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Characterization of the Purinergic Receptor
P2X7 and the NLRP3 Inflammasome
in the Inflammatory Response Induced
by Antimicrobial Peptides and Infections

Caracterización del Receptor Purinérgico
P2X7 y el Inflamasoma NLRP3 en la
Respuesta Inflamatoria Inducida por
Péptidos Antimicrobianos e Infecciones

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INDEX

INDEX

Introduction -- 1

1. Immune system-- 1
    1.1 General introduction------------------------------------- 3
    1.2 Cell types of the immune system------------------------ 4
        1.2.1 Myeloid peripheral blood mononuclear--------- 4
        1.2.2. Lymphocytes---------------------------------- 5
            1.2.2.1. T cells--------------------------------- 6
            1.2.2.1. B cells --------------------------------- 7
        1.2.3. Natural killer cells------------------------------- 8
        1.2.4. Granolcytes------------------------------------- 9
    1.3. Monocyte and macrophages---------------------------- 10
        1.3.1. Myeloid origin---------------------------------- 10
        1.3.2. Distribution------------------------------------- 11
        1.3.3. Functionality------------------------------------ 13
        1.3.4. Macrophages adhesion------------------------ 14

2. Inflammatory response--- 17
    2.1. Introduction-- 17
    2.2. Exogenous origin-inducers of the inflammatory response 17
    2.3. Exogenous origin-inducers of the inflammatory response 18
        2.3.1. Extracellular ATP---------------------------------- 19
        2.3.2.. HMGB-1-- 20
        2.3.3. Hyaluronan-------------------------------------- 20
        2.3.4. Crystals--- 20
    2.4. Inflammatory cytokines------------------------------------ 21
    2.5. Acute phase proteins-------------------------------------- 23
    2.6. Pattern recognition receptors----------------------------- 24
        2.6.1. Introduction to PRR ------------------------------- 24
        2.6.2. Toll-Like receptors-------------------------------- 24
    2.7. Nucleotide-binding domain and Leucine-rich repeat containing Receptor--- 28
    2.8. Types of inflammasomes----------------------------------- 30
    2.9. Effector mechanisms of inflammasomes----------------------- 34
    2.10. Non-canonical inflammasome------------------------------- 36

3. NLRP3 inflammasome--- 37
    3.1. Structure and components------------------------------------ 38
    3.2. NLRP3 activation--- 40

3.2.1. P2X7 receptor in NLRP3 inflammasome activation------ 41
3.2.2. NLRP3 inflammasome activation
by crystals and particles------------------------------------ 41
3.2.3. NLRP3 activation by antimicrobial peptides--------- 42
3.2.4. Infections and microbial products
activating the NLRP inflammasome------------------------ 44
3.2.5. NLRP3 activating cascade---------------------------- 44
3.3. Development of NLRP3 inhibitors---------------------------- 45
3.4. NLRP3 mutations and autoinflammatory syndromes----------- 46

4. Purinergic signalling-- 47

5. Sepsis-- 52
5.1. Definition of sepsis--- 52
5.2. Physiopathology of sepsis-------------------------------------- 53
5.3. Prognostic markers and severity scores of sepsis--------------- 57
5.4. Role of P2X7 receptor and NLRP3 inflammasome in sepsis----- 59

Objectives-- 61

Material and methods--- 65

1. Reagents-- 67

2. Abs-- 70

3. Buffers and cell culture media-------------------------------------- 72

4. Human samples and clinical determinations----------------------- 72
4.1. Human samples--- 72
4.2. Clinical determinations---------------------------------- 73
4.3. Sample reception--- 74
4.4. PBMCs isolation-- 74
4.5. PBMCs defrosting-- 76

5. Differentiation of mouse bone marrow-derived macrophages----- 76
5.1. Mice strains-- 76
5.2. Bone marrow extraction----------------------------------- 77
5.3. Bone marrow macrophage differentiation----------------- 77

6. Cell lines--- 78
6.1. L929--- 78

6.2. THP-1-- 78
6.3. HEK293T-- 79

7. Cell stimulation-- 79

8. Cell adhesion protocol-- 81

9. Flow cytometry-- 81
9.1. Determination of the P2X7 receptor surface expression--- 81
9.2. Detection of active caspase-1------------------------------- 82
9.3. Determination of
mitochondrial membrane potential assay---------------------- 83
9.4. Multiplexing cytokine detection---------------------------- 85
9.5. Detection of ASC specks in plasma------------------------ 86

