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Expression and function of the $P2X_7$ receptor in monocytes from patients with Behçet's disease

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Abbreviation

AECA	Anti-endothelial cell antibodies
ANA	Anti-nuclear antibody
AP-1	Activator protein 1
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine 5′ triphoshate
B2M	Beta-2-microglubulin
BD	Behçet's disease
BSA	Bovine serum albumin
BzATP	2′-3′-O-(4-benzoylbenzoyl)adenosine 5′ triphoshate
CARD	Caspase-recruitment domain
CHDP	Cationic host defence peptides
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
FMF	Familial Mediterranean fever
Fura-2AM	Fluorescent-2-acetoxymethyl ester
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-B51	Human leukocyte antigen

hsCRP	high sensitivity C-reactive protein
ICE	IL-1β-converting enzyme
IgG	Immunolgobulin
IL	Interleukin
KN62	1-[N,O-bis(5-isoquinoline sulfonyl)-N-methyl-L-tyrosyl] 4 phenylpiperazine
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MEFV	Familial Mediterranean fever gene
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP3	NACHT, LRR and PYD domains-containing protein 3
NFkB	Nuclear factor kappa B
OA	Osteoarthritis
oATP	Periodate-oxidized ATP
PAMPs	Pathogen-associated molecular patterns
panx1	Pannexin-1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PRR	Pattern recognition receptors
PS	Phosphatidylserine
PYD	Pyrin domain
qRT-PCR	quantitative Reverse Transcriptase-Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SLE	Systemic lupus erythematosus

SNP	Single-nucleotide polymorphisms
SN	Supernatant
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF-α receptor
UBC	Ubiquitin C
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Abstract

Background. $P2X_7$ receptor is a nucleotide-gated ion channel involved in the inflammatory response triggered by passive release of ATP from damaged cells. It is expressed in monocytes and plays a key role in promoting the release of IL-1β. Behçet's disease is a systemic immune-inflammatory disorder of unknown origin whose clinical manifestations include oral and genital ulcers, skin lesions, uveitis and arthritis. Since innate immunity activation and IL-1β release seem to play a relevant role in BD, we hypothesized a $P2X_7$R involvement in the pathogenesis of the disease.

Methods. PBMC, or isolated monocytes, were prepared from 18 BD patients and 17 healthy controls matched for age and sex. In these cells we evaluated: (i)$P2X_7$R expression, and (ii) function, induced by $P2X_7$ receptor stimulation, as determined by cytosolic free Ca^{2+} influxes measurements and IL-1β release.

Results. $P2X_7$R expression and the amount of Ca^{2+} influxes, induced by the selective $P2X_7$R agonist BzATP, were higher in BD patients than in healthy controls monocytes. Moreover, in BD patients BzATP stimulation significantly enhanced the release of IL-1β from LPS-primed monocytes. Interestingly, we noted that BD patients treated with anti-TNFα drugs approached the $P2X_7$R expression and function of healthy controls. In addition, TNFα incubation stimulated $P2X_7$R expression and $P2X_7$R-dependent Ca^{2+} uptake in monocytes from healthy controls.

Conclusions. Our results provide evidence that in monocytes from BD

patients both the expression and the function of the purinergic $P2X_7$ receptor are increased with respect to healthy controls. These results confirm our hypothesis of an involvement of $P2X_7$ receptors in the pathogenesis of BD, in which it takes part in the inflammatory amplification process probably through the establishment of an autocrine loop in which also the TNF-α is involved. On this basis, $P2X_7$ receptor may designate a new potential therapeutic target of the disease to enhance BD resolution.

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Chapter 1

Introduction

1.1 Purinergic signalling

The so called purinergic receptors are membrane receptors activated by extracellular nucleotides. They are divided in two families P1 and P2 first distinguished for adenosine and ATP/ADP activation respectively [1]. Four subtypes of P1 receptors have been cloned, namely A_1, A_{2A}, A_{2B} and A_3. All P1 adenosine receptors are typical G protein-coupled (metabotropic) receptors [2]. In 1985, Burstock and Kennedy proposed dividing P2 receptors into two subtypes on the basis of pharmacology, P2X, ligand-gated ion channels, and P2Y, G protein-coupled receptors [3, 4]. Currently, seven subtypes of the P2X family and eight subtypes of the P2Y family have been cloned and functionally characterized.

Members of the ionotropic $P2X_{1-7}$ receptors share less than 50% identity and a similar structural topology consisting of two transmembrane domains connected by a large extracellular loop containing the putative ATP binding site, and intracellular N and C termini of various lengths [5, 6]. Data from a variety of studies support the idea that P2X channels exist as homomeric and heteromeric trimers [7] and that they are channels selectively permeable

to cations allowing the influx of Na^+ and Ca^{2+} ions and the efflux of K^+ [8]. The eight metabotropic P2Y receptor subtypes cloned (PY1, PY2, PY4, PY6, PY11, PY12, PY13 and PY14) are characterized by an extracellular N-terminus, an intracellular C-terminus and seven transmembrane spanning regions [9]. They present a high level of sequence homology between some transmembrane spanning regions, but structural diversity of intracellular loop and C-terminus thus influencing the degree of coupling with different G proteins [2].

The role of P2X and P2Y receptors in physiology is gaining importance since they are widely distributed on a large array of cell types and they induce important events in cell including proliferation, differentiation, apoptosis or necrosis, and release of inflammatory and tissue regenerating molecules. However, a full characterization of the specific mechanisms that these receptors have in different functions has to be defined [7, 9, 10].

1.2 The $P2X_7$ receptor

The human $P2X_7$R gene is localized in a 55-kb region of chromosome 12q34 and comprises 13 exons [11]. The $P2X_7$R gene is highly polymorphic with more than 686 single-nucleotide polymorphisms (SNPs) [12, 13]. Most of SNPs characterized to date have been shown to cause loss-of-function, but gain-of-function SNPs have also been described [14, 15, 16, 17, 18, 19, 20]. However, the functional effect of most SNPs are unclear and recent findings suggest that future genetic association studies of $P2X_7$ SNPs and disease should consider $P2X_7$R haplotypes rather than single SNPs [17]. Moreover, the $P2X_7$ function is influenced by the presence of eight splice variants of the human $P2X_7$ channel [21].

The $P2X_7$ protein is a 595 amino acid sequence consisting of an intracellular N-terminus, two hydrophobic transmembrane domains, an extracellular

loop and an intracellular C-terminus [22] (Fig. 1.1).
The N- and C-termini of $P2X_7$R comprise amino acids 1-25 and 356-595 respectively and form intracellular complexes with several membrane proteins including α-actin, receptor like tyrosine phosphatase and heat shock proteins [23]. The main structural distinctive features of the $P2X_7$R is a long C-terminal tail that is essential for pore formation, receptor trafficking [24] and also stabilization of $P2X_7$R in the membrane [25, 26].
The first and the second transmembrane domains include amino acids 26-46 and 335-355 respectively.
The extracellular loop (amino acids 47-334) comprises the main binding site of ATP [22], contains 10 cysteine residues that may form disulfide bonds and also contains N-glycosylation sites [27].
Although other receptors of the P2X subfamily have been shown to form functional hetero-oligomers, the $P2X_7$ subunits seem to associate only with each other to form homotrimeric $P2X_7$ receptors [28].

The $P2X_7$ receptor is predominantly expressed on cells of the immune system, such as macrophages/monocytes, dendritic cells, lymphocytes, and mast cells, as well as on various types of glia within the periferal and central nervous system, including microglia, astrocytes, oligodendrocytes, and Schwann cells [11, 29, 30, 31, 32]. $P2X_7$ protein subunits are also expressed on epithelial cells, osteoblasts fibroblasts and human synoviocytes [33, 34, 35, 36].

Activation of the $P2X_7$ channel has been associated with multiple cellular functions [37]. In monocytes, macrophages, dendritic cells and microglia it is best characterized for its role in mediating the processing and release of mature, biologically active interleukin-1β and interleukin-18 [38, 39, 40]. Macrophages and microglia pretreated with the $P2X_7$ receptor antagonists KN-62 or periodate-oxidized ATP (oATP), or from $P2X_7$-deficient mice, fail to release IL-1β when challenged with ATP or BzATP [41, 42]. Lenertz et al [43] showed that $P2X_7$R mediates ROS production in primary human mono-

cytes and that generation of ROS most likely involves activation of ERK1/2 and NADPH oxidase complex.

Activation of $P2X_7$R can induce proliferation of certain cell types like $P2X_7$R-transfected leukemic, K562 and LG14 cells line, and normal and leukemic T- and B-lymphocytes [44, 45, 46].

The role of $P2X_7$ has also been investigated in models of spinal cord injury or cerebral ischemia to assess the role of $P2X_7$ in neurodegeneration and cell death [47, 48].

$P2X_7$ channels are also expressed on osteoblasts and osteoclasts where the physiological role of $P2X_7$ channels in bone development and remodelling is not entirely clear [49, 50].

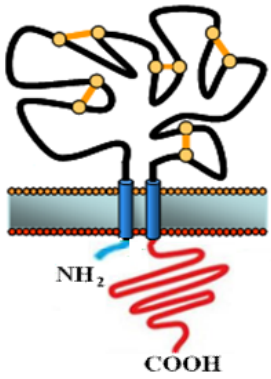

Figure 1.1: **Schematic representation of the $P2X_7$ receptor.**The $P2X_7$ protein is a 595 aminoacid sequence consisting of an intracellular N-terminus, two hydrophobic transmembrane domains, an extracellular loop containing cysteine residues and an intracellular C-terminus. Adapted from [12].

1.2.1 $P2X_7$R activation induces Ca^{2+} influxes

A specific property of the $P2X_7$R is that it exhibits two agonist-activated conductance modes.

Upon activation with ATP or potent agonist BzATP, the receptor functions as a non-selective cation channel permeant to small cations. After this activation, dependent on extracellular divalent cations, it allows the influx of Na^+ and Ca^{2+} ions and the efflux of K^+ [51, 52, 53]. Upon repeated or prolonged application of agonist, the $P2X_7$R becomes permeable to larger molecules like ethidium bromide and YO-PRO [51, 54, 55] (Fig. 1.2).

