed with LPS for different times, washed to remove accumulated TNF-α in the media and then the release of TNF-α was chased for 30 min in fresh medium (Figure 37A). We found a decrease of TNF-α release after 150 min of LPS stimulation (Figure 37A) and this decline was not due to a lack on gene transcription (Figure 37B).

This decrease was due to an impairment of TNF-α release, since P2X7R stimulation with 3 mM of ATP in pulse-chase experiments after 240 min of LPS treatment was able to increase TNF-α concentration on cell supernatants (Figure 37C). TNF-α release induced by ATP was independent on the upregulation of *Tnfa* gene expression (Figure 37D – adjunta con el e-mail). However, P2X7R stimulation did not affect TNF-α release in accumulative assays or pulse-chase experiments when TNF-α release was *maximum* (90 min) (Figure 37C). We then confirmed that the release of TNF-α upon P2X7R activation was not dependent on *de novo* gene transcription, since ATP treatment was not increasing gene expression of *Tnfa,* However, LPS modulate gene expression as expected while polarizing macrophages to M1 prior to ATP treatment (Figure 37D). Consistently, *P2rx7*$^{-/-}$ macrophages did not present a statistical significant impairment on TNF-α release when stimulated with ATP in accumulative assays, but deficiency of P2X7R resulted in an impaired release of TNF-α induced by ATP in pulse-chase experiments (Figure 37E). By contrast, the release of IL-1β, a cytokine with a highly dependent release on P2X7R activation, was found dependent on ATP treatment in both accumulative and pulse-chase experiments (Figure 37E,F). Similar results were found when different doses of LPS were used to prime macrophages, and ATP was unable to increase TNF-α release in pulse-chase experiments when LPS was used at a dose >100 ng/ml (Figure 37G). Again, this was not the case for IL-1β, which release was dependent on ATP and not on the LPS dose used to prime the macrophage (Figure 37G). These suggest that when TNF-α production and release is maximal due to early or strong LPS stimulation, P2X7R stimulation was unable to further regulate TNF-α release in macrophages.

3.2. P2X7 receptor controls TNF-α release in vivo and in extenuated blood monocytes from patients with severe sepsis

Therefore, the release of TNF-α *via* P2X7R activation could be relevant in pathological conditions where for example the TLR-signaling is extenuated and production of pro-inflammatory cytokines is dampened. This occurs during the immunosuppression phase of severe sepsis, one of the major mobility factors in sepsis (Hotchkiss and Karl, 2003). We then confirmed that P2X7R stimulation on

human peripheral blood monocytes was able to increase TNF-α release in pulse-chase experiments but not in accumulative experiments (Figure 38A). As expected, peripheral blood monocytes from acute sepsis patients presented a significant reduced TNF-α release after LPS treatment when compared to monocytes from healthy individuals (Figure 38B), suggesting they are extenuated to normally respond to LPS. Specific P2X7R antagonism with AZ10606120 prior ATP stimulation was able to reduce TNF-α release from septic patient blood monocytes (Figure 38C). This data suggests that P2X7R activation could be a strategy to boost immune system in sepsis-induced immunosuppression. As a proof of concept to study the involvement of P2X7R *in vivo* on TNF-α release, intraperitoneal challenge of mice with LPS and ATP resulted in an increase of peritoneal TNF-α, and as expected IL-1β, in WT, but not in *P2rx7*$^{-/-}$ mice (Figure 39A,B), without affecting the increase of infiltrated peritoneal Gr-1/Mac-1 positive cells (Figure 39C).

3.3. P2X7R controls the release of TNF-α through cathepsin B and metalloprotease activity

To study the mechanism of P2X7R controlling the classical release of TNF-α, when we treated macrophages with brefeldin A prior ATP stimulation blocked P2X7R induced TNF-α release, indicating that this process was mediated by exocytosis of vesicles classically trafficked from the endoplasmatic reticulum to Golgi (Figure 40A). However, microtubules or actin filaments were not required for P2X7R induced TNF-α release, since colchicine or cytokalasin B had no effect on extracellular TNF-α levels (Figure 40A). We further confirmed that P2X7R was not inducing *de novo* TNF-α gene expression *via* NF-κB, as the proteasome inhibitor MG132 or the translation blocker G418 did not alter extracellular levels of TNF-α upon ATP stimulation (Figure 40A). It was reported that cathepsin B mediates the release of TNF-α containing vesicles (Ha et al, 2008) and here we found that both the general cathepsin inhibitor E-64 and the specific cathepsin B inhibitor CA-074 Me were able to inhibit TNF-α release upon ATP stimulation, without affecting IL-1β release (Figure 40B). Both inhibitors also blocked the release of cathepsin B

upon P2X7R activation as measured by the activity of cathepsin B on macrophage supernatants (Figure 40C).

It is known that P2X7R activation induces the activation and release of metalloproteases (Gu and Wiley, 2006), and that TNF-α suffer a specific dependent shedding from the plasma membrane for its release by the metalloprotease TNF-α converting enzyme (TACE) (Moss et al., 1997). Treatment of macrophages with the general metalloprotease inhibitor GM6001 during ATP stimulation reduced the release of TNF-α (Figure 41A), without affecting the release of IL-1β (Figure 41B). Specific inhibition of metalloprotease 9 did not affect ATP induced release of TNF-α or IL-1β (Figure 41A,B), while specific inhibition of TACE with TAPI-0 completely abrogated P2X7R induced TNF-α release (Figure 41A).

Therefore we studied if P2X7R could be modulating TACE in macrophages to induce the release of TNF-α. Indeed, we found an increase of TACE activity in macrophages after 5 min of ATP stimulation and after 15 and 30 min in cell supernatants (Figure 41C), suggesting that TACE was not only activated upon P2X7R activation, but also released. Extracellular TACE activity was blocked using the specific inhibitor TAPI-0 (Figure 41D), and was absent when ATP was added to macrophages deficient on P2X7R or when the specific P2X7R antagonist A438079 was incubated with the cells prior ATP stimulation (Figure 41E). In macrophages, p38 MAPK controls activation of TACE (Scott et al., 2011) and it was reported that P2X7R activates MAPK *via* intracellular Ca^{2+} (Pfeiffer et al., 2004). Here we found that upon P2X7R activation extracellular TACE activity was reduced when macrophages were treated with the p38 inhibitor SB202190 or when intracellular Ca^{2+} rise was prevented using an extracellular medium with EGTA (Figure 41E). However, TACE activity induced by P2X7R activation was not affected by ERK or JNK inhibitors (not shown). Finally, we aimed to find a function of P2X7R-induced TACE release. We collected mouse macrophage supernatants after stimulation or not with ATP, we confirmed that supernatants from ATP treated macrophages contained TACE activity (data averaged on Figure 41C). We incubated these supernatatns with HEK293 cells expressing human TNF-α, and we found that supernatant from ATP-treated macrophages was able to increase

the amount of released human TNF-α from transfected HEK293 cells (Figure 41F). This suggests that soluble TACE released upon P2X7R activation was able to induce TNF-α release from cells that lack TACE but express TNF-α on the plasma membrane. As a control, we confirmed that the different transfected HEK293 treated with the different macrophage supernatants expressed similar TNF-α levels (Figure 41G).

3.4. Figures for the Results section: P2X7 receptor controls TNF-α release

Figure 37

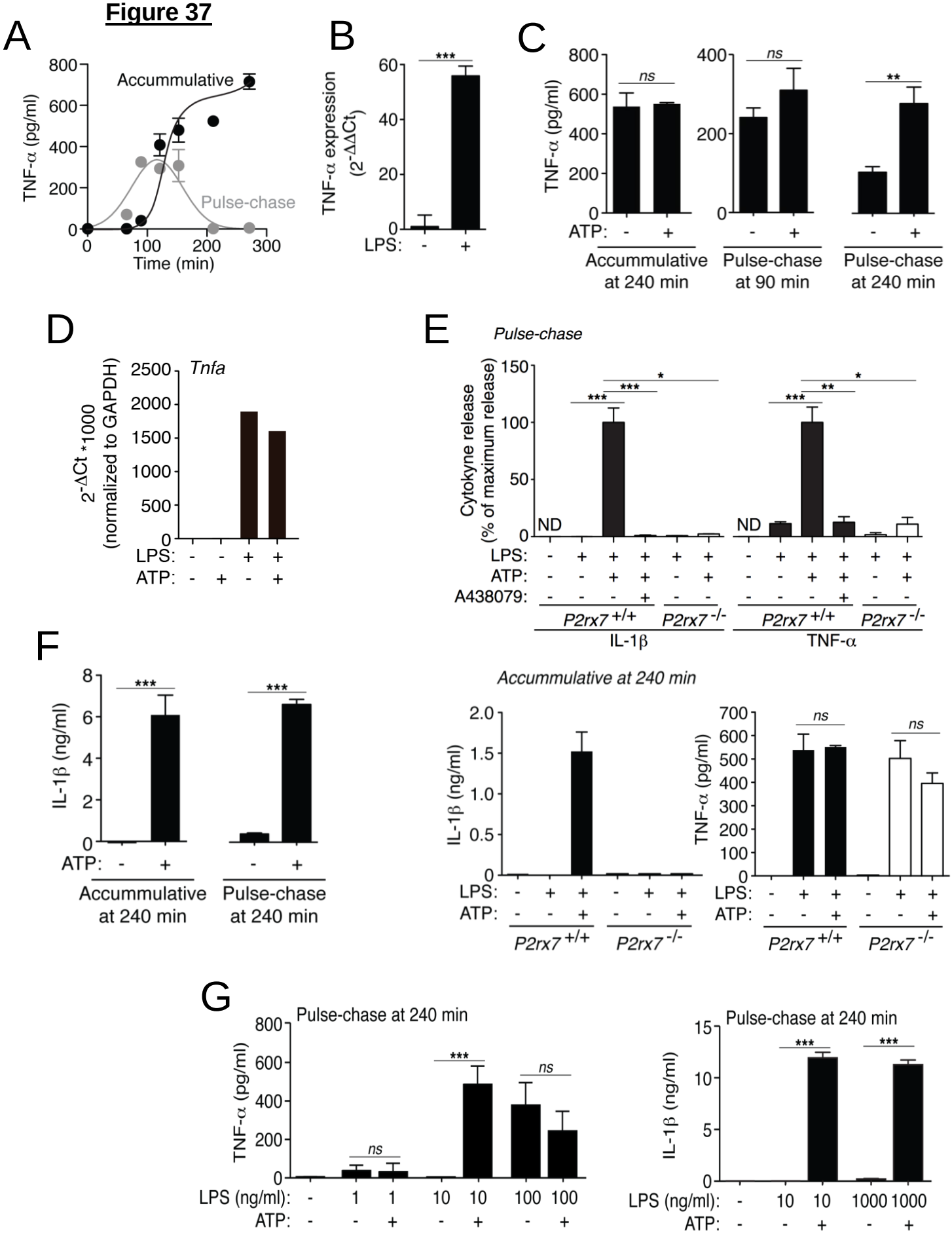
A
TNF-α (pg/ml)
Accummulative
Pulse-chase
Time (min)
B
TNF-α expression (2-ΔΔCt)
LPS:
C
TNF-α (pg/ml)
ATP:
Accummulative at 240 min
Pulse-chase at 90 min
Pulse-chase at 240 min
D
Tnfa
2-ΔCt *1000 (normalized to GAPDH)
LPS:
ATP:
E
Pulse-chase
Cytokyne release (% of maximum release)
ND
A438079:
P2rx7 +/+
P2rx7 -/-
IL-1β
TNF-α
F
IL-1β (ng/ml)
Accummulative at 240 min
Pulse-chase at 240 min
TNF-α (pg/ml)
G
Pulse-chase at 240 min
LPS (ng/ml):