10. Determination of mitochondrial membrane potential
using a plate reader-- 86

11.Enzyme-linked immunosorbent assays------------------------------ 86

12. Western-Blot determination-- 89
12.1. Sample processing-- 89
12.2. Acrylamide preparation------------------------------------ 90
12.3. Protein electrophoresis and transference----------------- 91
12.4. Ab Blotting and bioluminescence detection-------------- 91

13. Yo-Pro-1 cell staining-- 92
13.1. Yo-Pro-1 to quantify number of cells---------------------- 92
13.2. Yo-Pro-1 tostudy plasmamembrane permeabilization---- 92

14. MTT assay--- 92

15. Lactate dehydrogenase assay-------------------------------------- 93

16. Real time cell analysis for cell adhesion---------------------------- 94

17. Statistical analysis-- 95

Results

1. Inflammatory response induced by venoms and infections---------- 99
1.1. Melittin activates NLRP3 inflammasome--------------------- 99
1.1. FIGURES-- 103

1.2. The inflammatory response in sepsis----------------------- 121
1.2. FIGURES-- 127

2. Expression of the purinergic P2X7 receptor
in the inflammatory response--- 143
2.1. P2X7 receptor surface is increased
in leukocytes from septic patients------------------------------- 143
2.1. TABLES--146
2.1. FIGURES-- 163
2.2. P2X7 receptor function assotiates with
innate immunoparalysis during sepsis--------------------------- 191
2.2. FIGURES---195
2.3. P2X7 receptor activation affects cell adhesion-------------- 203
2.3. FIGURES--- 209

Discussion-- 229

Conclusions--- 243

Bibliography-- 249

Publications from the Thesis--- 299

Spanish summary-- 303

FIGURES INDEX

Figure 1	7
Figure 2	14
Figure 3	26
Figure 4	29
Figure 5	31
Figure 6	33
Figure 7	35
Figure 8	36
Figure 9	39
Figure 10	40
Figure 11	51
Figure 12	54
Figure 13	75
Figure 14	78
Figure 15	81
Figure 16	84
Figure 17	85
Figure 18	87
Figure 19	93
Figure 20	94

Figure 21 95
Figure 22 106
Figure 23 108
Figure 24 110
Figure 25 112
Figure 26 114
Figure 27 116
Figure 28 118
Figure 29 130
Figure 30 132
Figure 31 134
Figure 32 136
Figure 33 138
Figure 34 140
Figure 35 164
Figure 36 166
Figure 37 168
Figure 38 170
Figure 39 172
Figure 40 174
Figure 41 176
Figure 42 178
Figure 43 180
Figure 44 182

Figure 45	184
Figure 46	186
Figure 47	188
Figure 48	198
Figure 49	200
Figure 50	212
Figure 51	214
Figure 52	216
Figure 53	218
Figure 54	220
Figure 55	222
Figure 56	224
Figure 57	226

TABLES INDEX

Table 1	67
Table 2	70
Table 3	88
Table 4	146
Table 5	148
Table 6	150
Table 7	152

ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
ADP	Adenosine 5′-diphosphate
AIFEC	Autoinflammation with infantile enterocolitis
AIM2	Absent in melanoma 2 protein
AMP	Antimicrobial peptide
AP-1	activator protein 1
APACHE II	Acute physiology and chronic health evaluation-II
APC	Antigen presentation cell
APLAID	Antibody deficiency and immune dysregulation
APP	Acute phase protein
APR	Acute phase response
ART-2	ADP-ribosyltransferase 2
ATP	Adenosine 5′-triphosphate
BCR	B-cell antigen receptor
BIR	Baculovirus inhibitor of apoptosis repeat
BMDC	Bone marrow differentiated dendritic cell
BMDM	Bone marrow differentiated macrophages
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
BzATP	2′(3′)-O-(4-Benzoylbenzoyl)- adenosine 5′-triphosphate
cAMP	cyclic adenosine 5´ phosphate
CAPS	Cryopirin Associated Periodic Syndromes
CARD	caspase recruitment domain
CASP	Colon ascendens stent peritonitis
CCL13	Chemokine ligand 13
CCL14	Chemokine ligand 14