Several mechanisms have been proposed to explain this phenomenon [56]. It was hypothesized that other molecular components are necessary for the transition in the pore mode, like Pannexin-1 (panx1), a hemichannel protein that seems to be the large pore associated to $P2X_7$R [39, 57].

In contrast, other studies reported that sustained rise in $P2X_7$ current and permeability was caused by dilatation of the $P2X_7$R itself [55, 58]. Yan et al [55] suggest that channel pore opening of $P2X_7$R involves a two-step transition: from closed to opened and from opened to dilated, leading to the generation of a biphasic currents. The occurrence of biphasic current, response only at high agonist concentrations [55], is in accordance with a hypothesis of two distinct ATP activation sites at $P2X_7$R [59].

Alloisio et al [60] reported evidence of a $P2X_7$R complex in which the C-terminus domain modulate the interaction between two conductive pathways: one with the ATP binding site and permeable to Ca^{2+}, and the other permeable to monovalent and large cations.

Although the molecular basis of pore formation, $P2X_7$ activation causes massive upset of cytoplasmatic ion homoestasis. Since the $P2X_7$ is nondesensiting, the pore stays open as long it is bound by ATP.

The $P2X_7$ receptor is distinct from the other P2X family members requiring 10- to 100- fold higher ATP concentrations for its activation [37].

2'(3')-O-(4-Benzoylbenzoyl)adenosine-5'-triphospahate (BzATP) is a $P2X_7$R synthetic agonist that exhibits an order of magnitude greater potency than ATP, while a potent, non-competitive, human $P2X_7$R antagonist is 1-[N,O-bis(5-isoquinoline sulfonyl)-N-methyl-L-tyrosyl] 4 phenylpiperazine (KN-62) [61].

$P2X_7$R activation is also influenced by various extracellular ions. Virginio et al [51] carried out a comprehensive study of functional inhibition of $P2X_7$ by divalent cations. They showed that Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+} strongly inhibited both $P2X_7$-mediated currents and YO-PRO-1^{2+} dye uptake.

Extracellular Na^{2+} ions can also impair $P2X_7$ receptor function. Extracellular Na^+ removal was found to drastically change the $P2X_7$ ion channel kinetics [62]. In fact, the open probability, the mean open time as well as the activation and deactivation time constants considerably increased when Na^+ was replaced by other monovalent cations. Under conditions of hypoxia or tissue injury, ATP and K^+ are secreted into the extracellular space [37]. Therefore, in metabolically compromised tissue, a replacement of extracellular Na^+ by K^+ may enhance the efficacy and potency of ATP on $P2X_7$ receptors. The fact that the $P2X_7$ receptor-induced increase of the cell membrane permeability to organic cations is promoted in Na^+-free media can, therefore, be explained by an enhanced $P2X_7$ receptor activation. K^+ efflux may reinforce the ATP effect on $P2X_7$ receptor-expressing cells, and even low concentration of ATP may activate the $P2X_7$R to a substantial extent [63].

Gudipaty et al [64] showed that both the allosteric modulation by extracellular ionic composition, and the receptor surface density, have a critical role in the induction of nonselective pores by human blood monocytes. They showed that limited expression of cell surface $P2X_7$ receptor subunits is one mechanism for the lack of nonselective pore formation in freshly isolated blood

monocytes stimulated with high concentrations of extracellular ATP.

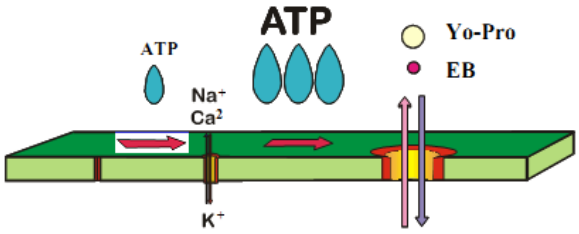

Figure 1.2: **Schematic representation of the $P2X_7$R activation.** ATP activation allows the influx of Na^+ and Ca^{2+} ions and the efflux of K^+. Upon repeated or high concentration of agonist, the $P2X_7$R becomes permeable to larger molecules like ethidium bromide and YO-PRO.

1.2.2 $P2X_7$R activation induces IL-1β maturation and release

Activation of the $P2X_7$ channel is characterized for its role in mediating the processing and release of mature, biologically active interleukin-1β from immune cells.

IL-1β is a pro-inflammatory cytokine predominantly released from monocytes, macrophages and dendritic cells [65, 66, 67]. IL-1β production by mononuclear phagocytoses is controlled at the transcriptional, translational, maturation and secretion level, [68]. Pathogen-associated molecular pattern molecules (PAMPs), such as LPS, activate the nuclear factor-kappa beta (NFkB) cascade to induce the IL-1β gene transcription and accumulation of the 31 kDa pro-cytokine in the cytosol, acting as only a very weak stimulator of IL-1β maturation and externalization [12, 38, 69]. A secondary stimulus is required for efficient processing and release of mature IL-1β. Maturation of pro-IL-1β is catalysed by caspase-1(casp-1), also known as IL-1β-converting enzyme (ICE) [70, 71]. In quiescent cells, casp-1 is normally present as an

inactive 45kDa precursor, procaspase (procasp)-1. Following cell stimulation with inflammatory stimuli, casp-1 undergoes activation. Proteolytical activation of procasp-1 occurs in a multimeric specialized structure named "inflammasome" [72] comprising at least four different proteins (casp-1, apoptosis-associated speck-like protein containing protein containing a CARD (ASC), and NACHT-, LRR-, and PYD-containing proteins) [73]. The inflammasome activation can be driven by several stimuli, one of those is the depletion of cytosolic K^+ [38, 74] caused by $P2X_7$R activation [75]. Activation of the $P2X_7$R by ATP leads to the loss of cytoplasmatic K^+, and the assembling and activation of the NALP3 (NLRP3) inflammasome, a multi-protein complex that recruits pro-caspase-1 and induces its proteolytic activation [12, 38]. Pannexin-1 is also critical for NALP3 inflammasome and $P2X_7$R-mediated IL-1β maturation and release even if the exact role of pannexin-1 remains to be fully determined [76]. Panx-1 participates in $P2X_7$ receptor-dependent inflammasome activation as a transducer of the perturbation in intracellular K^+ homeostasis caused by the $P2X_7$R even if, in the sequence leading to inflammasome activation, K^+ efflux is upstream of panx-1 and is required for panx-1 activation [39, 77] (Fig. 1.3).

Two models have been put forward to explain IL-1β externalization. Andrei et al [79] suggest that IL-1β is accumulated into endocytis vesicles (secretory lysosomes) together with casp-1, then a $P2X_7$R-induced loss of intracellular K^+ activates phosphatidylcholine-specific phospholipase C, which in turns causes an increase in cytosolic Ca^{2+}, Ca^{2+}-dependent phospholipase A_2 activation, and exocytosis of the IL-1β-containing lysosomes. According to this model, casp-1 activation and IL-1β processing are triggered by the K^+ loss-stimulated activation of a Ca^{2+}-independent phospholipase A_2 within the lysosomes.

Alternatively, MacKenzie et al [80], upon $P2X_7$R-mediated macrophages activation, proposed that IL-1β, in the mature as well as immature form, is

packaged into small plasma membrane blebs that are released into the extracellular space as microvescicles. Once the microvescicles approach the plasma membrane of the target cells, where the ATP concentration is higher than the bulk solution due to active cellular rise, the $P2X_7$R is activated, the microvescicles lyse and IL-1β is released [81]. Microvescicles budding and released are preceded by phosphatidylserine flip and loss of membrane

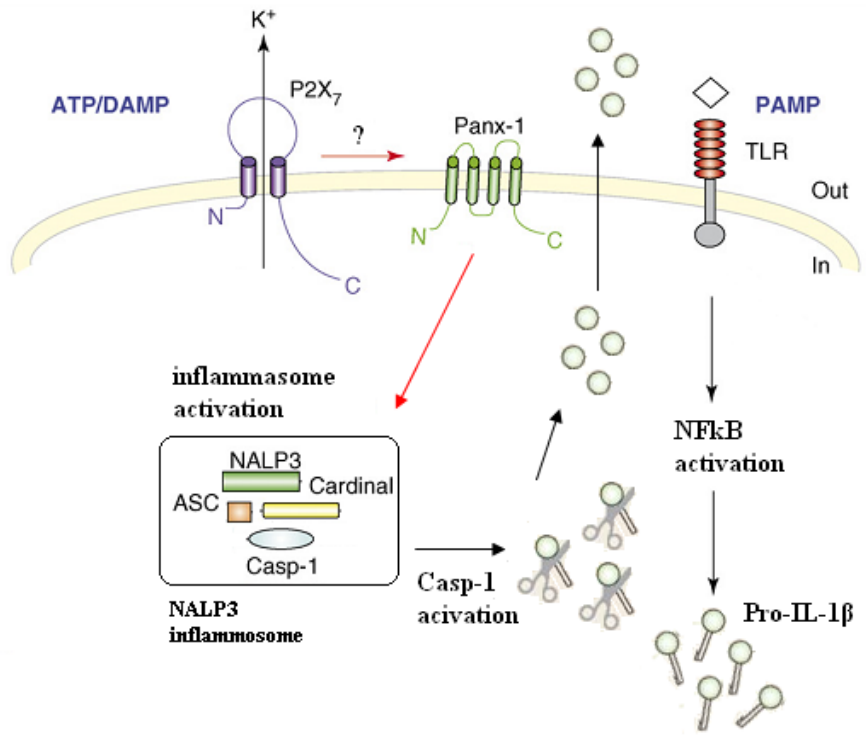

Figure 1.3: **$P2X_7$R activation induces IL-1β maturation and release.** Activation of $P2X_7$R by ATP results in K^+ efflux and the activation of pannexin-1 to activate the NALP3 complex containing the adaptor molecule ASC and cardinal. This complex aggregates pro-caspase-1 molecules to form an inflammasome complex that facilitate pro-caspase-1 activation and subsequent pro-IL-1β processing to its mature IL-1β form. Figure adapted from [78]

asymmetry [80].
In both of these models, the final trigger for release is stimulation of $P2X_7$ receptor.