Figure 37. P2X7R controls TNF-α release. A) ELISA analysis for released TNF-α from BMDMs primed with 10 ng/ml of LPS for different times as indicated (accumulative) or pulsed with LPS for the indicated time and then washed and chased in the absence of LPS for 30 min (pulse-chase); data is presented as mean and s.e.m. of *n* = 2 independent experiments. B) Relative gene expression ($2^{-\Delta\Delta Ct}$) for TNF-α determined by quantitative RT-PCR from BMDMs unprimed (−) or primed (+) for 4 h with LPS (1 µg/ml); data is the average of duplicate runs and representative of *n* = 3 independent experiments. C,F) ELISA analysis for released TNF-α (C) and IL-1β (F) from BMDMs primed as in A, but followed by no stimulation (−) or stimulation (+) for 20 min with ATP (3 mM); data is presented as mean and s.e.m. of *n* = 2 to 6 independent experiments. D) Relative gene expression of *Tnfa,* determined by quantitative RT-PCR from BMDMs unprimed (−) or primed (+) for 4 h with LPS (1 µg/ml), followed by no stimulation (−) or stimulation (+) for 20 min with ATP (3 mM); data is the average of duplicate runs and representative of n = 2 independent experiments. E) Pulse-Chase: ELISA analysis for released IL-1β and TNF-α from *P2rx7*$^{+/+}$ or *P2rx7*$^{-/-}$ BMDMs unprimed (resting) (−) or primed (+) for 4 h with 1 µg/ml of LPS for IL-1β or 10 ng/ml of LPS for TNF-α, followed by no stimulation (−) or stimulation (+) of P2X7R for 20 min with ATP (3 mM); when indicated BMDMs were treated 10 min before and during ATP stimulation with the selective P2X7R antagonist A438079 (25 µM). Maximum cytokine release detected in LPS+ATP treated macrophages and used for normalization was 13,459.22 pg/ml for IL-1β and 1,188.72 pg/ml for TNF-α. ND, not detected; data is presented as mean and s.e.m. of n = 2-3 independent experiments for *P2rx7*$^{+/+}$ and n = 1 for *P2rx7*$^{-/-}$ BMDMs. Accumulative assay: ELISA analysis for released TNF-α from *P2rx7*$^{+/+}$ or *P2rx7*$^{-/-}$ BMDMs primed for 4 h with LPS (1 µg/ml), followed by no stimulation (−) or stimulation (+) for 20 min with ATP (3 mM) added in the LPS-priming media; data is presented as mean and s.e.m. of *n* = 2 independent experiments. G) ELISA analysis for released TNF-α and IL-1β from BMDMs primed for 4 h with different concentrations of LPS as indicated, followed by no stimulation (−) or stimulation (+) for 20 min with ATP (3 mM) (pulse-chase); data is presented as mean and s.e.m. of *n* = 4 independent experiments. ns, p >0.05; *ns*, not significant (p>0.05); ***p <0.001; **p <0.01; *p <0.05

Figure 38

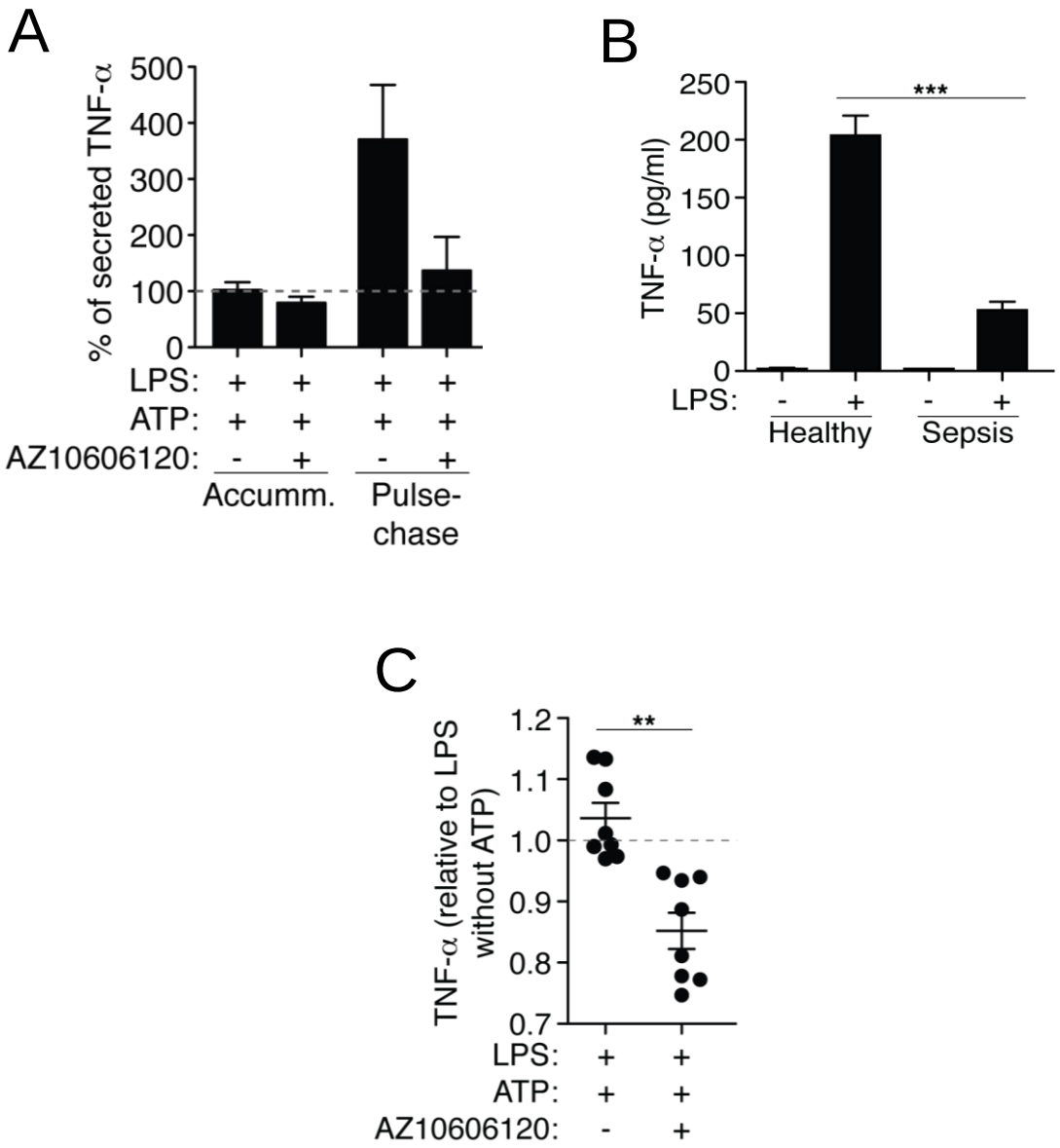
A
% of secreted TNF-α
500
400
300
200
100
0
LPS: + + + +
ATP: + + + +
AZ10606120: - + - +
Accumm.
Pulse-chase
B
TNF-α (pg/ml)
250
200
150
100
50
0
***
LPS: - + - +
Healthy
Sepsis
C
TNF-α (relative to LPS without ATP)
1.2
1.1
1.0
0.9
0.8
0.7
**
LPS: + +
ATP: + +
AZ10606120: - +

Figure 38. P2X7R controls TNF-α release in blood monocytes. A) ELISA analysis for released TNF-α from human blood monocytes isolated from healthy donors primed for 4 h with LPS (10 ng/ml), followed by stimulation for 20 min with ATP (3 mM) in the LPS-containing media (accumulative) or after washing LPS-priming media (pulse-chase); when indicated cells were treated 10 min before and during ATP stimulation with the selective human P2X7R antagonist AZ10606120 (10 μM); data is presented as mean and s.e.m. of 4 healthy donors for the accumulative experiments and 6 healthy donors for the pulse-chase experiment. B,C) ELISA analysis for released TNF-α from human blood monocytes isolated from healthy donors (B) or patients with severe sepsis (B,C), unprimed (−) or primed (+) for 4 h with LPS (1 μg/ml), followed by no stimulation (−) or stimulation (+) for 20 min with ATP (3 mM) (C); when indicated cells were treated 10 min before and during ATP stimulation with the selective human P2X7R antagonist AZ10606120 (10 μM); data is presented as mean and s.e.m. of 4 healthy donors and 8 patients with sepsis. ***p <0.001; **p <0.01; *p <0.05; *ns*, not significant, p>0.05.