CCL17	Chemokine ligand 17
CCL18	Chemokine ligand 18
CCL22	Chemokine ligand 22
CCL24	Chemokine ligand 24
CCR6	CC chemokine receptor type 6
CCR7	CC chemokine receptor type 7
CD11b	Cluster of differentiation 11b
CD11c	Cluster of differentiation 11c
CD123	Cluster of differentiation 123
CD127	Cluster of differentiation 127
CD13	Cluster of differentiation 13
CD135	Cluster of differentiation 135
CD14	Cluster of differentiation 14
CD15	Cluster of differentiation 15
CD16	Cluster of differentiation 16
CD163	Cluster of differentiation 163
CD19	Cluster of differentiation 19
CD1d	Cluster of differentiation 1d
CD20	Cluster of differentiation 20
CD206	Cluster of differentiation 206
CD25	Cluster of differentiation 25
CD27	Cluster of differentiation 27
CD28	Cluster of differentiation 28
CD3	Cluster of differentiation 3
CD38	Cluster of differentiation 38
CD39	Cluster of differentiation 39

CD4	Cluster of differentiation 4
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand
CD43	Cluster of differentiation 43
CD45RA	Cluster of differentiation 4 RA isoform
CD49d	Cluster of differentiation 49d
CD56	Cluster of differentiation 56
CD62L	Cluster of differentiation 62L
CD66b	Cluster of differentiation 66b
CD73	Cluster of differentiation 73
CD8	Cluster of differentiation 8
CD80/86	Cluster of differentiation 80/86
CD86	Cluster of differentiation 86
CDR	Complementary determining regions
CIITA	Major histocompatibility complex-class II transactivator
CINCA	Chronic infantile, neurological, cutaneous, and articular syndrome
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
CoA	Coenzyme A
CPP	Calcium pyrophosphate dehydrate
CRP	C-Reactive protein
CXCR3	CXC chemokine receptor type 3
DAMP	Danger associated molecular patterns
DCs	Dendritic cells
DMEM	High glucose Dulbecco's modified Eagle's medium media
DNA	Deoxyribonucleic acid

DOCK8	Dedicator of cytokinesis 8
ds	Double stranded
ECL	enhanced chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
ET	E total
FADD	Fas-associated protein with death domain
FAS	First apoptosis signal
FCAS	Familial cold autoinflammatory syndrome
FCS	Foetal calf serum
FLICA	Fluorochrome-labelled inhibitor of caspase-1
FMF	Familial Mediterranean fever
fMLP	Formyl-methionyl-leucyl-phenylalanine
FOXP3	Forkhead box P3
FSC	Forward side Scatter
GM-CSF	Granulocyte and monocytes-colony stimulating factor
GSDMD	Gasdermin D
H5N1	Hemagglutinin 5-neuraminidase 1
HA	Hyaluronan
HEK	Human embryonic kidney
HGNC	Human Genome Organisation Gene Nomenclature Committee
HIF-1α	hipoxia inducible factor-1α
HLA-DR	Human antigen Leukocyte DR
HMGB1	High-mobility group box 1
HUGO	Human Genome Organisation

IFN-α	Alpha interferon
IFN-β	Beta interferon
IFN-γ	Gamma interferón
IgD	Immunoglobulin D
IKKα	inhibitor of κB kinase alpha
IKKε	Inhibitor of κB kinase epsilon
IL-10	Interleukin-10
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-18	Interleukin -18
IL-18R	Interleukin -8 receptor
IL-1F	Interleukin -1 family
IL-1Ra	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-33	Interleukin -33
IL-33	Interleukin-33
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
iNOS	Inducible nitric oxide syntase
IP3	Inositol triphosphate
IRAK	N-terminal death domain of IL-1R-associated kinase

IRF	Interferon-regulatory factor
IκBα	inhibitor of κB alpha
JAK	Janus-kinase
KLRG1	Killer cell lectin-like receptor G1
LDH	Lactate dehydrogenase
LEU-CAM	Adhesion molecule of the leukocytes
LFA-1	Lymphocyte function associated antigen-1
LLO	Listeriolysin
LPS	Lipopolysaccharide
LTA	lipotechoic acid
Ly6C	Lymphocyte antigen 6C
Ly6G	Lymphocyte antigen 6G
MAC-1	Macrophage antigen-1
MAPK	Mitogen activated protein kinases
MARCO	Macrophage receptor with collagenous structure
M-CSF	Macrophage-colony stimulating factor
MDP	Muramyl-dipeptide
MHC	Major histocompatibility complex
MSU	Monosodium urate
MWS	Muckle–Wells síndrome
MyD88	Myeloid differentiation-primary response gene 88
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide
NAIP	Nucleotide-binding domain and leucine-rich repeat family apoptosis inhibitory protein
NET	Neutrophils extracellular traps