Several studies indicate that in addition to extracellular ATP, endogenous agonists, like CHDP (also known as antimicrobial peptide), could produce significant $P2X_7$R stimulation. The endogenous, inducible CHDP, LL-37 induces caspase-1 activation and secretion of mature IL-1β in LPS-primed monocytes, in absence of cytotoxicity, through $P2X_7$R activation [82, 83, 84].

IL-1β is a pleiotropic inflammatory cytokines that has a crucial role in the activation of both the innate and adaptive immune response. Its inappropriate secretion can drive consequences, as shown by acquired and hereditary chronic inflammatory diseases [68]. Thus, it is vital for the organism to exert a tight control on its secretion. This is achieved by a "two-key" mechanism of release [78]. Firstly, detection of PAMPS drives IL-1β gene transcription and accumulation of the pro-cytokine without any immediate consequences. If the foreign microorganism also causes call damage, DAMPs (such as intracellular ATP) are released, the $P2X_7$R is turned on and the IL-1β secreted.

IL-1β is produced by both monocytes and macrophages, but the rate and mechanism of release differ according to the differentiation status and the origin of these cells. Piccini et al [85] showed that, in human monocytes, agonists of different PRRs (pattern recognition receptors) trigger the release of endogenous ATP as a common response and that the autocrine stimulation of $P2X_7$R by the released ATP is then responsible for the cascade of events that leads to maturation and secretion of IL-1β. Ward et al [86] showed that human peripheral blood monocytes undergo PS (phosphatidylserine) exposure in response to $P2X_7$ receptor activation, which was independent of priming stimulus, caspase-1 activation, and IL-1β secretion and that 24h activation with LPS or stimulation with TLR 7/8 agonists leads to $P2X_7$ receptor-independent IL-β secretion only in primary human monocytes but

not in monocyte-derived macrophages. However, sequential addition of ATP in both cell types resulted in a fast accentuated release response to enhanced IL-1β secretion.

1.2.3 $P2X_7$R and inflammation

Inflammation is a complex response generated by an interacting network of stimulatory an inhibitory signals. Immune cells primed by soluble factors produced by infections or tissue damage may or may not progress to a full-activated phenotype depending on the additional signals that they receive by neighbouring cells [87]. Ferrari et al describe $P2X_7$R like a "sensor of danger" that monitors the release of danger signals (ATP) at inflammation sites and drives mononuclear phagocytes primed bacterial products into fully activated inflammatory effectors (IL-1-secreting cells). Thus, in the coordinate response to pathogens, $P2X_7$R might be a "checkpoint" where the choice is made to progress to a full-blown response or put inflammation on hold [12]. Accumulation of ATP into the pericellular space generates a microenvironment with a dual activity. A moderate ATP release, such as might occur during a low-level inflammation due to a weakly pathogenic microorganism, exerts an anti-inflammatory effect [88, 89]. On the other side, high ATP concentrations, activating the low-affinity $P2X_7$R, causes massive release of pro-inflammatory mediators and induces the antigen presenting cells to initiate the innate immune response [88]. Gudipaty et al [64] demonstrated that in human blood monocytes, where the $P2X_7$ receptor protein traffics to the plasma membrane, multiple mechanisms have evolved to minimize the adventitious activation of $P2X_7$ receptors in circulating monocytes while permitting these receptors to be rapidly unregulated and activated when monocytes leave the blood and enter the peripheral sites of tissue inflammation.

Innate immunity is able to recognise several pathogen-associated molecular

pattern molecules (PAMPs) that are detected by a small number of receptors, known as pattern recognition receptors (PRRs) whose the best known members are TLRs. PRRs function as "sensors" that alert the immune system of an impending danger. However, innate immune is initiated by a variety of cytokines such as IL-1β, IL-18, IL-6 and TNF-α, all of which can be produced by $P2X_7$R activation [90]. This channel is predominantly expressed on cells of haemopoietic origin such as monocytes, macrophages and microglia where activation of these cell types is associated with an increased expression of the $P2X_7$R that ultimately leads to an amplification of the downstream production of the pro-inflammatory cytokines IL-1β and IL-18, and in turn IL-6, IL-8 and TNF-α. As over-production of these cytokines is detrimental, particularly in chronic disease state [90].
These observations suggest that the $P2X_7$R has a putative role in redirecting the immune and or inflammatory cells response. $P2X_7$R antagonists can be useful to both decrease the cytokines over-production and inhibit inflammasome activation, since inflammasome dysregulation is known to produce inflammatory disorders [38]. New studies in $P2X_7$R knockout mice continue to indicate that this receptor plays a role in a number of conditions in addition to arthritis and include multiple sclerosis, hepatitis and pain [91, 92].

1.3 Behçet's disease

Behçet's disease is a systemic inflammatory vasculitis of unknown etiology with a diverse spectrum of clinical manifestations [93].
It is heterogeneous in onset, involves different organs and results in considerable morbidity and increased mortality [94, 95].
In 1937, Behçet, a Turkish dermatologist, identified the three major signs and grouped them in a clinical entity [96]. There are three key features of BD: recurrent oral ulcers, genital ulcers and ocular disease. In addition,

skin, joint, nervous, cardiac, vascular and gastrointestinal systems may be involved. BD can affect nearly every system of the body, following a course of relapses and remissions.

It usually develops between the third and the forth decades of life, rarely appearing before puberty and after the age of 50 [97, 98, 99] and it is slightly more frequent with a worse clinical course in men [100, 101].

As there are no pathognomic clinical or laboratory findings of BD, several diagnostics criteria have been developed during the years, all having in common the three major features of oral ulceration, genital ulceration and eye lesions. It is utmost importance to collect a detailed clinical history as it can help exclude other important conditions [96].

Main goals of the treatments are relieving symptoms, achieving a rapid resolution of inflammation, preventing or limiting tissue damage, reducing frequency and severity of attacks, and avoiding complications [102]. The treatments used are determined by which organ is affected and the extension and severity of the involvement. They include corticosteroids, colchicine, alkylating agents, metotrexate, calcineurin inhibitors, azathioprine, thalidomide, sulfasalazine, dapasone, pentoxyfilline, INF-γ and anti-TNF-α drugs [96].

1.3.1 Autoimmunity vs autoinflammation in Behçet's disease

The etiology of Behçet's disease is still unknown, but both the genetic and environmental factors are thought to be important in the pathogenesis of the disease. BD has been considered to be due to an autoimmune process triggered by an infectious or environmental agent in a genetically predisposed individuals [103, 104]. Various immunological studies show an immune hyperreactivity to streptococci in BD [105, 106]. However, as microbial antigens common to different species seem to drive a similar immune activation in BD,

not the specific microorganism itself but its presence and persistence might determine its role in BD pathogenesis [107].

Although Behçet's disease has been frequently considered as a Th1 type autoimmune disease, it was recently found that BD and autoinflammatory diseases share several clinical features. Behçet's disease does not have the classical clinical features of autoimmunity such as anti-nuclear antibody (ANA) positivity, female dominance and association with other autoimmune diseases such as Sjogren's syndrome [108]. However, it has various aspects that deserve to be evaluated as "autoimmune" like the MHC association, the autoantibodies against cell surface antigens presence and the classical immunosuppresant drugs efficacy. BD is associated with class I antigen HLA-B51 [107]. HLA-B51 allele, located in the MHC locus on chromosome 6p, has been the most strongly associated risk factor for BD [109]. A general B-cell activation and autoantibodies against cell surface antigens such as anti-endothelial cell (AECA) and anti-lymphocytes antibodies are demonstrated [110, 111] and some effective treatments in BD are classical immunosuppressives (azathioprine, cylophosphamide) and T-cell inhibitors (cyclosphorine A) [96]. However, TNF-α-antagonists are also an exception in this concept, as they act mainly as anti-inflammatory agents and are accepted to be contraindicated in autoimmune disease such as SLE and multiple sclerosis.

A recently introduced concept to BD is autoinflammation. Autoinflammatory diseases are described as a group of inherited disorders characterized by episodes of seemingly unprovoked recurrent inflammatory attacks due to activity of cells of the innate immunity, mainly neutrophils [112]. The prototype disorder in Middle Eastern populations is familial Mediterranean fever (FMF). Behçet's disease, with some of its clinical features such as recurrent non-scarring mucocutaneous lesions and non-deforming arthritis, and enhanced inflammatory response with the overexpression of pro-inflammatory cytokines, is described to be in this spectrum [113]. Increased activity of neu-

trophils and elevated levels of interleukin-1β are observed in both Behçet's disease and autoinflammatory diseases. Furthermore, MEFV mutations are also observed more frequently in BD and are associated with a more severe disease [114]. However, various clinical aspects differ between the two diseases, like the prolonged inflammatory skin response reported only in BD patients [115].

Although there are clinical and inflammatory response similarities between autoinflammatory disorders and BD, the presence of a prolonged inflammation such as non-specific (pathergy) or urate-induced skin responses suggests that innate and adaptive pathways are more integrated in BD [116]. A unifying hypothesis for BD requires the explanation of these links between the two arms of the immune system. One explanation might be an unprovoked, uncontrolled innate-related inflammation causing an adaptive system activation only as a secondary response, as in autoinflammatory disorders [113].
It is also known that a defective regulation in the inflammasome-driven interleukin-1β secretion leading to an uncontrolled release of the cytokine represents a key pathogenetic mechanism of the so-called "autoinflammatory diseases" [117] which probably can take part in BD.