Figure 39

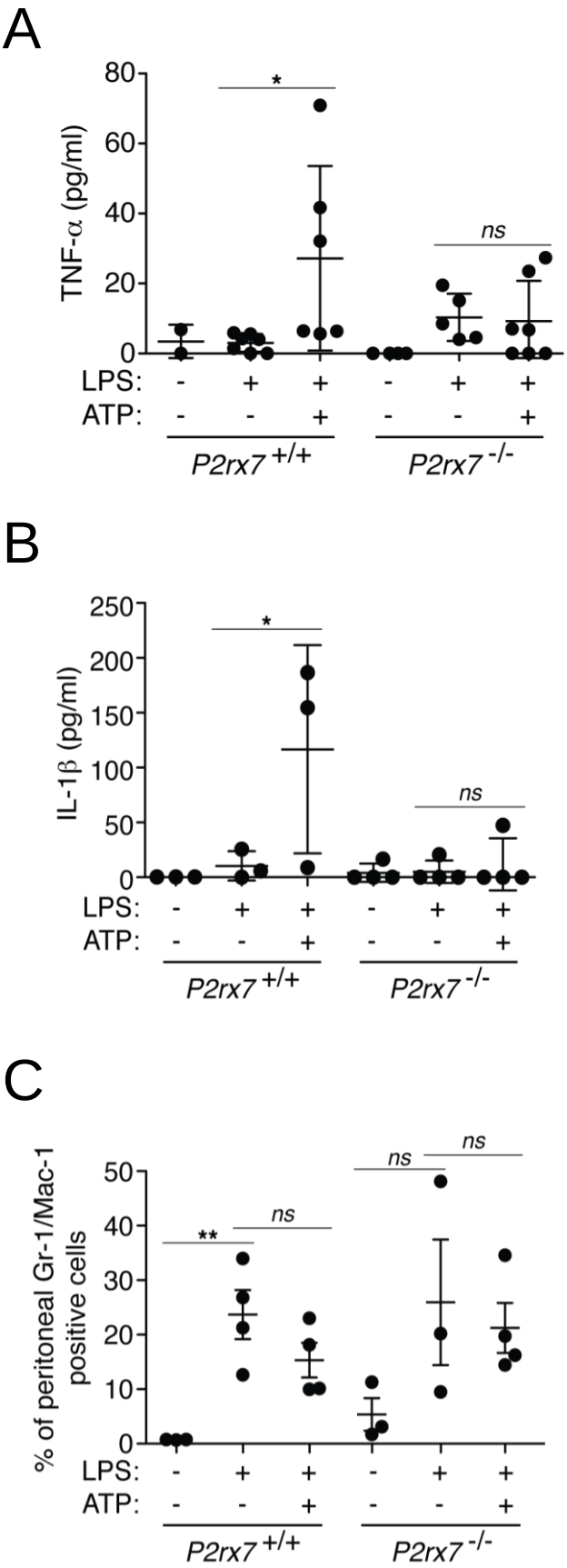
A
TNF-α (pg/ml)
80
60
40
20
0
*
ns
LPS: - + + - + +
ATP: - - + - - +
P2rx7 +/+
P2rx7 -/-
B
IL-1β (pg/ml)
250
200
150
100
50
0
*
ns
LPS: - + + - + +
ATP: - - + - - +
P2rx7 +/+
P2rx7 -/-
C
% of peritoneal Gr-1/Mac-1 positive cells
50
40
30
20
10
0
**
ns
ns
ns
LPS: - + + - + +
ATP: - - + - - +
P2rx7 +/+
P2rx7 -/-

Figure 39. P2X7R controls TNF-α release in a peritonitis mice model. A) ELISA of peritoneal TNF-α (A), IL-1β (B) or Gr-1/Mac-1 double positive cells (C) in *P2rx7*$^{+/+}$ or *P2rx7*$^{-/-}$ mice 4 h after intraperitoneal injection of vehicle (−) or 50 μg/kg of LPS (+) for 2 h followed by a second intraperitoneal injection of vehicle (−) or 1.5 M/kg ATP (+). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.e.m.). *ns*, not significant, p>0.05; **p <0.01; *p <0.05; ns, p >0.05.

Figure 40

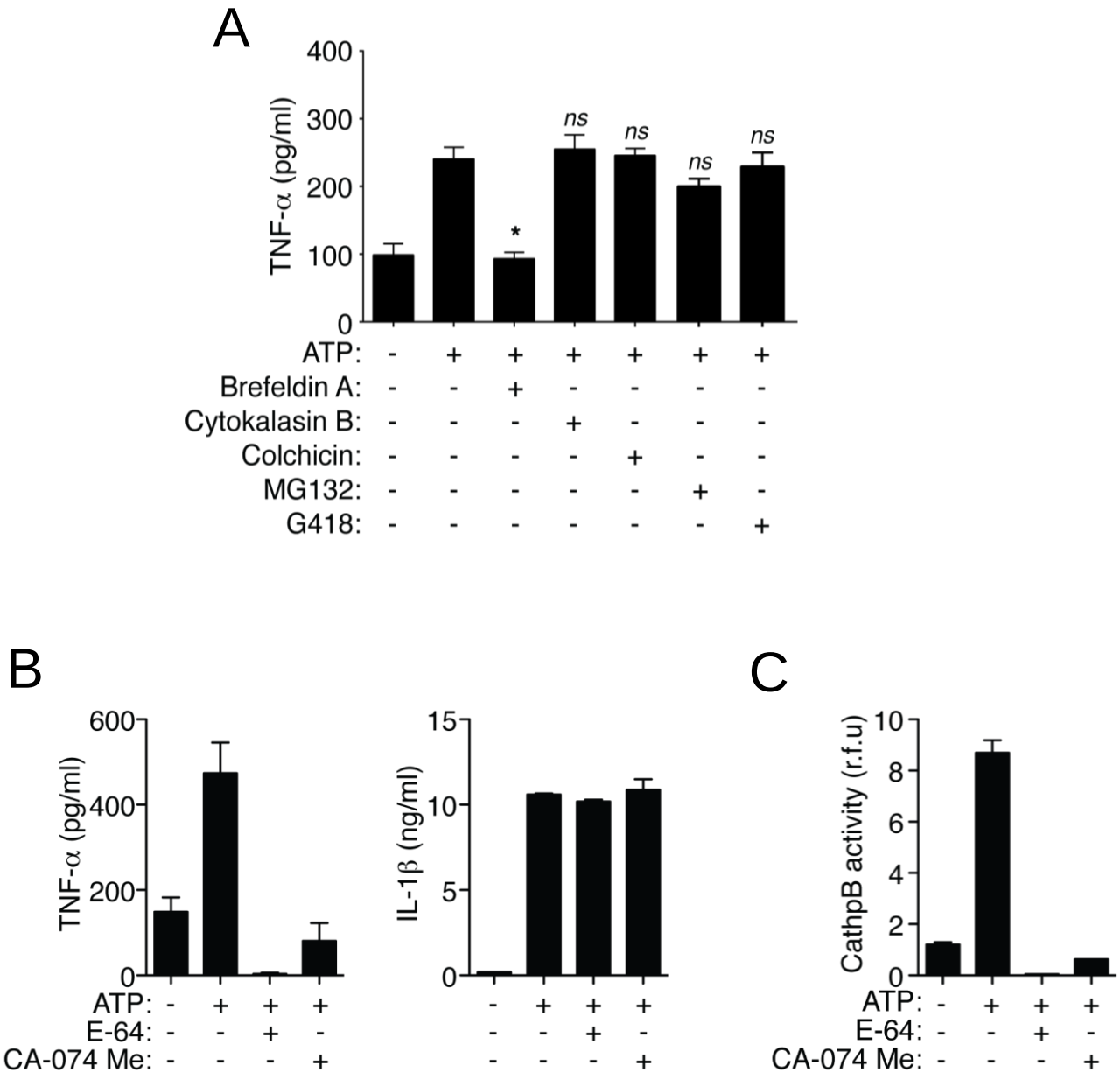
A
TNF-α (pg/ml)
400
300
200
100
0
ns
ns
ns
ns
*
ATP: - + + + + + +
Brefeldin A: - - + - - - -
Cytokalasin B: - - - + - - -
Colchicin: - - - - + - -
MG132: - - - - - + -
G418: - - - - - - +
B
TNF-α (pg/ml)
600
400
200
0
ATP: - + + +
E-64: - - + -
CA-074 Me: - - - +
IL-1β (ng/ml)
15
10
5
0
- + + +
- - + -
- - - +
C
CathpB activity (r.f.u)
10
8
6
4
2
0
ATP: - + + +
E-64: - - + -
CA-074 Me: - - - +

Figure 40. P2X7R control TNF-α release via cathpesin activity. A) ELISA analysis for released TNF-α from BMDMs primed for 4 h with LPS (10 ng/ml), followed by no stimulation (−) or stimulation (+) of P2X7R for 20 min with ATP (3 mM); when indicated BMDMs were treated 10 min before and during ATP stimulation with the endoplasmic reticulum-Golgi blocker brefeldin A (1 µg/ml), with the β-actin cytoskeleton blocker cytokalaisin B (2.5 µg/ml), the microtubule blocker colchicine (50 µM), the proteasome blocker MG132 (50 µM) or the transcriptional blocker G418 (0.8 mg/ml); data is presented as mean and s.e.m. of n = 3 independent experiments. B,C) ELISA analysis for released TNF-α and IL-1β (B) and cathepsin B activity (C) in cell-free supernatants from BMDMs primed for 4 h with LPS (1 µg/ml), followed by no stimulation (−) or stimulation (+) of P2X7R for 20 min with ATP (3 mM); when indicated BMDMs were treated 10 min before and during ATP stimulation with the general cathepsin inhibitor E-64 (100 µM) or the selective cathepsin B inhibitor CA-074 Me (50 µM); data is presented as mean and s.e.m. of duplicate experiments. *$p < 0.05$; ns, $p > 0.05$.