NF-κB	Nuclear factor kappa B
NK	Natural killer
NKT	Natural killer T
NLR	Nucleotide binding domain leucine-rich repeat containing receptors
NLRC	Nucleotide-binding domain and leucine-rich repeat containing caspase recruitment domain receptor
NLRP	Nucleotide-binding domain and leucine-rich repeat containing pyrin domain receptor
NOD	nucleotide-binding and oligomerization domain
NOMID	Neonatal-onset multisystem inflammatory disease
NTPDase	Nucleoside triphosphate diphosphohydrolase
NT-proBNP	N-terminal brain natriuretic propeptide
oATP	Oxidized adenosine 5′-triphosphate
oxPAC	Oxidized phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine
P2X1R	Purinergic P2X1 receptor
P2X2R	Purinergic P2X2 receptor
P2X4R	Purinergic P2X4 receptor
P2X7	Purinergic receptor P2X7
P2XR	Purinergic P2X receptor
P2Y11R	Purinergic P2Y11 receptor
P2Y12R	Purinergic P2Y12 receptor
P2Y13R	Purinergic P2Y13 receptor
P2Y14R	Purinergic P2Y14 receptor
P2Y1R	Purinergic P2Y1 receptor
P2Y2R	Purinergic P2Y2 receptor

P2Y4R	Purinergic P2Y4 receptor
P2Y6R	Purinergic P2Y6 receptor
P2YR	Purinergic P2Y receptor
PAAND	Pyrin-associated autoinflammation with neutrophilic dermatosis
PAMP	Pathogens associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PCT	Procalcitonin
PDC	Pyruvate dehydrogenase
PDTC	Pyrrolidindythiocarbamate
PGE2	Prostaglandin E2
PLCG2	Phospholipase Cγ2
PRR	Pattern recognition receptor
PWM	Pokeweed mitogen
PYD	pyrin domain
qSOFA	Quick sequential organ failure assessment
RAGE	Receptor for advanced glycation end products
RIG-1	Retinoic acid-inducible gene I
RLH	Retinoic acid-inducible gene I-like RNA helicase
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SAA	Serum amyloid A
SAP	Serum amyloid P
SDS	Sodium dodecyl sulphate
SIRS	Systemic inflammatory response síndrome
SLO	Streptolysin

SOFA	Sequential organ failure assessment
STING	Stimulator of interferon genes protein
TAB1	Transforming grown factor beta -activated kinase 1-binding protein 1
TAB2	Transforming grown factor beta -activated kinase 1-binding protein 2
TAK1	Transforming grown factor beta -activated kinase 1
TBK1	Tumor necrosis factor -receptor-associated factor family member-associated NF-kappa-B activator-binding kinase 1
Tc	T cytotoxic
TCA	Tricarboxylic acid cycle
TCR	T cell antigen receptor
TEMED	Tetramethylethylenediamine
TGFβ	Transforming grown factor beta
Th	T helper
Th1	T helper 1
Th17	Interleukin-17 secreting T helper
Th2	T helper 2
TIR	Toll–Interleukin-1-resistence domain
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor -receptor-associated factor 6
Treg	T regulatory
TRIF	Toll–Interleukin-1-resistence domain-containing adaptor protein inducing beta interferon
TRX-1	Thioredoxin-1
TXNIP	Thioredoxin-interacting protein

UTP	Uridine 5′-triphosphate
UV	Ultraviolet
WB	Western-Blot
ΔSOFA	Sequential organ failure assessment variation

Introduction

1. Immune system

1.1. General introduction

The defence mechanisms are found in all living being including plants and viruses, functioning appropriately for their level of evolution (Cooper 2010). The immune system is response of the organism to protect it against infections and injuries. This task is mainly carried out by the specific cells of the innate and adaptive immune system (Janeway, C A; Murphy, K; Travers, P; Walport 2012). Also some proteins activities as lysozyme, as well as commensal bacteria that inhabits the entire gut, small and large intestine and colon has been observed as a part of the immune system (Smith and Dyrynda 2015; Kosiewicz, Zirnheld, and Alard 2011).

or tissue injury. (Janeway, C A; Murphy, K; Travers, P; Walport 2012). For a long time, innate immunity was considered non-specific (Yeretssian, Labbé, and Saleh 2008). However, the innate immune system recognizes infections and cellular damage through specific pattern recognition receptors (PRRs) that recognise evolutionary conserved microbial estructures such as flagellin and components of the bacterial cell wall or viral envelope (V. Kumar and Sharma 2009).