The serum levels of several cyokines including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-18 and TNF-α are elevated in BD patients [104, 118]. Studies showed that sophisticated intercalating cytokine network is closely implicated in the onset, evolution and even organ damages of the disease [119].
IL-1 has been found elevated in sera of patients with BD [120, 121]. The study of Salish et al. demonstrated a significantly higher level of IL-1β in synovial fluid of BD patients as compared to that in OA patients [122]. Moreover, a rapid and sustained responses to IL-1β blockade with anakinra, a recombinant non-glycosylated human IL-1 receptor antagonist, have been reported [123, 124].

Monocytes are the principal source for some of these cytokines so they may take part in the pathogenesis of chronic inflammation in this disorder. The presence of functionally active monocytes and also monocytes in the stage of transition to macrophages in Behçet's patients have been shown [125]. Several studies suggested that they may play a role in BD pathogenesis by the overproduction of proinflammatory cytokines that result in severe inflammation [126, 127, 128].

1.3.2 Anti-TNF-α therapies

TNF-α is a potent cytokine produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges [129]. It is a fundamental cytokine in the establishment and maintenance of the inflammatory response.

TNF-α exerts its effects through two distinct receptors, TNFR1 and TNFR2 [130]. Binding of the inherently TNF-α to TNFR1 and TNFR2 induces receptor trimerization and recruitment of several signalling proteins to cytoplasmatic domains of the receptors. Exposure of cells to TNF-α can result in the activation of a caspase cascade leading to apoptosis [131]. The binding of TNF-α to its receptors causes, also, activation of two major transcription factors, AP-1 and NF-kB, that in turn induce genes involved in chronic and acute inflammatory responses [132, 133].

TNF-α is considered to be involved in BD pathogenesis based on several findings: high levels of TNF-α have been found in the aqueous humour and serum of BD patients with uveitis; the number of TNF-α producing cells is increased in the active phases of the disease; TNF-α has been involved in experimental animal models of uveitis [96, 134, 135].

At present there are 3 TNF-α inhibitors available: infliximab, a recombinant monoclonal antibody; adalimumab, a humanized monoclonal antibody; and

etanercept, the fusion protein human p75 TNF-α receptor IgG [134]. All TNF-α inhibitors share a common molecular mechanisms of action, which is to competitively inhibit ligand-binding to their cognate receptors. However, the inhibitors differ in their physical and functional properties, including valency, effector functions, pharmacokinetics, and antigenicity [136].
A number of reports verifying the therapeutic efficacy of anti-TNF-α agents in BD have been published, denoting that TNF-α partakes probably in somehow the disease onset and or evolution [137, 138, 139]. The essential role of TNF-α has been substantiated since numerous proinflammatory cytokines have been observed down-regulated after the *in vivo* administration of infliximab [140, 141, 142].

1.4 Aim of the study

This thesis aims to examine the $P2X_7$ involvement in BD pathogenesis.
Specifically the aims are to:
(1) Analyse $P2X_7$R expression in human peripheral blood monocytes from BD patients and healthy controls;
(2) Study the $P2X_7$R function, as determined by calcium influxes and IL-1β release in human peripheral blood monocytes from BD patients and healthy controls;
(3) Detect a possible relation between $P2X_7$R and TNF-α.

Chapter 2

Materials and Methods

2.1 Study population

Study population consisted in 18 patients affected with BD (7 females) and 17 age- and sex-matched healthy volunteers. Demography, clinical history, laboratory data and ongoing therapy of the subjects are reported in Table 4.1. The diagnosis of BD was based on the criteria of the International Study Group [143]. Local Ethical Committee approved the study, and patients gave their written informed consent in accordance with the Principles of the Declaration of Helsinki.

2.2 Peripheral blood cells isolation and monocytes preparation

PBMC were isolated from 20 ml of venous blood from each subject by a density gradient centrifugation. Blood was diluted 1:3 with phosphate-buffered saline (PBS) pH 7.2, supplemented with 0,5% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA), and layered over 15ml $Lympholyte^{®} - H$ ($Cederlane^{®}$) and centrifuged at 400xg for 35 minutes.

Cells were washed twice with PBS pH 7.2, 0,5% BSA and 2mM EDTA to obtain PBMC. Then, cells were incubated with MACS CD14 MicroBeades and monocytes were isolated by using a Magnetic Separation with Positive Selection Columns (all from Miltenyi Biotec). Both PBMC and monocytes were cultured in RPMI 1640 with stable L-Glutamine, supplemented with 10% fetal bovine serum (FBS), 50U/ml penicillin and 50μg/ml streptomycin (Euroclone).

2.3 Analysis of $P2X_7$ receptor mRNA by qRT-PCR

Total RNA was isolated from monocytes using the RNeasy Plus Mini kit (Qiagen). For cDNA synthesis the QuantTect Reverse Transcription kit (Qiagen) were used according to the manufacturer's instructions. Real-time quantitative PCR was performed in the LightCycler system using the 2x QuantiFast SYBR Green PCR Mixture (Qiagen) and pre-designed primers for human $P2X_7$(Hs_P2RX7_1_SG -QuantiTect PrimerAssay-Qiagen). Human UBC (Hs_UBC_1_SG), YWHAZ (Hs_YWHAZ_1_SG) and B2M (Hs_B2M_1_SG) all from Qiagen were used as housekeeping genes. The cycling conditions comprised 5 min polymerase activation at $95°C$ and 40 cycles at $95°C$ for 10 sec and $60°C$ for 30 sec. The relative expression of analysed mRNAs from BD patients and healthy controls was normalized to the level of the three housekeeping genes in the same cDNA sample using the comparative CT method ($2^{-\Delta\Delta Ct}$).

2.4 Flow cytometry analysis of $P2X_7$ receptor expression in monocytes

PBMC ($2x10^5$) were incubated with an anti-$P2X_7$R (extracellular) antibody fluorescein isothiocyanate (FITC) conjugated (Sigma) for 30 minutes at 4°C and immunostained for CD14 using phycoerythrin (PE)-labelled monoclonal antibodies (BD Pharmigen). Mouse IgG2a-PE and Rabbit IgG-FITC isotype control antibodies were used for assessing the level of background staining of cells. Cells were analysed in a Dako Galaxy Flow Cytometry System using the FlowMax software.

2.5 $P2X_7$ receptor-induced calcium influx measurement

PBMC ($2x10^6$) were loaded with the cell-permeant fluorescent calcium dye Fura-2 acetoxymethyl ester (AM; 3 μM; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 20 minutes at 37°C. After loading, the cells were kept at room temperature in a medium containing 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) plus 1% BSA. Just prior to the experiment, a 1 ml aliquot of the cell suspension was rapidly centrifuged and resuspended in fresh medium. For experiments performed in the absence of extracellular calcium, the medium contained 0.2 mM ethylene glycol tetraacetic acid (EGTA) with no added Ca^{2+}. Fluorescence was measured with a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA; excitation wavelengths 340 and 380 nm, emission 510 nm) equipped with magnetic stirring and temperature control, and the images were acquired using digital imaging techniques. To minimize leakage of trapped Fura-2, the assay temperature was $30°C$, and 200 μM sulfinpyrazone was included in the medium.

Cells were stimulated with 100-500 μM BzATP in the presence and the absence of extracellular calcium or 1 μM KN62, all from Sigma-Aldrich. At the end of each incubation, 50 μg/ml digitonin and 20 mM EGTA were added to measure maximal (Rmax) and minimal (Rmin) fluorescence ratio (340/380) values, respectively.

For single cell $[Ca^{2+}]_i$ experiments, PBMC ($2x10^6$) were plated on glass coverslips (13mm diameter) at the density of $2.5x10^4$ per well in complete RPMI medium. After aspiration to remove non adherent cells, monocytes were loaded with the cell-permeant fluorescent calcium dye Fura-2 acetoxymethyl ester (AM; 3 μM; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 20 minutes at 37°C. Calcium measurements were carried out at room temperature. Fura-2-loaded cells were placed on a fluorescence image microscope and perfused with a medium containing 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) plus 1% BSA in the presence of 100-500 μM BzATP, or 1 μM KN62; all from Sigma-Aldrich). The digital fluorescence-imaging microscopy system was mounted on a Nikon Diaphot 300 (Nikon, Tokyo, Japan) inverted microscope. Fluorescence images were collected through a Nikon oil immersion $40x13$ numerical aperture objective and acquired by a cooled charge-coupled device (CCD) camera (Photometrics, Roper Scientific, USA) and a MetaMorph imaging system (Universal Imaging, Downingtown, PA, USA).

To describe the features of Ca^{2+} influx we analysed two parameters: AUC (4′) of $[Ca^{2+}]_i$ and $\Delta t\frac{1}{2}$ to $[Ca^{2+}]_i max$. The Area Under the curve (AUC) represents the area enclosed between the curve of the $[Ca^{2+}]_i$ versus the time for 4 minutes after the addition of the stimulus (AUC (4′)) and describes the extent of Ca^{2+} uptake both through the channel that following the pore opening. The half time necessary to reach the maximum Ca^{2+} concentration in the cytosol ($\Delta t\frac{1}{2}$ to $[Ca^{2+}]_i max$) is used to describe the time course of the

response. We also consider the ratio between this two parameters (AUC and $\Delta t\frac{1}{2}$) in order to analysed the phenomenon of Ca^{2+} influx overall.

2.6 $P2X_7$R expression and $P2X_7$R-induced calcium influx after stimulation with TNFα

$1x10^6$ PBMC isolated from healthy controls were plated in 6 wells plate and incubated in completed RPMI medium with or without 1-5,5ng/ml TNF-α (Miltenyi Biotec) for 18 hours. After incubation the cells were collected in order to analyse $P2X_7$R expression by flow cytometry. PBMC ($2x10^6$) isolated from healthy controls were plated on glass coverslips (13 mm diameter) at the density of $2.5x10^4$ per well in complete RPMI medium. After aspiration to remove non adherent cells, monocytes were incubated in completed RPMI medium with or without 1-5,5ng/ml TNF-α (Miltenyi Biotec) for 18 hours and then loaded with the cell-permeant fluorescent calcium dye Fura-2 acetoxymethyl ester to measure the Ca^{2+} influxes.