Figure 41

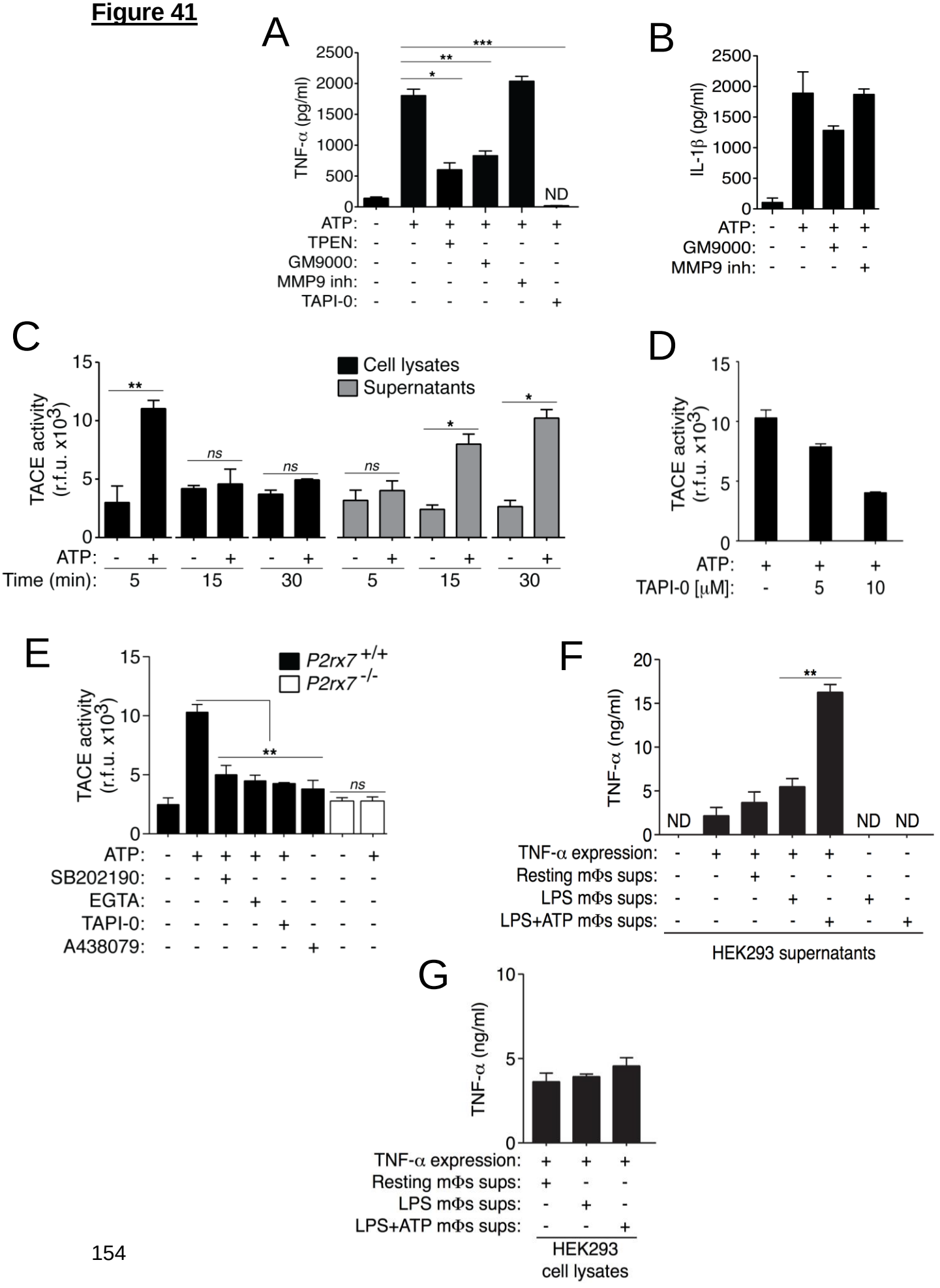
A
TNF-α (pg/ml)
2500
2000
1500
1000
500
0
***
**
*
ND
ATP: - + + + + +
TPEN: - - + - - -
GM9000: - - - + - -
MMP9 inh: - - - - + -
TAPI-0: - - - - - +
B
IL-1β (pg/ml)
2500
2000
1500
1000
500
0
ATP: - + + +
GM9000: - - + -
MMP9 inh: - - - +
C
TACE activity (r.f.u. x10^3)
15
10
5
0
Cell lysates
Supernatants
**
ns
ns
ns
*
*
ATP: - + - + - + - + - + - +
Time (min): 5 15 30 5 15 30
D
TACE activity (r.f.u. x10^3)
15
10
5
0
ATP: + + +
TAPI-0 [μM]: - 5 10
E
TACE activity (r.f.u. x10^3)
15
10
5
0
P2rx7 +/+
P2rx7 -/-
**
ns
ATP: - + + + + - - +
SB202190: - - + - - - - -
EGTA: - - - + - - - -
TAPI-0: - - - - + - - -
A438079: - - - - - + - -
F
TNF-α (ng/ml)
20
15
10
5
0
**
ND
ND
ND
TNF-α expression: - + + + + - - -
Resting mΦs sups: - - + - - - - -
LPS mΦs sups: - - - + - + - -
LPS+ATP mΦs sups: - - - - + - + -
HEK293 supernatants
G
TNF-α (ng/ml)
10
5
0
TNF-α expression: + + +
Resting mΦs sups: + - -
LPS mΦs sups: - + -
LPS+ATP mΦs sups: - - +
HEK293 cell lysates

Figure 41. P2X7R control the release of TNF-α through metalloproteases. A,B) ELISA analysis for released TNF-α (A) or IL-1β (B) from BMDMs primed for 4 h with LPS (1 µg/ml), followed by stimulation with 20 min of ATP (3 mM) stimulation and additionally 10 min before and during ATP stimulation cells were incubated with the Zn^{2+} chelator TPEN (50 µM) or a TACE inhibitor (TAPI-0, 1 µM); data is presented as mean and s.e.m. of *n* = 3 independent experiments. C,E) Quantification of TACE activity from *P2rx7*$^{+/+}$ (C,E) or *P2rx7*$^{-/-}$ (E) BMDMs cell lysates (C) and supernatants (C,E) primed for 4 h with LPS (1 µg/ml), followed by stimulation, with ATP (3 mM) for different times as indicated (C) or for 20 min (E); data is presented as mean and s.e.m. of *n* = 3 independent experiments. D) Quantification of TACE activity from BMDMs supernatants primed for 4 h with LPS (1 µg/ml), followed by stimulation with ATP (3 mM) during 20 min. The specific TACE inhibitor TAPI-0 (5 or 10 µM) was added to the supernatant after collecting from the cells and before TACE substrate was added; data is presented as mean and s.e.m. of *n* = 3 independent experiments. F,G) Release of TNF-α detected by ELISA from supernatants (F) or cell lysates (G) of HEK293 cells expressing human TNF-α and incubated during 30 min with supernatants from BMDMs activated as in B; Mφs: macrophages; data is presented as mean and s.e.m. of n = 3 independent experiments. ND, not detected; ***p <0.001; **p <0.01; *p <0.05; *ns*, p >0.05.

Discussion

Most of the effects of ATP and adenosine on myeloid cell biology have been studied on mature macrophages, in this Thesis we first examined how purinergic signaling affects macrophage differentiation from bone marrow precursors. We found that ATP and adenosine were able to decrease the number of differentiated macrophages, resulting in less mature macrophages. However, macrophages maturated in the presence of adenosine presented upregulation of certain M2 markers, including the lectin Ym1.

Macrophages are highly plastic cells, they adapt to different environments and produce cytokines to regulate the inflammatory response during both the initiation (*via* M1 macrophage polarization) and the resolution (*via* M2 macrophage polarization) (Gordon and Taylor, 2005; Martinez et al., 2008). Adenosine is able to synergistically increase *Arg1* gene expression when added to mature macrophages in combination with IL-4 or IL-10, enhancing M2 macrophage phenotype (Csóka et al., 2012; Haskó et al., 2007). Our findings are in agreement with these studies, and we confirmed that adenosine was able to increase the expression of *Arg1* in mature macrophages treated with IL-4. In addition, we also found that adenosine is able to upregulate other M2-related genes, such as *Chi3l3* (Ym1) and *Mrc1* (MRC1), but not *Retnla* (FIZZ1). On the other hand, similar to other studies (Ramanathan et al., 2009), adenosine inhibited the expression of the proinflammatory gene *Tnfa*, but surprisingly in

our study we found *Il1b* gene expression upregulated by adenosine treatment. These results suggest that adenosine promotes a characteristic phenotype of macrophage and question the broad anti-inflammatory or M2-promoting effect of adenosine arising from studies that measure just one gene as a general marker of M1 or M2 phenotype (Csóka et al., 2012).

Macrophage differentiation from mouse bone marrow precursors leads to a resting mature macrophage, which could then be polarized towards M1 or M2 upon the type of priming signal that challenge those mature macrophages (Loke et al., 2002; Welch et al., 2002). However, this model could be different when bone marrow precursors integrate different signaling to increase or decrease the production of macrophages, such as when differentiation occurs within an environment containing extracellular nucleotides. Our study found that both, ATP and adenosine, are able to arrest macrophage growth from bone marrow precursors, confirmed previous study identifying this receptor signaling through cAMP production and subsequent $p27^{kip-1}$ activation to inhibit macrophage proliferation (Xaus et al., 1999). Here we rule out the possible effect of ATP activating P2X7R and inducing cell death, and found that macrophages differentiated in the presence of ATP or adenosine were mature macrophages, expressing the mouse mature marker of macrophages F4/80 (Austyn and Gordon, 1981). Cell surface ectonucleotidases present on myeloid cells are able to degrade ATP into adenosine (Dull et al., 1992; Mello et al., 2014), our data found that ATP is quickly degraded by bone marrow precursors, and resulting adenosine could explain the inhibition of macrophage proliferation. From the different M1 and M2 markers analyzed in our study, macrophages differentiated in the presence of adenosine resulted in the upregulation of *Chi3l3*, the gene that encodes for the lectin Ym1, a well-established marker of murine M2 macrophages (Ho and Sly, 2009; Raes et al., 2002; Welch et al., 2002). Ym1 is a secretory protein synthesized by murine macrophages that binds to heparin and glucosamine (Chang et al., 2001) and its expression is strongly induced by IL-4 and IL-13 as compared with classical M1 stimulation of murine macrophages (Nair et al., 2003; Raes et al., 2002). Ym1 expression induced by IL-4 treatment involves the activation of the nuclear factor STAT6 (Welch et al., 2002).

Despite Ym1 being associated to M2 macrophages, it has been also identified as a protein forming crystals in chronic inflamed lungs and associated with macrophage deregulation (Guo et al., 2000; Harbord et al., 2002). Here we found the expression of Ym1 associated to the inhibition of macrophage proliferation induced not only by the anti-inflammatory signal adenosine, but also during macrophage senescence and in response to pro-inflammatory signals such as LPS that also arrest M-CSF macrophage differentiation. The expression of *Chi3l3* gene was opposite to macrophage proliferation, being early expressed by bone marrow precursors. These results are in agreement with a study that reported a transiently expression of Ym1 in early myeloid precursor cells of hematopoietic tissues, initially in the yolk sac and subsequently in fetal liver, spleen, and bone marrow (Hung et al., 2002). Therefore, Ym1 lectin may be involved in both hematopoiesis and inflammation and it is tempting to speculate that Ym1 production could be involved in the inhibition of macrophage proliferation after adenosine or LPS treatment.

The release of the proinflammatory cytokine IL-1β by mature macrophages is key for the initiation of the innate immune response and is a tightly regulated process, which comprises an initial priming step (signal 1, usually bacterial endotoxins) and a second inflammasome formation step (signal 2, usually a danger signal) (Pelegrín, 2011a). The nature of signal 2 differs from crystals to pathogens, but extracellular ATP is the most studied endogenous danger signal acting through the purinergic P2X7R (Ferrari et al., 2006; Pelegrín, 2011a). Pathophysiological production of PGE2 is tightly coupled to COX-2 expression levels, and COX-2 expression is a well-established marker of inflammation (Williams et al., 1999). COX-2 expression is negligible in unprimed cells, but after endotoxin (classically LPS) priming, there is a rapid induction of the levels of COX-2 (Goppelt-Struebe, 1995). Long endotoxin exposure led macrophages to the release of COX-2-dependent PGE2 compared with unprimed cells (Goppelt-Struebe, 1995). In this Thesis we found that short-time (4 h) LPS priming induces in macrophages a 600-fold increase in COX-2 expression levels and a 4-fold increase of PGE2 release. However, LPS-induced PGE2 release was small if compared with the release after P2X7R stimulation, which resulted in a further increase in PGE2 release of

up to 8-fold. Therefore, similar to IL-1β release, we found that the lipidic mediator PGE2 is also subjected to a 2-step release mechanism, where signal 1 induces the synthesis of COX-2 and signal 2 increases the enzyme substrate: the arachidonic acid. Here we have found that signal 2 could be mediated by P2X7R activation, but recently it has been reported that other inflammasome activators, such as silica crystals or aluminum salts, can also regulate the production of PGE2, acting as signal 2 (Kuroda et al., 2011). Surprisingly, PGE2 release induced by signal 2 is independent of the inflammasome activation, and the use of a casapse-1 inhibitor (present study) or macrophages deficient in the inflammasome adapter protein ASC or NLRP3 (Kuroda et al., 2011) showed no altered release of PGE2.