In mammals, the primary innate immune constitutive defences include the commensal microbial flora that compete with microbial pathogens; physical barriers of the skin and internal epithelial layers; mechanical defenses of cilia and chemical defences such as the acidic pH of the stomach (Yeretssian, Labbé, and Saleh 2008). Innate immunity is initiated by monocytes, macrophages, dendritic cells, granulocytes, NK cells other reduced group of lymphocytes (Bain and Mowat 2014; Schraml and Reis e Sousa 2015; Pillay et al. 2013; Rapp, Wiedemann, and Sun 2018; Artis and Spits 2015). Upon cell death or microbial sensing by PRR, these cells initiate the inflammatory response by secreting cytokines and chemokines. As a consequence there is a specific migration of immune cells to the site of infection and tissue injury, that will initiate the adaptive immune response (Saïd-Sadier and Ojcius 2012).

Adaptive immunity is initiated by lymphocytes **(Boehm and Swann 2014)**, and is the most recent system on the evolutionary landscape, (Charles A. Janeway and Medzhitov 2002). The immune systems of jawless vertebrates, such as hagfish and lamprey, include an adaptive immune system **(Boehm and Swann 2014)**. However, the cellular components of the adaptive immune system of hagfish and lampreys do have populations of cells that have similar function to B and T lymphocytes, but lack the characteristic markers of B or T cell receptors of jawed vertebrates **(Ito et al. 2012)**. Adaptive immunity is responsible for allergy, autoimmunity, and the rejection of tissue grafts (Charles A. Janeway and Medzhitov 2002), and can more effectively focus its resources to respond specifically against pathogens than the innate immunity. Specific antigen-recognizing antibodies and activated cells produced by the adaptive immune response can continue in the system after infection o tissue damage has been eliminated. They help to prevent immediate reinfection and also provide for long lasting immunity, allowing a faster and more intense response to a second exposure of the anigen during years However, antibodies against self-proteins produced in conditions of cell death, could develop autoimmune diseases **(Janeway, C A; Murphy, K; Travers, P; Walport 2012)**.

1.2. Cell types of the Immune System

The innate and adaptive immune responses depend on the activities of specific cells or leukocytes. The majority of these cells originate in the bone marrow, and many of them also develop and mature there **(Janeway, C A; Murphy, K; Travers, P; Walport 2012; Epelman, Lavine, and Randolph 2015)**. Once the cells are matured, they disperse through the organism using the circulation and/or migrate throught the endothelium to the peripheral tissues **(Janeway, C A; Murphy, K; Travers, P; Walport 2012).**

1.2.1. Myeloid Peripheral Blood Mononuclear Cells

The myeloid cells play major roles in development, scavenging, inflammation, and anti-pathogen defenses **(van Furth 1976)**. They are grouped lineage-committed bone marrow precursors, circulating monocytes, resident macrophages, and dendritic cells (DCs) **(Taylor and Gordon 2003)**. Considering the importance of myeloid peripheral blood mononuclear cells for this Thesis, they will be extensively described in the following section.

1.2.2. Lymphocytes

Lymphoctes essentially participate in the adaptive immunity (Crotty 2015). Although some special types of lymphocytes, termed as "innate lymphoid cells", are able to participate in innate immunity by secretion of effector cytokines (Artis and Spits 2015). The most abundant populations of lymphocytes in peripheral blodd are: T lymphocytes (T cells) and B lymphocytes (B cells) (Janeway, C A; Murphy, K; Travers, P; Walport 2012; Crotty 2015)

1.2.2.1. T cells

T cells are characterized to present the cluster of differentiation (CD)3 or T cell receptor (TCR) (Maecker, McCoy, and Nussenblatt 2012). In the inflammatory response, TCR binds with the major histocompatibility complex (MHC)-class II from myeloid cells as a first signal of recognition. These cells also present CD40L and CD28, that binds with CD40 and CD80/86 from myeloid cells respectively (Figure 1) (Muñoz 2016). The most important T cells populations are: T cytotoxic (Tc) cells and T helper (Th) cells. The major part of these cells originate in bone marrow and mature on the thymus (Janeway, C A; Murphy, K; Travers, P; Walport 2012; Maecker, McCoy, and Nussenblatt 2012).