2.7 Measurement of BzATP-induced release of IL-1β

Isolated monocytes were plated in a 24-well culture plates in completed RPMI 1640 medium and seeded at $37°C$ over night. Adherent monocytes were cultured for 2 hours in complete culture medium containing 1 μg/ml LPS (Sigma), washed and then stimulated with 100 μM BzATP in RPMI culture medium for 30 minutes. Finally, cell culture supernatants were collected, and the concentration of IL-1β was determined by ELISA (R&D Sytems or Invitrogen).

2.8 Statistical analysis

Results were expressed as the arithmetic mean ± standard deviation. Statistical analysis was performed using the GraphPad-InStat (version 3.06 for Windows 2000, GraphPad, San Diego, CA, USA; Microsoft Corp., Redmond, WA). Parametric analysis was performed using two-tail unpaired t test, one-way analysis of variance (ANOVA) and one-way analysis of variance for repeated measurements (RM-ANOVA) with the Tukey-Kramer test for multiple comparisons. The Mann-Whitney U test, the Kruskal-Wallis test, the Friedman test with the Dunn's Multiple Comparison test and Spearman Rank Correlation were used when data were not normally distributed. Values of $p < 0.05$ were considered significant.

Chapter 3

Results

3.1 $P2X_7$ expression

3.1.1 Monocytes from BD patients display an enhanced expression of cell surface $P2X_7$ receptor

Monocytes represent immune effector cells, equipped with chemokine receptors and adhesion receptors that take part in the inflammatory process [144]. Behçet's disease (BD) is a systemic inflammatory disorder [96] where both autoimmunity and autoinflammation may peculiarly play a key pathogenic role. Previous work demonstrated expression of cell surface $P2X_7$ receptors in $> 80\%$ of human blood monocytes [11, 145]. Therefore, the possibility that $P2X_7$ receptor expression is higher in BD patients than in healthy controls was tested.

PBMC, isolated from venous blood, were double immunostained for $P2X_7$R and CD14 in order to analyse the $P2X_7$ receptor expression in monocytes. Fig. 4.1 illustrates the FACS dot-plot of PBMC obtained from a representative BD patient and confirms the previous results that almost all $CD14^+$ cells express $P2X_7$ receptors [145]. In fact, Fig. 4.2 shows that only $\sim 10\%$

of $CD14^-$ cells expressed $P2X_7$ while $\sim$ 85% of $CD14^+$ cells were $P2X_7$ positive both in BD patients and healthy controls. Based on these results, we compared the $P2X_7$R expression between BD patients and healthy controls looking at blood monocytes ($CD14^+$ cells). Although the percentage of monocytes expressing $P2X_7$R did not change between patients and healthy controls, the mean fluorescence intensity (MFI, an index of receptor number per cell) of surface labelled $P2X_7$ receptors increased significantly in BD patients compared with healthy controls (Fig. 4.3).

However, quantitative qRT-PCR analysis of P2RX7 gene expression in monocytes indicated that the levels of $P2X_7$ receptor mRNA were similar in BD patients and in healthy controls blood monocytes (Fig. 4.4), suggesting that $P2X_7$ receptor gene transcription or stability did not increase in patients.

3.2 $P2X_7$ function

3.2.1 $P2X_7$ receptor-mediated calcium influx is increased in monocytes from BD patients.

The function of $P2X_7$R was assessed by analysing the effect of BzATP agonist on Ca^{2+} influx.

We first analysed Ca^{2+} influx using $2x10^6$ PBMC from healthy controls and BD patients. The application of 500 μM BzATP in calcium-free medium did not induce calcium rise, while the readdition of extracellular calcium (1.2 mM) resulted in a light $[Ca^{2+}]_i$ increase, putatively attributed to the extracellular calcium entry through ligand gated $P2X_7$ receptor. When PBMC were incubated with 1 μM KN62, a $P2X_7$ antagonist, the calcium mobilization was inhibited confirming the specific role of $P2X_7$ receptor (Fig. 4.5).

However, since it was found that $P2X_7$R is expressed mostly in mono-

cytes, the Ca^{2+} influx was evaluated on isolated monocytes using a single-cell calcium measurement and a lower concentration of BzATP (100 μM). Despite a certain variability in the response was observed, BD patients showed an higher sensitivity to the $P2X_7$R stimulation, in terms of calcium influx, in comparison with healthy controls (Fig. 4.6).

In fact, not only the large majority of BD monocytes responded to the lowest BzATP concentration used (with only 5% of non-responders vs. 20% in the controls), but also the amount of calcium influx throughout the time was higher in the patients than in controls (Fig. 4.7). Although the extent of calcium entry in monocytes, as assessed by AUC, was not significantly different between the two groups, the half-time to reach the cytosolic calcium peak ($\Delta t\frac{1}{2}$) and the AUC/$\Delta t\frac{1}{2}$ ratio were significantly reduced and increased, respectively, in BD patients compared with healthy controls (Fig. 4.7).

In BD patients $P2X_7$R expression, as assessed by MFI, was significantly correlated with both $\Delta t\frac{1}{2}$ (inversely; $r = -0.64$, $p = 0.01$) (data not shown) and AUC/$\Delta t\frac{1}{2}$ ratio (directly; $r = 0.63$, $p = 0.01$) (Fig. 4.8), while this correlation is not significant when healthy controls were considered. This means that BD monocytes, unlike healthy controls, increased the amount of calcium influxes also upregulating the $P2X_7$ receptor trafficking to the plasma membrane.

3.2.2 $P2X_7$ receptor activation markedly increases interleukin-1β release in LPS-stimulated monocytes from BD patients

To further explore the functional status of $P2X_7$R in BD patients, we assessed the effect of BzATP, a $P2X_7$ agonist, on the release of IL-1β by freshly isolated monocytes. As shown in Fig. 4.9, we observed that monocytes in

culture spontaneously released IL-1β, without any significant difference between BD patients and healthy controls. Differently to LPS, not able to increase cytokine levels in supernatant per se, LPS and BzATP co-incubation significantly enhanced IL-1β release with respect to both unstimulated and LPS-stimulated cells. The extent of IL-1β release after LPS and BzATP co-incubation was markedly higher in BD patients when compared with healthy controls.

3.3 Tumor necrosis factor-α up-regulates $P2X_7$R expression and function in monocytes

Increasing evidence suggests that anti-TNFα drugs, including neutralizing monoclonal antibodies (infliximab, adalimumab, golimumab) and soluble decoy receptors (etanercept), are very effective in BD patients non-responders to conventional therapies [146, 147, 148].

In our study population, a significant proportion of BD patients were under chronic treatment with these anti-TNFα drugs (12/18, 66%). Interestingly, we noted that untreated BD patients showed the highest values of $P2X_7$R expression and function (evaluated as calcium fluxes). In fact, both the MFI, that describes the $P2X_7$R expression, and the $P2X_7$receptor-mediated Ca^{2+} influxes were significantly higher only in untreated BD patients when compared with healthy controls. Instead, BD patients treated with anti-TNFα drugs approached the expression and function of healthy controls (Fig. 4.10).

These data suggested a possible stimulating effect of TNFα on $P2X_7$R expression and function as counteracted by anti-TNFα drugs. An investigation on the effect of this cytokine was conducted in ex-vivo experiments on monocytes from five healthy controls. The preincubation of monocytes with 1 or 5.5 ng/ml TNFα for 18 hours [149] was associated with a significant, dose-

dependent, increase in $P2X_7$R function, as assessed by $\Delta t_{\frac{1}{2}}$ and AUC/$\Delta t_{\frac{1}{2}}$ ratio measurement (Fig. 4.11a). The TNFα stimulation induced also an enhanced protein expression of cell surface $P2X_7$ receptors (Fig. 4.11b).

Chapter 4

Discussion

The aim of this thesis was to study the $P2X_7$R involvement in BD pathogenesis. We hypothesized that an overexpression/overactivity of the $P2X_7$R, that plays a key role in promoting the release of the pro-inflammatory cytokine IL-1β, may contribute to the inflammatory activation characterizing BD.

The first part of this work enlighten that monocytes from BD patients express more cell surface $P2X_7$ receptors than those from healthy controls. Furthermore, the $P2X_7$R function, analysed in terms of Ca^{2+} influxes and IL-1β release, is higher in BD patients when compared with healthy controls.

Concerning $P2X_7$R expression, we detected that $P2X_7$R is expressed in almost all $CD14^+$ cells ($\sim$ 85%) both in BD patients and healthy controls. However, the number of cell surface receptors, indicated by the MFI value, was higher in monocytes from BD patients than healthy controls. These results show that, among PBMC, $P2X_7$R is mostly expressed in monocytes, as already known [145], and that monocytes from BD patients express more $P2X_7$R than those from healthy controls. In fact, the prolonged inflammation state of BD cause the presence of functionally active monocytes and also monocytes in the stage of transition to macrophages [116, 125], that increase the number of $P2X_7$R as they develop into fully differentiated macrophages

[64].
We show that the $P2X_7$R expression upregulation do not involve the gene transcription but only the cell surface expression. The $P2X_7$R overexpression is connected with the trafficking of the $P2X_7$R protein to the plasma membrane that is known to increase in activated monocytes, as when monocytes leave the blood and enter the peripheral sites of the tissue inflammation [64].

The presence of active monocytes in the state of transition to macrophages in BD patients was supported by the detection of an higher Ca^{2+} influxes and IL-1β release in BD patients when compared with healthy controls.