Therefore, the mechanism underlying P2X7R activation and PGE2 release in mature macrophages differs from the inflammasome pathway, and we found that it was strongly dependent on the sustained rise of intracellular calcium, permeating directly through P2X7R pores. This calcium-dependent signaling pathway for P2X7R induction of PGE2 release is similar to the recently described pathway for P2X7R induction of cathepsin release or T-cell proliferation (Lopez-Castejon et al., 2010; Yip et al., 2009) and is in line with P2X7R inflammasome activation, a calcium-independent process (Brough et al., 2003; Pelegrin et al., 2008). P2X7R strongly activates MAPK signaling (Donnelly-Roberts et al., 2004; Lenertz et al., 2011; Shiratori et al., 2010), and we found that JNK activity was required for P2X7Rdependent PGE2 release. It is well known that JNK modulates COX-2 expression (Hunot et al., 2004; Nieminen et al., 2006), but ATP did not alter COX-2 or PGE-synthase transcript levels, ruling out a synthesis step in the P2X7R-PGE2 release pathway. A nonmetabolizing analog of arachidonic acid blocked the release of PGE2 after P2X7R stimulation, suggesting that the rise in intracellular calcium and activation of MAPK leads to an increase of free arachidonic acid through cPLA2 activation, since the enzymatic activity of cPLA2 is highly controlled by intracellular calcium and phosphorylation (Gijón et al., 2000; Kuroda et al., 2011; Ulmann et al., 2010; Xia and Zhu, 2011). Also, intracellular calcium rise after P2X7R stimulation is known to activate cPLA2 in rat submandibular glands (Alzola et al., 1998), and our data validate this model in macrophages. The

modulation of arachidonic acid by P2X7R leads us to study whether ATP and P2X7R could also induce the release of other important lipidic mediators. We found, for the first time, that P2X7R could also couple to the release of other arachidonic acid mediators, such as TXs and leukotrienes, from primed macrophages and monocytes.

In this Thesis, we used primary human macrophages and monocytes to corroborate mouse data; it has been reported that *in vitro* maturation of human macrophages from monocytes increase the expression of *P2RX7* (Gudipaty et al., 2001). Our data using human fresh-blood monocytes and primary mature peritoneal macrophages from a cohort of different individuals show similar expression of the *P2RX7* gene, but monocytes presented a higher release rate (from 10- to 100-fold) of IL-1β and autacoids in response to P2X7R activation. This finding is in agreement with previous data supporting the conclusion that P2X7R is more active in monocytes than in macrophages and that the endogenous ATP release from monocytes can activate P2X7R induced IL-1β release (Netea et al., 2009b). Our mouse macrophage *in vitro* data support previous observations for a role of P2X4R in PGE2 release due to low extracellular ATP concentrations (Ulmann et al., 2010), but P2X4R contribution to PGE2 release was negligible when P2X7R was activated. Despite the fact that primary human monocytes and peritoneal macrophages presented higher expression of P2X4R compared to P2X7R, low ATP concentrations alone or in combination with ivermectin could not potentiate the release of PGE2, suggesting that P2X4R could be arrested in intracellular compartments in human monocytes and macrophages and be non-functional at the plasma membrane level, as P2X4R presents a potent internalization sequence (Royle et al., 2005).

All these findings have enormous implications, since autacoids control key signaling steps in many physiological and pathophysiological processes, where purinergic signaling and P2X7R can now be novel master regulators. For example, it is well known that PGE2 sensitizes nociceptors during inflammation, and therefore increases pain sensation (Portanova et al., 1996; Samad et al., 2002). Experiments with *P2rx7*$^{-/-}$ mice and with selective P2X7R antagonists have revealed P2X7R as a key mediator of pain sensation in models of

neuropathic pain, thermal hyperalgesia, and hypersensitivity postinflammatory bowel disease (Chessell et al., 2005; Donnelly-Roberts et al., 2008; Honore et al., 2006; Keating et al., 2011). The antinociceptive effects of P2X7R inhibition have been attributed to an impairment of IL-1β release, since IL-1β has been described as a mediator of pain (Honore et al., 2009). However, after P2X7R blockage there is a reduced noxious evoked activity of neurons (Itoh et al., 2011; Keating et al., 2011), which directly correlates with the effect of PGE2 promoting nociceptor sensitization and hyperexcitability (Portanova et al., 1996; Samad et al., 2002).

One part of this Thesis focus on the involvement of P2X7R on the febrile response, one of the five classical signs associated with the inflammatory process. Fever is a primary systemic response to infection, coordinated by the induction of different endogenous pyrogens (Bartfai and Conti, 2010). Actually, it is well characterized that IL-1β is one of the intermediate endogenous pyrogens transducing pathogen-derived signals. IL-1β signaling activates the production of COX-2, and therefore increases the levels of PGE2, which has been postulated as one of the final local endogenous pyrogens that acts on the thermoregulatory area of the hypothalamus, thereby increasing body temperature (Bartfai and Conti, 2010; Cao et al., 1996; Li et al., 2001). It is known that P2-receptor blockade attenuates fever induced by LPS in rats (Gourine et al., 2005). Among P2 receptors, P2X7R controls the release of both pyrogens IL-1β and PGE2, and here we found that its deficiency or specific blockage with A438079 decrease the febrile response in mice in response to LPS. However, such decrease could be due to either an impairment of IL-1β and/or PGE2 production. We successfully found that P2X7R deficiency was related to impairment on local and systemic IL-1β levels; however, we failed to detect a reliable increase on PGE2 *in vivo* after LPS challenge. This could be due to the very short half-life of PGE2 in vivo (≈20 s) and to local changes in PGE2 production during fever, which are unlikely to be detected systemically (Cao et al., 1996; Davidson et al., 2001; Eguchi et al., 1992; Ivanov and Romanovsky, 2004). We therefore used IL-1β to induce fever and found that the increase in body temperature was significantly shorter in *P2rx7*$^{-/-}$ animals. We can suggest either that P2X7R deficiency alters the IL-1β positive-feedback

loop or alters PGE2 release by exogenous IL-1β. We also found that ATP, the endogenous ligand for P2X7R, was accumulated *in vivo* after i.p. LPS administration. This result is in accordance with recent publications where ATP has been found to be release as a danger molecule *in vivo* during graft-*vs*.-host disease and contact hypersensitivity (Weber et al., 2010; Wilhelm et al., 2010). Altogether, we can speculate that bacterial endotoxin causes release of the endogenous danger signal ATP, which in turn activates P2X7R to cause fever.

Different pharmaceutical companies are actively producing drug-like P2X7R antagonists; some of them have been extensively used in animal models of inflammation and pain, and some of them are under clinical trials (Arulkumaran et al., 2011; Pelegrin, 2008). These molecules have successfully passed phase I clinical trials, and some of them are in phase II/III for rheumatoid arthritis, osteoarthritis, Crohn's disease, and chronic obstructive pulmonary disease (Arulkumaran et al., 2011). We simultaneously compared the inhibitory effects of P2X7R antagonism with caspase-1 inhibitors (inflammasome blockers) and with inhibitors of COX-2 as representative of NSAIDs. As expected, caspase-1 inhibition only decreased proinflammatory cytokine IL-1β release, without affecting the release of lipidic mediators. COX-2 inhibitors belong to the NSAID family and comprise one of the most widely used types of drugs, mainly due to their potent anti-inflammatory and painkiller actions (Rainsford, 2007). However, COX-2 inhibitor was only effective in blocking the release of PGE2 and TXB4, without affecting LTB2 or IL-1β release. P2X7R antagonism could block autacoids and IL-1β release, conferring to these novel drug-like antagonists a wider anti-inflammatory and antipain spectrum than classical NSAIDs, emerging as important therapeutics for inflammation, pain, and fever.

Once we identified that P2X7R could induce the release of other inflammatory mediators (autacoids) besides the well characterized release of IL-1β (Ferrari et al., 1997), we found that in M1 macrophages P2X7R is able to control the classical release of TNF-α cytokine, another important proinflammatory mediator (Dinarello, 2000; Movat and Cybulsky, 1987). There have been recent reports demonstrating TNF-α induction after P2X7R activation in microglia (Shieh et al., 2014b). However, while this previous work found that

P2X7R induces release of TNF-α by inducing *de novo* gene expression, in this Thesis our results suggests that P2X7R potentiates the release of TNF-α from macrophages without affecting gene expression. In fact, our results show that TNF-α was not upregulated at gene expression level upon P2X7R activation, showing that TNF-α release depended on proteases (cathepsins and TACE) regulation.

In macrophages, p38 mitogen-activated protein kinase (MAPK) controls activation of TACE (Scott et al., 2011). The activation of p38 MAPK induces reactive oxygen species, that is essential for innate immune responses induced by LPS (Matsuzawa et al., 2005) and both, the induction of LPS or reactive oxygen species, enhance TACE activity indirectly *via* the p38 MAPK pathway (Scott et al., 2011). It is also known that P2X7R activates MAPK *via* intracellular calcium rise (Pfeiffer et al., 2004). In this Thesis we demonstrate that upon P2X7R activation extracellular TACE activity was reduced when macrophages were treated with a p38 MAPK inhibitor or when intracellular Ca^{2+} rise was blocked exposing macrophages to an extracellular medium without calcium and with EGTA. Obviously, this release was dependent on the intracellular trafficking of TNF-α containing vesicles to the plasma membrane (Lieu et al., 2008; Shurety et al., 2001). Moreover, we found that P2X7R induced the release of TACE having a physiological role inducing the shedding of TNF-α from cells that are deficient on TACE activity (Hiraoka et al., 2008; Reddy et al., 2000). TNF-α release is a complex mechanism and recently it has been demonstrated that also involves the activity of cathepsin B through an independent pathway and the extent of cathepsin B involvement in TNF-α secretion appears to be varied in different macrophages (Ha et al., 2008). It has been determinated that is intracellular cathepsin B activity in the cytosol, which is involved in the process of TNF-α release, rather than cathepsin B activity in pericellular space (Ha et al., 2008). Since P2X7R controls the release of cathepsins (Lopez-Castejon et al., 2010), we found this additional level of regulation on P2X7R induced release of TNF-α.