Tc cells present CD8 receptor (Maecker, McCoy, and Nussenblatt 2012). Their function is to kill other cells that are infected with viruses or other intracellular pathogens through synthesis and exocytosis of cytotoxic components such as granzyme B or first apoptosis signal (FAS)-ligand. These components bind to perforin B and FAS respectively on the target cell and activate the apoptotic cell death (Janeway, C A; Murphy, K; Travers, P; Walport 2012; Golstein and Griffiths 2018).

Th cells express the CD4 receptor (Maecker, McCoy, and Nussenblatt 2012). These cells provide of essential additional signals such as cytokines and chemokines that influence the behavior and activity of other cells. For example, they can regulate the B cells and the production of antibodies. Also Th cells can regulate macrophages activity (Janeway, C A; Murphy, K; Travers, P; Walport 2012). Th cells include Th1, Th2, interleukin (IL)-17-secreting helper T (Th17), and regulatory T (Treg) cells (Maecker, McCoy, and Nussenblatt 2012):

- Th1 can be identifiyed by the expression of CXC chemokine receptor type (CXCR)3 in the cell surface. They also are negative for CCR6 marker (Maecker, McCoy, and Nussenblatt 2012). These cells are activated after interleukin (IL)-12 production from myeloid cells of innate immune system (Martinez, Helming, and Gordon 2009; Saito et al. 2010). Th1 cells produce IL-2 and gamma interferon (IFN-γ) and are involved in the activation of cellular immunity (T R Mosmann 1989; Saito et al. 2010).

- Th2 cells are $CXCR3^-CCR6^-$ (Maecker, McCoy, and Nussenblatt 2012). Th2 cells are activated after they recognise the IL-10 produced by myeloid cells of the innate immune system (Martinez, Helming, and Gordon 2009; Saito et al. 2010). Th2 cells produce IL-4, IL-5,IL-10 and IL-13 that are involved in the immune response regulation (T R Mosmann 1989; Saito et al. 2010; McKenzie et al. 1993). Indeed, Th2 cells secrete IL-10 to supress the Th1 response (T R Mosmann 1989).

- Th17 cells are $CXCR3^-CCR6^+$ (Maecker, McCoy, and Nussenblatt 2012). These cells release IL-17 when they are activated by IL-6 or IL-1β from myeloid cells of innate immune system, and promote the activation of the inflammatory response (Saito et al. 2010).

- Treg cells Treg cells can be divided into two subgroups according to the site of their maturation: natural Treg cells and inducible Treg cells. Natural Treg cells maturate in the thymus meanwhile Inducible Treg cells mature in peripheral lymphoid organs (Noack and Miossec 2014). Treg are $CD25^+$, and differentially to other Th cells, they are (forkheadbox P3) $Foxp3^+$ and with a CD127 low expression (Saito et al. 2010; Maecker, McCoy, and Nussenblatt 2012). Treg cells suppress the activity of other pro-inflammatory lymphocytes, for example, Tregs inhibit the Th17 response and help to control immune responses (Saito et al. 2010; Charles A. Janeway and Medzhitov 2002).

These T cells that execute their function are considered as effector T cells, wich are $CCR7^-$ $CD45RA^+$. Before activation, these cells are naïve T cells, and they present the expression of CCR7 and CD45RA in the surface. T effector cells can change the phenotype to memory T cells, which present a lack of CD45RA expression (Maecker, McCoy, and Nussenblatt 2012). When a new infection developes, memory T cells aimplify the antibodies production by B cells (Vitetta et al. 1991).

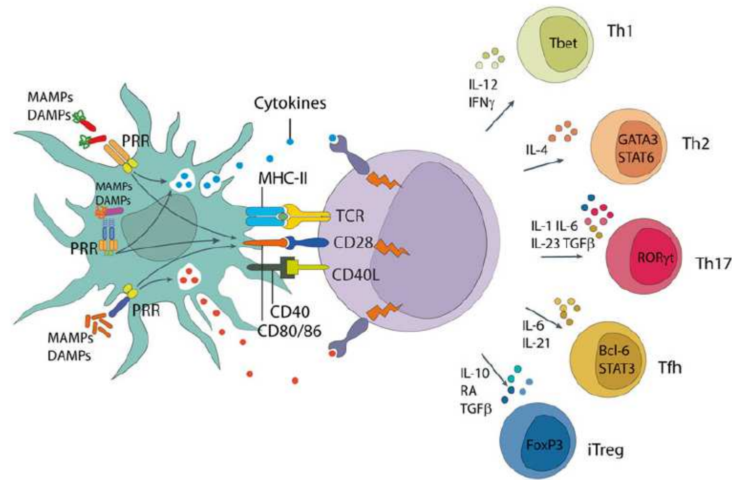


Figure 1. Three-signal model of Th cell activation (Muñoz 2016). First at all, the MHC-class II recognition and TCR activation. Secondly CD40-CD40 ligand and CD80/86-CD28 myeloid-Th cell interaction. Finally, cytokine release for Th1, Th2, Th17, Tfh or Treg response.