To analyse the $P2X_7$R-dependent Ca^{2+} influxes we used BzATP, the most potent synthetic agonist for this receptor [150]. In our experimental setting 100 and 500 μM BzATP incubation gave rise to Ca^{2+} uptake both in BD patients and healthy controls.
It is known that the Ca^{2+} influx after the $P2X_7$R activation trigger to a $P2X_7$R opening that involves a two step transition leading to a generation of a biphasic current [55]. The first step opening, from closed to open, was detected measuring the half-time necessary to reach the cytosolic calcium peak after $P2X_7$R activation, while the second step opening, from opened to dilated, was evaluated analysing the extent of calcium entry over the time. Using these parameters we show that the maximum Ca^{2+} concentration in the cytosol is quickly reached in BD patients. The amount of Ca^{2+} influx overall is also higher in monocytes from BD patients than those from healthy controls.
The greater ability of BD patients to uptake Ca^{2+} after $P2X_7$R agonist stimulation when compared to healthy controls is connected either with the presence of active monocytes that develop into differentiated macrophages [90] and with an increased $P2X_7$R surface density [64]. In fact, the $P2X_7$R-mediated calcium influxes and the cell surface $P2X_7$R expression were sig-

nificantly and positive correlated only among the BD patients sample.

The better characterized $P2X_7$R function is its role in mediating the processing and release of mature interleukin-1β (IL-1β) from immune cells like monocytes [12, 151, 152, 153]. Primary human monocytes have multiple regulators of IL-1β release, in which a TLR-specific response induces proIL-1β synthesis and a secondary stimulus, like ATP, processing and release mature IL-1β [86].

In this work we exhibit that purified human primary peripheral blood monocytes both from healthy controls and BD patients release IL-1β after LPS-priming and BzATP stimulation, confirming the known mechanism of IL-1β secretion [38, 41]. Interestingly, we also noted that BD patients released higher amount of IL-1β in the SN (supernatant) when compared with healthy controls. The enhanced release of IL-1β by monocytes from BD patients in response to BzATP confirms the previous results showing the $P2X_7$R over-activity. Since IL-1β has been found elevated in sera and in synovial fluid of patients with BD [120, 121, 122], the enhanced release of IL-1β from BD patients monocytes in response to BzATP further supports the possible involvement of $P2X_7$ in the pathogenesis of this inflammatory condition.

Despite BD patients showed an higher $P2X_7$R expression and function when compared to healthy controls, we detected a certain variability among the patients sample. For this reason demographic, clinical, laboratory and therapeutic characteristics of BD patients studied were analysed.

A significant portion of the BD patients population was under chronic treatment with anti-TNFα drugs (12/18, 66%). Interestingly, we noted that untreated BD patients showed the highest values of $P2X_7$R expression and function (evaluated as calcium influxes), while treated BD patients approached the expression and function of healthy controls.

TNFα is a pleiotropic cytokine that regulates a broad range of biological activities, including inflammation, innate and adaptive immune responses, and

tissue development [129]. It is involved in acute inflammation and plays a key role in chronic inflammation in autoimmune diseases [154, 155]. TNF-α stimulation of healthy human monocytes activates signalling events that culminate in the induction of an acute inflammatory state increasing the proinflammatory cytokines production. It has been showed that human macrophages treated for 24 hours with TNF-α induced the IL-1β gene expression [156]. Moreover Yarilina et al showed that long-term exposure to TNF on primary human macrophages activates a "feed-forward" loop that sustains inflammation and identify a new signalling pathway activated by TNF that mediates delayed and sustained signalling responses important for macrophage differentiation and chronic inflammation [157, 158].
Starting from these data we hypothesized that the presence or absence of TNF-α was responsible for the variability of $P2X_7$R expression and function among BD patients. For this reason the possible stimulating effect of TNF-α on $P2X_7$R expression and function was investigated *in vitro*. Monocytes from five healthy controls treated with TNF-α for 18h increased significantly both the cell surface $P2X_7$R expression, analysed by flow cytometry, and $P2X_7$R function, as assessed by Ca^{2+} influxes detection. These results show that monocytes from healthy controls after TNF-α stimulation become similar to monocytes from BD patients since the $P2X_7$R expression and function increase.

One of the main limit of the study is the lower number of patients enrolled, due to the fact that BD is a are disease. Moreover, for the same reason, the enrolled patients were observed in different phases of the disease (at the diagnosis or after treatment with anti-TNFα).

In conclusion, we can produce evidence that the inflammatory state of BD, characterized by hight levels of proinflammatory cytokines, is connected with an increase of the $P2X_7$R expression and function, in term of Ca^{2+} influxes and IL-β release, on monocytes. Moreover, it seems that the use of

anti-TNF-α drugs in BD patients decrease the $P2X_7$R overexpression and overfunction on monocytes. On the other side, the TNF-α treatment induced healthy controls monocytes to express more $P2X_7$R and to increase the Ca^{2+} influxes after the $P2X_7$R activation.

These results confirm our hypothesis of an involvement of $P2X_7$ receptors in the pathogenesis of BD. $P2X_7$R takes part in the inflammatory amplification process probably through the establishment of an autocrine loop in which both TNF-α and IL-1β are involved. However, further studies should be done in order to understand the specific connection between TNF-α and $P2X_7$R activity. The development of $P2X_7$ antagonists could represent a new class of anti-inflammatory compounds able to enhance BD resolution through the block of the inflammatory process amplification.

Figures

Table 4.1: **Study population characteristics.**

Demographic, clinical, laboratory characteristics and ongoing immunosuppressive treatment in BD patients and healthy controls.

$\oint$ Datum not available in three patients.

$\oint\oint$ Including infliximab ($n = 11$) or etanercept ($n = 1$)

	BD patients	Healthy controls	p
Patients,n	18	17	
Age, years	41.3±11.4	38.9±11.0	n.s.
Sex, F/M	7/11	7/10	n.s.
Disease duration, years	12.6±5.8	-	-
HLA-B51 positivity$^{\oint}$	12.6±5.8	-	-
hsCRP, mg/dl (n.v.<0.5)	0.43±0.53	-	-
ESR, mm/h (n.v.<25)	19.3±11.5	-	-
Ongoing immunosoppresive treatment			
anti-TNFα drugs $^{\oint\oint}$	12	-	-
Steroids	4	-	-
Cyclosporine	1	-	-
Methotrexate	1	-	-
Leflunomide	1	-	-
No therapy	3	-	-

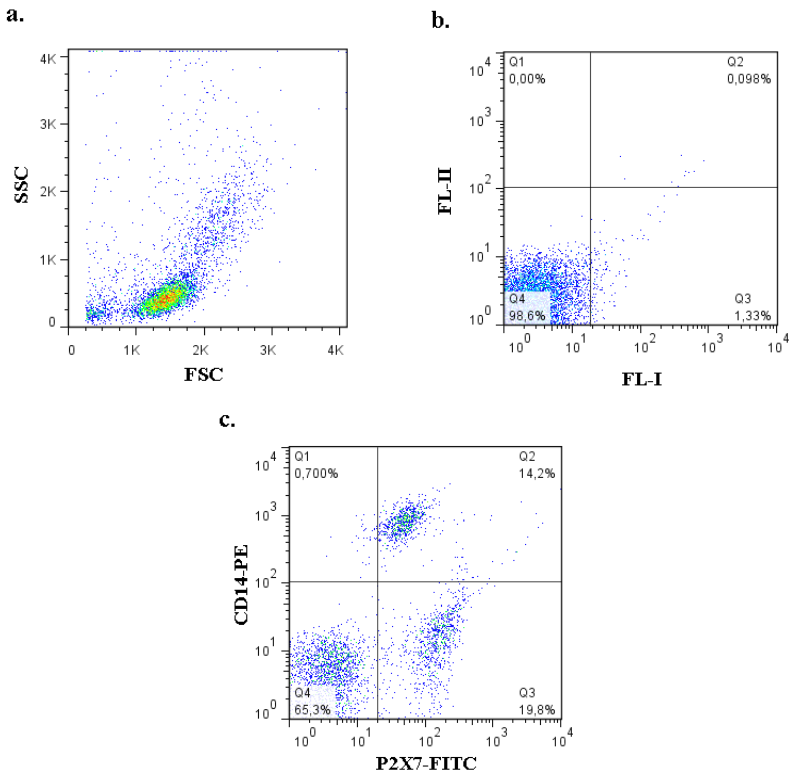

Figure 4.1: $P2X_7$ **receptor protein expression in PBMC by FACS analysis.**

Representation of PBMC obtained from one BD patient. (a) Characteristic forward (FSC, x-axis) and side (SSC, y-axis) scatter profiles of PBMC.
Dot plot for FLI (fluorescein, FITC) and FLII (phycoerithrin, PE) obtained from PBMC stained with mouse IgG2a-PE and rabbit IgG-FITC isotype control antibodies (negative control) (b) and with $P2X_7$-FITC and CD14-PE antibodies (c).

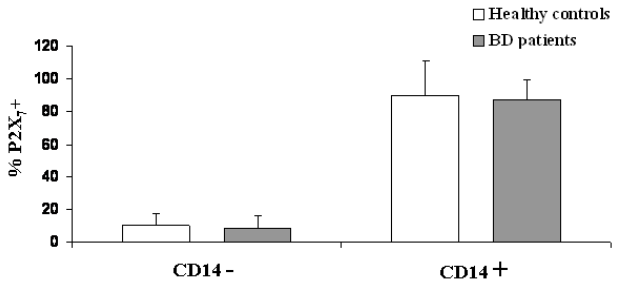

Figure 4.2: **Percentage of $P2X_7$-positive $CD14^+$ and $CD14^-$ cells.** PBMC from BD patients ($n = 14$) and healthy controls ($n = 14$) were double immunostained with $P2X_7$R-FITC and $CD14^+$-PE and analysed by flow cytometry. Arithmetic mean and SD are indicated.