Different recent studies support the pro-inflammatory importance of extracellular ATP and P2X7R in the systemic inflammatory response observed in a mouse sepsis model (Cauwels et al., 2014; Santana et al., 2015; Sumi et

al., 2014). Animal models of sepsis only reproduce partially the human pathophysiology of sepsis syndrome, where after an overstimulation of the immune system an immunosuppressive state is developed in the patients (Hotchkiss and Karl, 2003). The high mortality of patients suffering severe sepsis is due to secondary infections araising during this immunosuppressive phase that follow the strong initial cytokine storm in response to the systemic infection (Hotchkiss and Karl, 2003). We found that during this immunosuppression, P2X7R activation could boost the immune response through the fine-tuning release of TNF-α in situations when the immune cells are extenuated. This was evident in our pulse-chase experiments to study the regulation of TNF-α release by P2X7R. Therefore, potentiating P2X7R, rather than blocking it, could be beneficial for pathologies such as severe sepsis or HIV infection, and this has been recently suggested in arecent report using a mice model of sepsis (Csoka et al., 2015).

Overall, this Thesis demonstrates that purinergic signaling is able to reduce the number of differentiated macrophages, which display upregulation of certain M2 markers, as Ym1, resulting in a specific phenotype of macrophage. In mature macrophages, ATP activating P2X7R induces the release of the lipidic mediator PGE2 and we found that P2X7R is important in the febrile response, a classical sign associated with the inflammatory process. Furthermore, P2X7R signaling in extenuhated M1 macrophages is able to control the classical release of TNF-α cytokine. These findings have future clinical implications, since targeting of P2X7R emerge as a promising therapy for fever and its potentiation during immunosupression could boost immunity in septic processes.

Conclusions

1. Macrophage proliferarion during differentiation from bone marrow precursors is arrested upon purinergic stimulation.
2. Macrophages differentiated in the presence of adenosine results in alternative M2-like phenotype with increased expression of Ym1.
3. Activation of P2X7 receptor in macrophages enhances the release of eicosanoids.
4. Extracellular peritoneal ATP increases after LPS injection in mice.
5. P2X7 receptor is involved in the febrile response triggered by LPS or IL-1β.
6. TNF-α release is boosted after P2X7 receptor stimulation in LPS-extenuated macrophages.
7. Extracellular TACE activity increases upon P2X7 receptor activation in macrophages.

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Spanish summary

1. Introducción

En esta Tesis hemos trabajado con receptores purinérgicos, principalmente con P2X7, analizando sus diferentes funciones en células presentadoras de antígeno de la estirpe mieloide mononuclear fagocítica (monocitos, macrófago peritoneales y macrófagos derivados de médula ósea). Estudiando como la señalización purinérgica afecta a la diferenciación de los macrófagos, que asociación existe entre la activación del receptor P2X7 en macrófagos y la producción de eicosanoides, y por último identificar cómo este receptor regula la liberación de TNF-α durante la inmunosupresión. Para ello, hemos trabajado con diferentes estímulos celulares; como señales de peligro (PAMPs) principalmente con LPS, señal que es específicamente reconocida a través de TLR4; y señales de daño celular (DAMPs) principalmente con el nucleótido ATP y el nucleósido Adenosina.

2. Objetivos

1. Estudiar cómo afectan la señalización purinérgica a la diferenciación de los macrófagos.

2. Analizar la relación entre la activación del receptor P2X7 en macrófagos y la producción de eicosanoides.

3. Determinar cómo el receptor P2X7 regula la liberación de TNF-α en macrófagos extenuados.

3. Material y métodos

Los experimentos fueron realizados empleando: muestras humanas, macrófagos peritoneales obtenidos de lavados peritoneales en el campo quirúrgico y monocitos de sangre periférica de pacientes sanos y pacientes que sufrían un proceso séptico severo de origen abdominal; muestras no humanas, se emplearon ratones, de 8-10 semanas de edad, de la cepa C57 BL/6 y derivados modificados de esta cepa carentes de los diferentes genes que codifican para los receptores que se quería estudiar (*P2rx7*$^{-/-}$ y *P2rx4*$^{-/-}$), estos animales se emplearon para estudios tanto *in vivo* como *in vitro*; de estos ratones se diferenciaron macrófagos derivados de médula ósea, sobre los que se realizarán la gran mayoría de los estudios de esta Tesis.

Los diferentes tipos celulares fueron sometidos a protocolos de estímulos determinados para cada tipo de estudio: para estudios de respuesta ante situaciones de inflamación se sometió a las células a diferentes dosis de LPS durante 4 horas a 37 ºC con 5 % de CO_2; para estudios de polarización de macrófagos se estimuló a las células con 100 ng/ml de LPS en combinación con 20 ng/ml IFNγ (para polarizar a M1) y con 20 ng/ml de IL-4 (para polarizar a M2); para estudiar el efecto del flujo de iones a través de la membrana plasmática se emplearon tampones con altas concentraciones de potasio; para el estudio del efecto del calcio extracelular se empleó un tampón EGTA.

Los resultados de estos estudios *in vitro* fueron analizados mediante diferentes técnicas: la cantidad liberada al medio de IL-1β, TNF-α, PGE2, TXA2 y LTB4, se determinó empleando la técnica ELISA sobre los sobrenadantes de las células estimuladas mediante los métodos anteriormente descritos; con la finalidad de valorar la muerte celular sufrida por las células sometidas a los estímulos descritos, se cuantificó la presencia de LDH en los sobrenadantes

celulares y se comparó con la presencia de esta en los lisados celulares totales, expresándose los resultados en porcentaje total de contenido en LDH; la viabilidad de macrófagos derivados de médula ósea (BMDM) a lo largo de su diferenciación fue analizada mediante un ensayo de viabilidad (MTT) a diferentes días; se empleó la técnica de PCR reversa a tiempo real para cuantificar la expresión relativa de los genes que codifican para los diferentes receptores y marcadores citoquímicos de interés; mediante diversas técnicas de citometría de flujo se analizó la cuantificación de marcadores de membrana específicos de macrófagos, la muerte celular y la determinación de la etapa de ciclo celular en BMDM a diferentes tiempos de diferenciación tras la administración de ATP o adenosina; mediante técnicas fluorométricas se determinó la actividad de la enzima convertidora de TNF-α (TACE) y la actividad catepsina B, así como la determinación del calcio intracelular empleando Fura 2-acetoxymethyl (AM).

Los estudios *in vivo* realizados analizaron: la caracterización de la actividad el receptor P2X7 en modelo murino, mediante la administración intraperitoneal de LPS y el posterior análisis de los lavados peritoneales para la determinación de IL-1β y TNF-α empleando la técnica ELISA; la degradación de ATP extracelular en el peritoneo de diferentes cepas de ratón, esta medición se llevó a cabo mediante la cuantificación de la luminiscencia emitida por células HEK293-pmLUC que poseen en su membrana una luciferasa que degrada el sustrato, luciferina, en presencia de ATP; otro de los estudios realizados *in vivo* fue la medición de la fluctuación de la temperatura en diferentes cepas de ratón, tras la administración intraperitoneal de LPS, IL-1β recombinante con o sin tratamiento previo con el antagonista específico del receptor P2X7 (A438079).

4. Resultados y Discusión

La mayor parte de los efectos del ATP y la adenosina en la biología de células mieloides se han estudiado en los macrófagos maduros, en esta Tesis se estudió por primera vez cómo la señalización purinérgica afecta a la

diferenciación de los macrófagos a partir de precursores de la médula ósea. Se encontró que el ATP y la adenosina fueron capaces de disminuir el número de macrófagos diferenciados, pero los macrófagos resultantes fueron. Sin embargo, los macrófagos madurados en presencia de adenosina presentaron regulación positiva de ciertos marcadores M2, incluyendo la lectina YM1.

Los macrófagos son células altamente plásticas, que se adaptan a diferentes entornos y producen citoquinas para regular la respuesta inflamatoria tanto durante la iniciación (a través de M1 macrófagos polarización) y la resolución (a través de M2 polarización de macrófagos) (Gordon y Taylor, 2005;. Martínez et al., 2008). La adenosina es capaz de aumentar sinérgicamente la expresión génica *Arg1* en macrófagos maduros en combinación con IL-4 o IL-10, mejorando M2 fenotipo de macrófagos (Csóka et al., 2012;. Hasko et al., 2007). Nuestros resultados se corresponden con estos estudios, y nos confirman que la adenosina fue capaz de aumentar la expresión de *Arg1* en macrófagos maduros tratados con IL-4. Además, también encontramos que la adenosina es capaz de regular positivamente otros genes relacionados con el M2, como *Chi3l3* (YM1) y *Mrc1* (MRC1), pero no *Retnla* (FIZZ1). Por otro lado, de forma similar a otros estudios (Ramanathan et al., 2009), encontramos que la adenosina inhibe la expresión del gen proinflamatorio *Tnfa*, pero, sorprendentemente, en nuestro estudio hemos encontrado la expresión génica de *Il1b* se ve incrementada por el tratamiento con adenosina. Estos resultados sugieren que la adenosina promueve un fenotipo característico de los macrófagos y cuestionan el amplio efecto anti-inflamatorio o pro-M2 de la adenosina que surge de estudios que miden un solo gen como un marcador general de los fenotipos M1 o M2 (Csóka et al., 2012).