Natural killer T (NKT) cells is an special subset of T cells that recognise and kill target cells expressing lipid antigens presented by the MHC-class I-like molecule CD1d (Bhan et al. 2016a). These cells express CD56, as NK cells, but differentially, they highly express CD3, and can express CD8 or CD4, as T cells (C. Wang et al. 2015; Nakamura and Sonoda 2003). NKT cells can also be activated by cytokines such as IL-12 or IL- 18 from innate immune system cells, with help of antigen presentation cells (APCs) by CD1d. NKT cells can express several Toll-Like receptors (TLRs) that allows them to respond immediately to a variety of pathogens (Bhan et al. 2016a). Invariant NKT cells is an specific type of NKT cells that stimulate DCs to produce inflammatory mediators without the presence of microbial compounds, and promote neutrophil responses (Xu et al. 2016).

1.2.2.2. B cells

The major part of B cells originate in the bone marrow and mature in the spleen (Pieper, Grimbacher, and Eibel 2013). Mature B cells are negative for CD3 but they can be detected by their B-cell receptor (BCR) CD19 and CD20^{+} (Maecker, McCoy, and Nussenblatt 2012). The main role of B cells resides in the specific-antibodies production through their activation mediated by the Th response or antigen recognition. After antigen binds to a B-cell antigen receptor, or BCR on the B-cell surface, the lymphocyte will proliferate and differentiate into plasmatic cells or plasmablasts. These are the effector form of B cells that produce antibodies, which are a secreted form of the B-cell receptor with an identical antigen specificity. The antigen receptor of B cells is also known as surface

immunoglobulin, and differes of secreted antibodies in the small portion of the C-terminus of the heavy-chain constant region. In the case of the B-cell receptor the C-terminus is a transmembrane hydrophobic domain, and in the case of antibodies it is a hydrophilic sequence that allows secretion. **(Janeway, C A; Murphy, K; Travers, P; Walport 2012)**.

Nowadays, it is known that B cells also can secrete IL-10, or IL-6 to regulate or amplify the Th response (respectively). B cells also secrete IL-3 that by autocrine signalling, produces the granulocyte and monocyte-colony stimulating factor (GM-CSF), and promotes neutrophils and macrophages production **(Chousterman and Swirski 2015)**. Plasmablasts can be observed by anti-CD38, as well as transitional B cells that also are $CD24^{+}$. As T cells, effector B cells can change their phenotype and function to memory B cells to amplify the effectiveness of antibodies secretion on the next infection **(Vitetta et al. 1991)**. Memory B cell populations are $CD38^{-}IgD^{+}$. CD27 can also be used to define naive ($CD27^{-}$) and memory ($CD27^{+}$) B cells **(Maecker, McCoy, and Nussenblatt 2012)**.

1.2.3. Natural killer cells

Natura Killer (NK) cells are specific lymphocytes that are considered as a part of the innate immune system that responds to the presence of infection but is not specific for antigen **(Janeway, C A; Murphy, K; Travers, P; Walport 2012; Artis and Spits 2015; Rapp, Wiedemann, and Sun 2018)**. These cells originate in the bone marrow and maturate in the secondary lymphoid tissues **(Freud, Yu, and Caligiuri 2014)**. They are activated after sensing of innate immune system cells and recognition of IL-12, IL18, IL-33, or IFN-α/β proinflammatory factors, released by innate immunity cells **(Rapp, Wiedemann, and Sun 2018)**. NK cells have a large variety of activating and inhibitory receptors expressed by overlapping subsets of cells that vary widely between individuals. However, NK cells can be subdivided into two major categories based only on the markers Fc gamma receptor III (CD16) and CD56. The major part of peripheral blood NK cells are $CD16^{+}CD56^{low}$, whereas a smaller subset is $CD16^{+}CD56^{++}$ **(Maecker, McCoy, and Nussenblatt 2012)**. NK cells are capable to recognize and kill some tumor cells as well as infected cells with viruses **(Janeway, C A; Murphy, K; Travers, P; Walport 2012)**. NK cells also coordinate early responses to bacterial infections by amplifying the antimicrobial functions of myeloid cells (especially macrophages, by producing IFN-γ) (Y. Guo, Luan, et al. 2017). Recently, some evidences suggest that NK cells also present many characteristics similar to cells of the adaptive