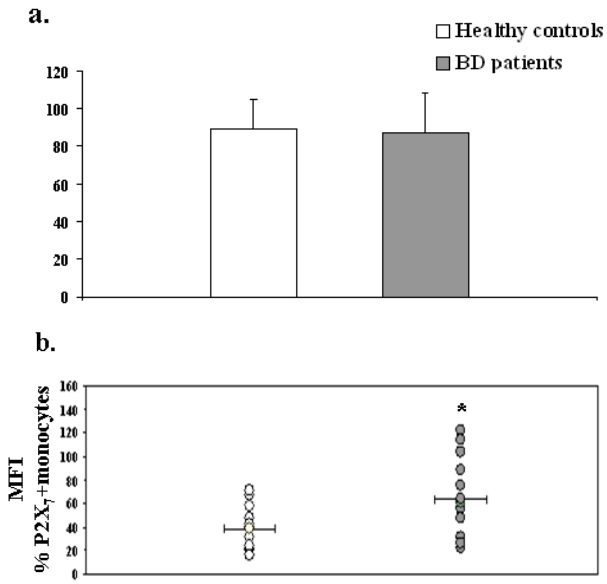

Figure 4.3: **Expression of $P2X_7$R in monocytes from BD patients and healthy controls.**

PBMC from BD patients ($n = 14$) and healthy controls ($n = 14$) were double immunostained for the detection of $P2X_7$R in monocytes ($CD14^+$) and were analysed by flow cytometry, as stated in Chapter 2. (a) Percentage of $P2X_7$-positive cells in monocytes. (b) Mean fluorescence intensity. Arithmetic mean and SD are indicated. $*p< 0.05$, two-tail unpaired t test.

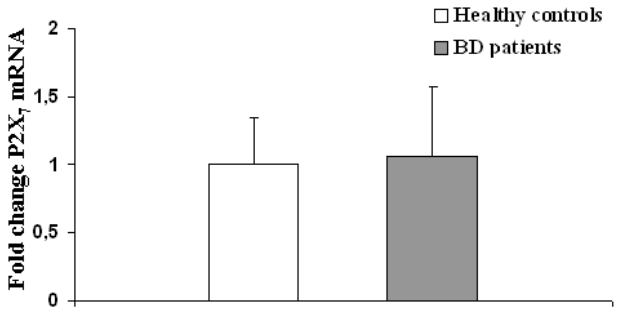

Figure 4.4: $P2X_7$**R mRNA levels of monocytes from BD patients and healthy controls.**

The relative $P2X_7$R mRNA levels were determined from 17 BD patients and 10 healthy controls monocytes by quantitative real-time PCR and normalized to three housekeeping genes (UBC, YWHAZ and B2M) mRNA expression. Data are the mean of triplicate PCRs obtained from each RNA preparation. Arithmetic mean and SD are indicated.

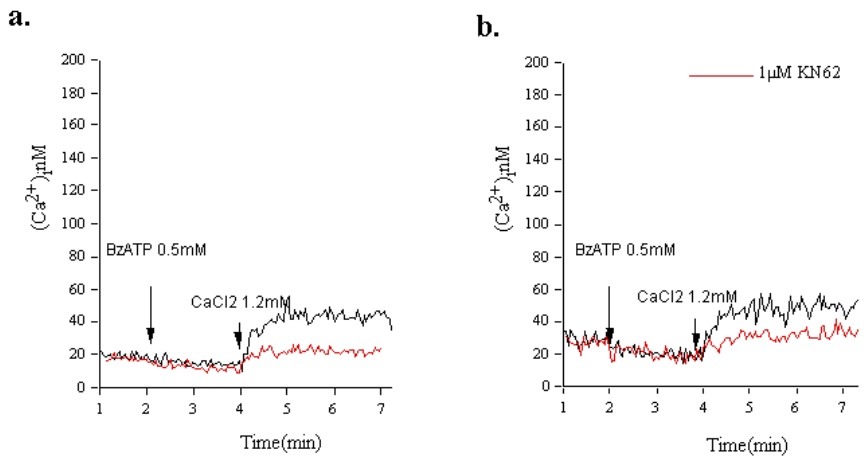

Figure 4.5: $P2X_7$**R-mediated calcium influx in PBMC analysed by fluorescence spectrophotometer.**

Representative images of $[Ca^{2+}]_i$ changes in the presence of 500 μM BzATP with or without preincubation with 1 μM KN62 of one healthy control (a) and one BD patient (b). Cells were loaded with the Ca^{2+} indicator Fura-2/AM and stimulated with the agonist and/or antagonist. For Ca^{2+}-free conditions, cells were incubated in Ca^{2+}-free, 0.2 mM EGTA-supplemented buffer.

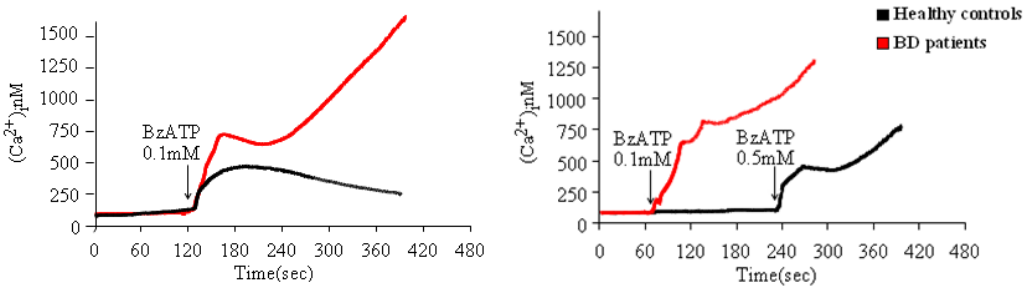

Figure 4.6: $P2X_7$**R-mediated calcium influx in monocytes analysed by single cell calcium experiments.**

The image is representative of the response of at least 30 cells of the same culture dish both from patients and controls. Cells were loaded with the Ca^{2+} indicator Fura-2/AM and stimulated with the agonist BzATP 100 or 500 μM. For each experimental condition, the traces are representative of similar results obtained from four distinct experiments. The arrow indicates the time of agonist addition.

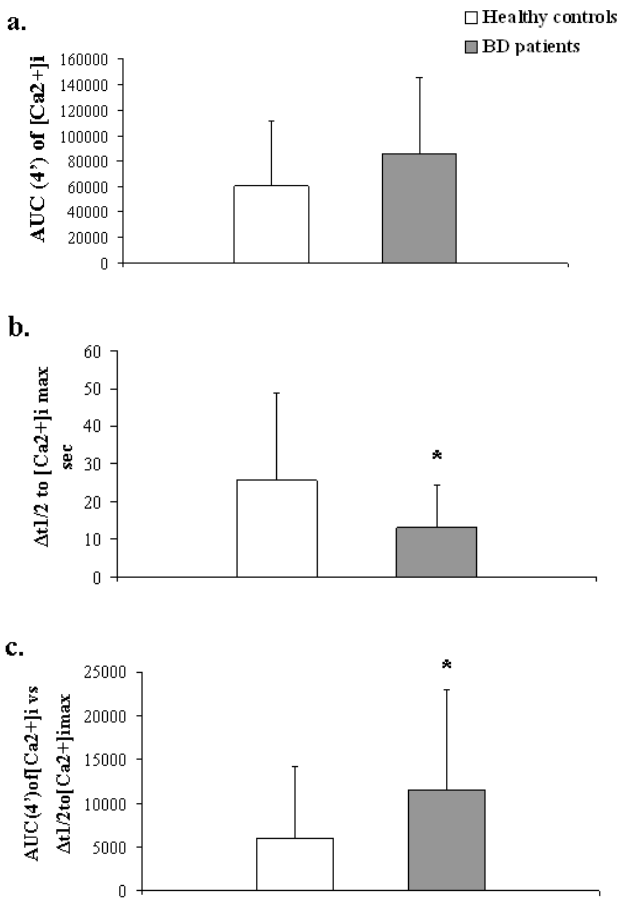

Figure 4.7: **Difference of $P2X_7$R-induced calcium influx between BD patients and healthy controls.**

Characterization of Ca^{2+} influx from 14 healthy controls and 17 BD patients by single cell calcium experiments. For each subject the average response of 25 ± 10 cells of the same culture dish, loaded with Ca^{2+} indicator Fura-2AM were analysed after stimulation with BzATP 100 μM, as described in Chapter 2. (a) The Area Under the Curve (AUC) of the mean cytosolic Ca^{2+} concentration upon 4 minutes of BzATP 100 μM stimulation (AUC (4′) of $[Ca^{2+}]_i$); (b) the half time necessary to reach the maximum Ca^{2+} concentration in the cytosol ($\Delta t\frac{1}{2}$ to $[Ca^{2+}]_i max$) and (c) the ratio between the two parameters AUC and $\Delta t\frac{1}{2}$ is reported. Arithmetic mean and SD are indicated. $*$p< 0.05, Unpaired t test.

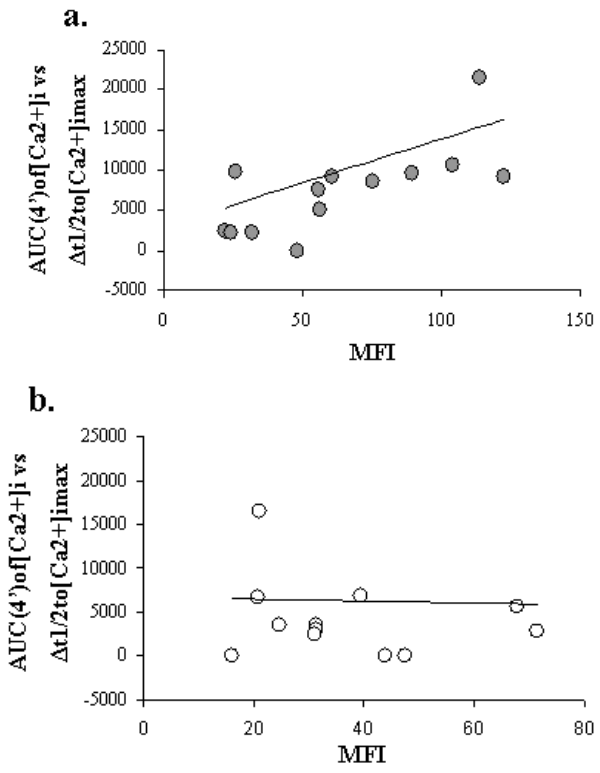

Figure 4.8: **Correlation between $P2X_7$R expression and $P2X_7$ receptor-induced calcium influx in monocytes from BD patients and healthy controls.**

Correlation between $P2X_7$R expression, as assessed by MFI, and $P2X_7$R-induced calcium influx (AUC/$\Delta t\frac{1}{2}$) of 14 BD patients (a) and 13 healthy controls (b).