La diferenciación de los macrófagos a partir de precursores de médula ósea de ratón producen macrófagos maduro en reposo, que podrían ser polarizados hacia M1 o M2 dependiendo del tipo de señal de estímulo que se administre a esos macrófagos maduros (Loke et al., 2002; Welch et al., 2002). Sin embargo, este modelo podría ser diferente cuando los precursores de la médula ósea integran diferentes señales para aumentar o disminuir la producción de macrófagos, como sucede cuando la diferenciación se produce

dentro de un entorno que contiene nucleótidos extracelulares. Nuestro estudio encontró que ambos, ATP y adenosina, son capaces de detener el crecimiento de los macrófagos a partir de precursores de la médula ósea, confirmó el estudio anterior que identifica la señalización de este receptor a través de la producción de AMP cíclico y posterior activación de p27kip-1 para inhibir la proliferación de macrófagos (Xaus et al., 1999). Aquí descartamos que el posible efecto de la activación de P2X7R por ATP y la inducción de la muerte celular, y hallamos que los macrófagos diferenciados en presencia de ATP o adenosina eran macrófagos maduros, expresando el marcador de macrófagos maduros de ratón F4 / 80 (Austyn y Gordon, 1981). Las ectonucleotidasas de la superficie celular presentes en las células mieloides son capaces de degradar el ATP a adenosina (Dull et al., 1992;. Mello et al., 2014), nuestros datos indicaron que el ATP se degrada rápidamente por los precursores de la médula ósea, y el dar como resultado la adenosina podría explicar la inhibición de la proliferación de los macrófagos. A partir de los diferentes marcadores M1 y M2 analizados en nuestro estudio, los macrófagos diferenciados en presencia de adenosina tienen como resultado el incremento de *Chi3l3*, el gen que codifica para la lectina YM1, un marcador bien establecido de macrófagos M2 murinos (Ho y Sly, 2,009 ; Raes et al., 2002; Welch et al., 2002). YM1 es una proteína secretora sintetizada por los macrófagos murinos que se une a la heparina y la glucosamina (Chang et al., 2001) y su expresión está fuertemente inducida por IL-4 e IL-13, en comparación con la clásica estimulación M1 de macrófagos murinos (Nair et al., 2003; Raes et al., 2002). La expresión de YM1 inducida por el tratamiento con IL-4 incluye la activación del factor nuclear STAT6 (Welch et al., 2002).

A pesar de que YM1 este asociada a los macrófagos M2, se ha identificado también como una proteína que forma de cristales en inflamación pulmonar y crónica asociadas con la desregulación de macrófagos (Guo et al., 2000; Harbord et al., 2002). Encontramos que la expresión de YM1 está asociada a la inhibición de la proliferación de macrófagos inducida, no sólo por la señal anti-inflamatoria adenosina, sino también durante la senescencia de los macrófagos y en respuesta a las señales proinflamatorias tales como el LPS que también detienen la diferenciación de los macrófagos con M-CSF. La

expresión de gen *Chi3l3* fue opuesta a la proliferación de los macrófagos, que se expresa de manera inicial por precursores de la médula ósea. Estos resultados están en línea con un estudio que informó que existe una expresión transitoria de YM1 en las primeras células precursoras mieloides de tejidos hematopoyéticos, inicialmente en el saco vitelino y, posteriormente, en el hígado fetal, bazo y médula ósea (Hung et al., 2002). Por lo tanto, la lectina YM1 puede estar implicada en la hematopoyesis y la inflamación, por lo que es tentador especular que la producción YM1 podría estar implicado en la inhibición de la proliferación de los macrófagos después del tratamiento con adenosina o LPS.

La liberación de la citoquina proinflamatoria IL-1β por los macrófagos maduros es la clave para la iniciación de la respuesta inmune innata y es un proceso estrechamente regulado, que comprende una etapa inicial de estímulo (señal 1, endotoxinas normalmente bacterianas) y una segunda etapa de formación del inflamasoma (señal 2, por lo general una señal de peligro) (Pelegrín, 2011a). La naturaleza de la señal 2 va desde cristales a patógenos, pero el ATP extracelular es la señal de peligro endógena más estudiada que actúa a través del receptor purinérgico P2X7 (Ferrari et al., 2006;. Pelegrín, 2011a). La producción fisiopatológica de PGE2 está estrechamente unida a los niveles de expresión de COX-2, siendo esta un marcador bien establecido de la inflamación (Williams et al., 1999). La expresión de COX-2 es insignificante en células sin estimular, pero después de un estímulo con una endotoxina (clásicamente LPS), se produce una rápida inducción de los niveles de COX-2 (Goppelt-Struebe, 1995). En esta Tesis, se encontró que un estímulo a tiempos cortos (4 h) con LPS induce en los macrófagos un aumento de 600 veces en la expresión de los niveles de COX-2 y un aumento de 4 veces de la liberación de PGE2. Sin embargo, la liberación de PGE2 inducida por LPS era pequeña si se compara con la liberación después de la estimulación del receptor P2X7, lo que resultó en un incremento adicional en la liberación de PGE2 de hasta 8 veces. Por lo tanto, similar a la liberación de IL-1β, se encontró que el mediador lipídico PGE2 también se somete a un mecanismo de liberación de 2 pasos, donde la señal 1 induce la síntesis de COX-2 y la señal 2 aumenta el sustrato de la enzima: el ácido araquidónico. Aquí hemos encontrado que la señal 2

podría estar mediada por la activación del receptor P2X7, pero recientemente se ha encontrado que otros activadores del inflamasoma, tales como cristales de sílice o sales de aluminio, que también pueden regular la producción de PGE2, la cual actúa como señal 2 (Kuroda et al., 2011). Sorprendentemente, la liberación de PGE2 inducida por la señal 2 es independiente de la activación inflamasoma, y el uso de un inhibidor de caspasa-1 (en este estudio) o los macrófagos deficientes en la proteína adaptadora inflamasoma ASC o NLRP3 (Kuroda et al., 2011) no mostraron alteración en liberación de PGE2.

Por lo tanto, el mecanismo subyacente a la activación del receptor P2X7 y la liberación de PGE2 en los macrófagos maduros difiere de la vía inflamasoma, y encontramos que era altamente dependiente del aumento sostenido de calcio intracelular, penetrando directamente a través del receptor P2X7. Esta vía de señalización dependiente de calcio a través receptor P2X7, es similar a la vía recientemente descrita para la proliferación de células T o la inducción de la liberación de catepsinas inducidas por el receptor P2X7 (López-Castejon et al., 2010;. Yip et al., 2009) y en línea con la activación del inflamasoma por el receptor P2X7, un proceso independiente del calcio (Brough et al., 2003; Pelegrin et al., 2008). El receptor P2X7 activa fuertemente la señalización por MAPK (Donnelly-Roberts et al., 2004; Lenertz et al., 2011;. Shiratori et al., 2010), y encontramos que la actividad de JNK era necesaria para la liberación de PGE2 dependiente del receptor P2X7. Es bien sabido que JNK modula la expresión COX-2 (Hunot et al., 2004;. Nieminen et al., 2006), pero el ATP no alteró los niveles de COX-2 o la transcripción de la sintasa de PGE, descartando una etapa de síntesis en la vía de liberación de PGE2 por el receptor P2X7. Un análogo no metabolizable del ácido araquidónico bloqueó la liberación de PGE2 después de la estimulación del receptor P2X7, lo que sugiere que el aumento de calcio intracelular y la activación de MAPK conduce a un aumento de ácido araquidónico libre probablemente a través de la activación de cPLA2, ya que la actividad enzimática de cPLA2 está muy controlada por el calcio intracelular y la fosforilación (Gijón et al., 2000; Kuroda et al., 2011; Ulmann et al., 2010; Xia y Zhu, 2011). Además, previamente ya se había descrito que el aumento del calcio intracelular después de la estimulación del receptor P2X7 activa a cPLA2

en glándulas submandibulares de rata (Alzola et al., 1998), y nuestros datos apoyan este modelo en macrófagos. La modulación del ácido araquidónico por el receptor P2X7 nos llevó a estudiar si el ATP y el receptor P2X7 también podrían inducir la liberación de otros mediadores lipídicos importantes. Nos encontramos, por primera vez, con que el receptor P2X7 también podría llevar a cabo la liberación de otros mediadores del ácido araquidónico, como tromboxanos y leucotrienos, en macrófagos y monocitos estimulados.

En esta Tesis, hemos utilizado los macrófagos y monocitos humanos primarios para corroborar los datos obtenidos en ratón; se ha informado que en la maduración *in vitro* de macrófagos humanos a partir de monocitos aumenta la expresión del gen *P2RX7* (Gudipaty et al., 2001). Nuestros datos utilizando monocitos sanguíneos humanos y macrófagos peritoneales primarios maduros muestran que existe una expresión similar del gen *P2RX7*, pero los monocitos presentan una tasa de liberación más alta (de 10 a 100 veces) de IL-1β y autacoides en respuesta a la activación del receptor P2X7. Esta conclusión está en sintonía con los datos anteriores que permitían concluir que el receptor P2X7 es más activo en los monocitos que en los macrófagos y que la liberación de ATP endógeno a partir de monocitos puede activar liberación de IL-1β inducida por el receptor P2X7 (Netea et al., 2009b). Nuestros datos *in vitro* en macrófagos de ratón apoyan estudios previos sobre el papel del receptor P2X4 en la liberación de PGE2 debido a concentraciones de ATP extracelular bajas (Ullmann et al., 2010), pero la contribución del receptor P2X4 a la liberación de PGE2 fue insignificante cuando se activó el receptor P2X7. A pesar del hecho de que los monocitos humanos primarios y los macrófagos peritoneales presentaron mayor expresión del receptor P2X4 en comparación con el receptor P2X7, las concentraciones de ATP bajas solas o en combinación con ivermectina no pudieron potenciar la liberación de PGE2, lo que sugiere que el receptor P2X4 podría estar confinado en compartimentos intracelulares en los monocitos y macrófagos humanos y no ser funcional a el nivel de la membrana plasmática, ya que el receptor P2X4 presenta una secuencia de internalización (Royle et al., 2005).

Todos estos hallazgos tienen enormes implicaciones, ya que los autacoides controlan pasos de señalización clave en muchos procesos fisiológicos y fisiopatológicos, donde la señalización purinérgica y el receptor P2X7 pueden ser novedosos reguladores. Por ejemplo, es bien sabido que PGE2 sensibiliza nociceptores durante la inflamación, y por tanto aumenta la sensación de dolor (Portanova et al., 1996; Samad et al., 2002). Los experimentos con ratones deficientes para el receptor P2X7 y con antagonistas selectivos del receptor P2X7 han revelado que el receptor P2X7 es un mediador clave de la sensación de dolor en modelos de dolor neuropático, la hiperalgesia térmica, y la hipersensibilidad en enfermedad intestinal postinflamatoria (Chessell et al., 2005; Donnelly-Roberts et al., 2008; Honore et al., 2006; Keating et al., 2011). Los efectos antinociceptivos de la inhibición del receptor P2X7 se han atribuido a un deterioro de la liberación de IL-1β, ya que IL-1β ha sido descrito como un mediador del dolor (Honore et al., 2009). Sin embargo, después del bloqueo del receptor P2X7 existe una reducida actividad nociva de las neuronas (Itoh et al., 2011; Keating et al., 2011), que se correlaciona directamente con el efecto de PGE2 promoviendo la sensibilización de los nociceptores e hiperexcitabilidad (Portanova et al., 1996; Samad et al., 2002).