(a) BD patients. Direct correlation (Spearman Rank correlation); $r = 0.63$, $p = 0.01$.

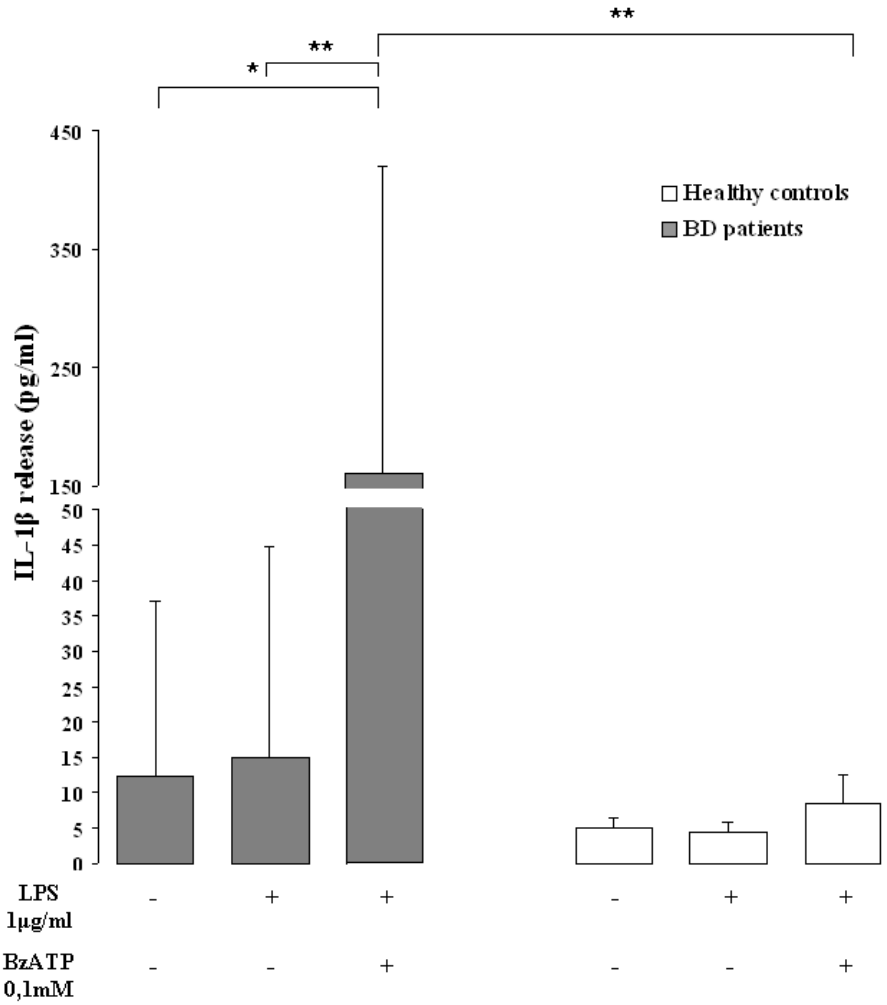

Figure 4.9: **Effect of BzATP on the release of IL-1 β from monocytes.** Freshly isolated monocytes were primed with 1 μg/ml LPS and then treated with 100μM BzATP for 30 minutes at 37°C. At the end of the incubation, levels of IL-1β were measured in the cell culture supernatant by ELISA, as described in Chapter 2. Arithmetic mean and SD are shown. $*p< 0.05$ $**p< 0.01$. RM-ANOVA. BD patients, Dunn's test. Healthy controls, Tukey-Kramer test. Mann-Whitney test.

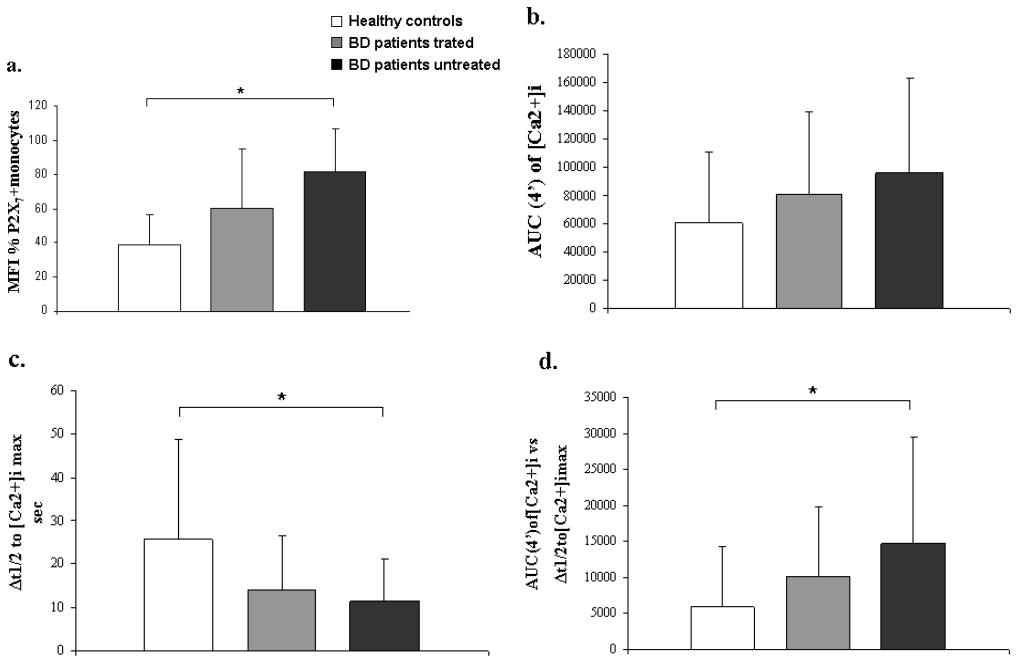

Figure 4.10: $P2X_7$**R expression and** $P2X_7$**R-mediated calcium influx in monocytes from healthy subjects and BD patients treated or untreated with anti-TNF**α **drugs.**

(a) $P2X_7$R expression. Mean fluorescence intensity obtained by FACS analysis of $P2X_7$R-positive monocytes from 14 healthy controls, 9 BD patients treated with anti-TNFα drugs and 4 BD patients not treated with anti-TNFα drugs. Arithmetic mean and SD are indicated. One-way ANOVA method.

(b,c,d) $P2X_7$R-mediated calcium influx. Characterization of Ca^{2+} entry in BzATP 100 μM stimulated monocytes from 14 healthy controls, 13 BD patients treated with anti-TNFα drugs and 4 BD patients not treated with anti-TNFα drugs. (b) the Area Under the Curve (AUC) of Ca^{2+} influx upon 4 minutes after BzATP 100 μM stimulus; (c) the half time necessary to reach the maximum Ca^{2+} concentration in the cytosol and (c) the AUC/$\Delta t_{\frac{1}{2}}$ ratio (d) were analysed. Arithmetic means and SD are reported. Ordinary ANOVA tests were used.

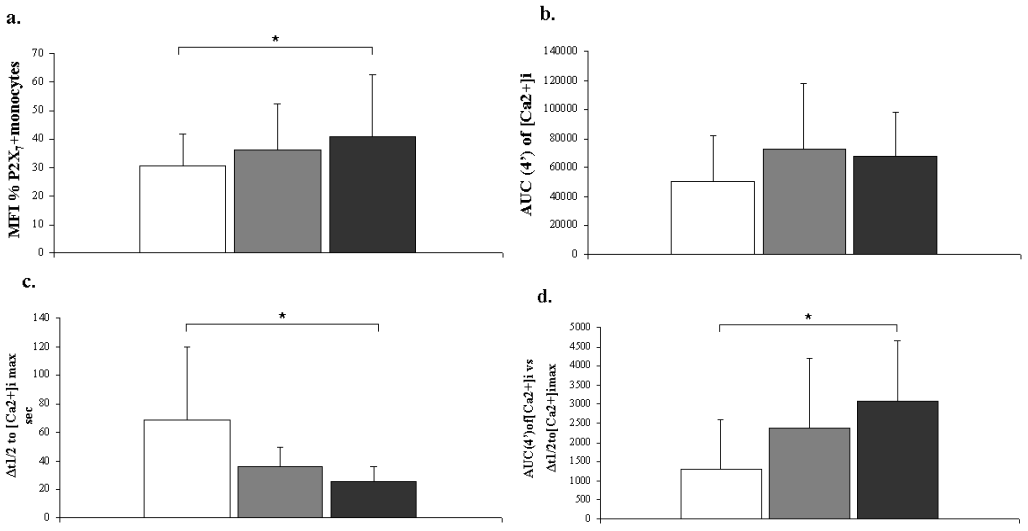

Figure 4.11: **Effect of TNF-α on $P2X_7$R expression and $P2X_7$R-induced calcium influx in monocytes from healthy controls.**
$P2X_7$R expression was analysed by flow cytometry. PBMC from 5 healthy controls, after TNF-α treatment, were immunostained by double labelling for detection of $P2X_7$R in monocytes ($CD14^+$) population. The Mean fluorescence intensity of $P2X_7$+ monocytes is reported (a). $P2X_7$R function was analysed by the measurement of $P2X_7$R-induced calcium influxes in monocytes isolated from 5 healthy controls treated with or without TNF-α, as described in Chapter 2 . The Area Under the Curve (AUC) of cyosolic Ca^{2+} concentration upon 4 minutes after BzATP 100 μM (a), the half time necessary to reach the maximum Ca^{2+} concentration in the cytosol (b) and the ratio between this two parameters (AUC and $\Delta t\frac{1}{2}$) (c) are showed.
Arithmetic mean and SD are reported. RM-ANOVA. (a) Tukey-Kramer test. (b,c,d) Friedman and Dunn's test. $*p< 0.05$.

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Publication during the PhD program

Adenosine A2A receptor activation stimulates collagen production in sclerodermic dermal fibroblasts either directly and through a cross-talk with the cannabinoid system.

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