Una parte de esta Tesis se centra en la implicación del receptor P2X7 en la respuesta febril, uno de los cinco signos clásicos asociados con el proceso inflamatorio. La fiebre es una respuesta sistémica a la infección primaria, coordinada por la inducción de diferentes pirógenos endógenos (Bartfai y Conti, 2010). En realidad, está bien caracterizado que la IL-1β es uno de los pirógenos endógenos intermedios de transducción de señales derivados de patógenos. La señalización de IL-1β activa la producción de COX-2, y por lo tanto aumenta los niveles de PGE2, que ha sido postulado como uno de los pirógenos finales endógenos locales que actúa sobre el área termorregulador del hipotálamo, lo que aumenta la temperatura corporal (Bartfai y Conti, 2.010; Cao et al., 1996;. Li et al., 2001). Se sabe que el bloqueo de los receptores P2 atenúa la fiebre inducida por LPS en ratas (Gourine et al., 2005). Entre los receptores P2, el receptor P2X7 controla la liberación de ambos pirógenos IL-1β y PGE2, y encontramos que su deficiencia o bloqueo específico con

A438079 disminuye la respuesta febril en ratones en respuesta a LPS. Sin embargo, esta disminución podría deberse tanto a un deterioro de la IL-1β como a la producción de PGE2. Encontramos con éxito que la deficiencia del receptor P2X7 se relaciona con el deterioro en los niveles de IL-1β locales y sistémicos; sin embargo, no hemos podido detectar un aumento fiable de PGE2 *in vivo* después de estimular con LPS. Esto podría ser debido a que la vida media de PGE2 es muy corta *in vivo* (≈20 s) y a que los cambios locales en la producción de PGE2 durante la fiebre, son difíciles de detectar sistémicamente (Cao et al., 1996; Davidson et al., 2001; Eguchi et al., 1992; Ivanov y Romanovsky, 2004). Por ello, se utilizó IL-1β para inducir fiebre y encontramos que el aumento de la temperatura corporal fue significativamente menor en los animales deficientes para el receptor P2X7. Podemos sugerir que la deficiencia del receptor P2X7 bien altera el bucle de retroalimentación positiva de IL-1β o altera la liberación de PGE2 inducida por IL-1β exógena. También se encontró que el ATP, ligando endógeno para el receptor P2X7, fue acumulado *in vivo* después de la administración intraperitoneal de LPS. Este resultado coincide con publicaciones recientes donde el ATP se ha visto que es liberado como una molécula de peligro *in vivo* durante la patología de injerto contra huésped y en la hipersensibilidad por contacto (Weber et al., 2010; Wilhelm et al., 2010). En conjunto, podemos especular que la endotoxina bacteriana causa la liberación de la señal de peligro, ATP endógeno, que a su vez activa al receptor P2X7 para causar fiebre.

Diferentes compañías farmacéuticas están produciendo activamente antagonistas del receptor P2X7 como fármacos; algunos de ellos se han utilizado ampliamente en modelos animales de inflamación y el dolor, y algunos de ellos son usados en ensayos clínicos (Arulkumaran et al., 2011; Pelegrín, 2008). Estas moléculas han superado con éxito la fase I de ensayos clínicos, y algunos de ellos están en fase II / III para la artritis reumatoide, la osteoartritis, la enfermedad de Crohn, y la enfermedad pulmonar obstructiva crónica (Arulkumaran et al., 2011). Simultáneamente comparamos los efectos inhibidores de los antagonistas del receptor P2X7 con los inhibidores de caspasa-1 (bloqueadores inflamasoma) y con inhibidores de la COX-2 como representante de los anti-inflamatorios no esteroideos (AINE). Como era de

esperar, el bloqueo de caspasa-1 solamente disminuyó la inhibición de la liberación de citoquinas proinflamatorias IL-1β, sin afectar a la liberación de mediadores lipídicos. Inhibidores de la COX-2 que pertenecen a la familia de los AINE y comprenden uno de los tipos más utilizados de fármacos, principalmente debido a sus acciones antiinflamatorias y calmantes (Rainsford, 2007), solo fueron eficaces en el bloqueo de la liberación de PGE2 y TXB4, sin afectar a la liberación de LTB2 o IL-1β. Finalmente, demostramos que los antagonistas del receptor P2X7 podían bloquear los eicosanoides y la liberación de IL-1β, esto es lo que confiere a estos antagonistas un espectro anti-inflamatorio y analgésico más amplio que los AINE clásicos, emergiendo como agentes terapéuticos importantes para la inflamación, dolor y fiebre.

Una vez que hemos identificado que el receptor P2X7 puede inducir la liberación de otros mediadores de la inflamación (eicosanoides), además de la liberación bien caracterizado de IL-1β (Ferrari et al., 1997), encontramos que en macrófagos M1 el receptor P2X7 es capaz de controlar la liberación clásica de TNF-α, otro importante mediador proinflamatorio (Dinarello, 2.000; Movat y Cybulsky, 1987). Una publicación reciente demostró la inducción de TNF-α tras la activación del receptor P2X7 en la microglía (Shieh et al., 2014b). Sin embargo, este trabajo previo, demostró que en microglia el receptor P2X7 induce la liberación de TNF-α mediante la inducción de la expresión de genes de *novo*, en esta Tesis nuestros resultados sugieren que el receptor P2X7 potencia la liberación de TNF-α en los macrófagos, sin afectar a la expresión génica. De hecho, nuestros resultados demuestran que la liberación de TNF-α tras activar a P2X7 depende de proteasas (catepsinas y TACE).

En los macrófagos, la proteína quinasa activada por mitógeno (MAPK) p38 controla la activación de TACE (Scott et al., 2011). También se sabe que el receptor P2X7 activa a MAPK a través de aumento de calcio intracelular (Pfeiffer et al., 2004). En esta Tesis se demuestra que tras la activación del receptor P2X7 se redujo la liberación de TNAF-α cuando los macrófagos fueron tratados con un inhibidor de p38 MAPK o cuando el aumento de calcio intracelular fue bloqueado al exponer a los macrófagos a un medio extracelular sin calcio y con EGTA. Obviamente, esta liberación fue dependiente sobre el

tráfico intracelular de vesículas con TNF-α hacia a la membrana plasmática (Lieu et al., 2008; Shurety et al., 2001). Por otra parte, se encontró que el receptor P2X7 indujo la liberación de TACE, teniendo un papel fisiológico en la liberación de TNF-α en células que son deficientes en la actividad de TACE (Hiraoka et al., 2008; Reddy et al., 2000). La liberación de TNF-α es un mecanismo complejo y recientemente se ha demostrado que también implica la actividad de catepsina B a través de una vía alternativa, el grado de implicación de la catepsina B en la secreción de TNF-α parece ser variar en diferentes macrófagos (Ha et al., 2008). Se ha determinado que es la actividad de la catepsina B intracelular, la cual está implicada en el proceso de la liberación de TNF-α, en lugar de la actividad de la catepsina B del espacio pericelular (Ha et al., 2008). Como el receptor P2X7 controla la liberación de catepsinas en macrófagos (López-Castejón et al., 2010), encontramos que el receptor P2X7 induce la liberación de TNF-α a través de catepsinas.

Diferentes estudios apoyan la importancia proinflamatoria del ATP extracelular y del receptor P2X7 en la respuesta inflamatoria sistémica, Y P2X7 es importante en modelos de sepsis en ratón (Cauwels et al., 2014; Santana et al., 2015; Sumi et al., 2014). Los modelos animales de sepsis sólo reproducen parcialmente la fisiopatología del síndrome de sepsis humana, donde después de una sobreestimulación del sistema inmune se desarrolla un estado de inmunosupresión en los pacientes (Hotchkiss y Karl, 2003). La alta mortalidad de pacientes que padecen sepsis grave se debe a infecciones secundarias incrementadas durante la fase inmunosupresora que siguen a la fuerte tormenta inicial de citoquinas en respuesta a la infección sistémica (Hotchkiss y Karl, 2003). Encontramos que durante esta inmunosupresión, la activación del receptor P2X7 podría aumentar la respuesta inmune a través de la liberación de TNF-α en situaciones en las que se extenuan las células inmunes. Esto fue evidente en experimentos de pulso y captura para estudiar la regulación de la liberación de TNF-α por el receptor P2X7. Por lo tanto, la potenciación del receptor P2X7, podría ser beneficiosa para patologías como la sepsis grave o la infección por VIH donde existe una inmunosupresión, habiéndose ya sugerido recientemente en un artículo donde se emplea un modelo de sepsis en ratón (Csoka et al., 2015).

En general, esta Tesis demuestra que la señalización purinérgica es capaz de reducir el número de macrófagos diferenciados de médula ósea, resultando en una regulación positiva de ciertos marcadores M2, como YM1, lo que resulta en un fenotipo específico de macrófago. En los macrófagos maduros, la activación de P2X7 induce la liberación del mediador lipídico PGE2 y siendo P2X7 importante en la respuesta febril, un signo clásico asociado con el proceso inflamatorio. Además, la señalización mediante el receptor P2X7 en los macrófagos M1 extenuados es capaz de controlar la liberación clásica de TNF-α. Estos resultados tienen implicaciones clínicas, ya que el receptor P2X7 emerge como una diana terapéutica prometedora para la fiebre y su potenciación durante inmunosupresión podría aumentar la inmunidad en los procesos sépticos.

5. Conclusiones

Finalmente podemos concluir que en esta Tesis se demuestra que:

1. El crecimiento de los macrófagos durante su diferenciación desde precursores de la médula ósea, se ve inhibido tras estimulación purinérgica.

2. Los macrófagos diferenciados en presencia de adenosina desarrollan un fenotipo alternativo similar a M2, con un aumento de la expresión de Ym1.

3. La activación de los receptores P2X7 en los macrófagos aumenta la liberación de eicosanoides.

4. El ATP extracelular del peritoneo aumenta tras inyectar LPS en ratones.

5. El receptor P2X7 está implicado en la respuesta febril provocada por LPS o IL-1β.

6. La liberación de TNF-α se incrementa tras la estimulación del receptor P2X7 en macrófagos extenuados por LPS

7. Encontramos TACE de forma extracelular tras la activación del receptor P2X7 en los macrófagos.

Publications resulting from this Thesis

1. Published

1. Baroja-Mazo A, Barberà-Cremades M, Pelegrín P. *The participation of plasma membrane hemichannels to purinergic signaling*. Biochim Biophys Acta. 2013 Jan;1828(1):79-93. doi: 10.1016/j.bbamem.2012.01.002. Epub 2012 Jan 12. Review.

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2. Barberà-Cremades M*, Amores-Iniesta J*, Martínez CM, Di Virgilio F, Baroja-Mazo A, Pelegrín P. *Extracellular ATP as an early danger signal to allotransplants leading to graft rejection) during allograft rejection.* (*Share first authorship).