immune system. Indeed, as T and B cells, NK cells also can change its phenotype to memory cells. These memory NK cells express several markers in the cell surface: they highly express the killer cell lectin-like receptor G1 (KLRG1), Lymphocyte antigen (Ly)6C, CD11b, CD43, and exhibit a low expression of CD62L and CCR7 **(Rapp, Wiedemann, and Sun 2018)**.

1.2.4. Granulocytes

Granulocytes are cells that belongs to the innate immune system. Granulocytes are myeloid cells. The major part of these cells originate and maturate in the bone marrow **(da Silva, Massart-Leën, and Burvenich 1994; Kolaczkowska and Kubes 2013)**. They share the granulocyte-macrophage progenitor in common with monocytes and macrophages, an maturate from the common granulocytic progenitor **(da Silva, Massart-Leën, and Burvenich 1994; Akashi et al. 2000)**. Granulocytes derive from and present densely granules in their cytoplasm and a nuclei with several lobes, that is why they are also called polymorphonuclear leukocytes. There are three types of granulocytes (neutrophils, eosinophils, and basophils) which are distinguished by the different staining properties of the granules. In comparison with macrophages they are all relatively short-living, only living few days **(Janeway, C A; Murphy, K; Travers, P; Walport 2012).**

Neutrophils present a key role in the innate immune response against infections. CD11b, CD15, CD16, and CD66b are markers consistently expressed on human neutrophils independent of the cell location. On the contrary, CD11b and Ly6G in combination with CD15 and CD16 are used targets to identify murine neutrophils **(Pillay et al. 2013; Lakschevitz et al. 2016)**. They are produced in increased numbers during the infection, and they migrate to the site of inflammation by extravasation due to endothelium adhesion by L-Selectins in the cell surface **(Rowe et al. 2002; Kolaczkowska and Kubes 2013)**. This recruitment is initiated by the tissue-resident macrophages that secret chemokines and increases the permeability of local blood vessels. One they are in the infection site, neutrophils participate in the pathogen clearance. These cells present three different pathways of pathogen killing: phagocytosis; degranulation (by secreting the cytoplasmic granules that contain a variety of enzymes and toxic proteins); and NETosis (by secreting neutrophils extracellular traps (NETs) composed by DNA and enzymes such as myeloperoxidase, and neutrophil elastase) **(Kolaczkowska and Kubes 2013)**. Neutrophils also are important in the resolution of

inflammation when they are recruited in the inflammatory responses during a steril injury (Kolaczkowska and Kubes 2013). Neuthrophils are cleared quickly and they die by caspase-dependent apoptosis, which is highly susceptible to be induced by host cytokines, including IL-1β (Rowe et al. 2002).

Eosinophils and basophils are less abundant cells than neutrophils, but like neutrophils, they have granules that contain enzymes and toxic proteins, which are released when the cells are activated. Eosinophils and basophils are important in defense against parasites, which are too large to be ingested by macrophages or neutrophils (Janeway, C A; Murphy, K; Travers, P; Walport 2012). They can also contribute to allergic inflammatory reactions trough IL-33 secretion. (Janeway, C A; Murphy, K; Travers, P; Walport 2012; Nadif et al. 2013). Eosinophils markers profile is very similar to neutrophils. However, eosinophils express CD49d in cell surface, meanwhile neutrophils are negative for this marker (Na et al. 2012). Eosinophils are considered as effector cells involved in host protection against helminth infections. Activated eosinophils release toxic granule proteins and proinflammatory mediators, which may cause tissue damage and dysfunction. Eosinophils can synthesize, store, and secrete inflammatory and immunoregulatory cytokines, chemokines, and growth factors. These factors may play roles in the regulatory functions of eosinophils in several eosinophil-associated human diseases (Kobayashi, Kouzaki, and Kita 2010). Basophils express CD11b, CD13 and CD33 and they are negative for HLA-DR and CD15 (Han et al. 2008) and also can participate as regulators of the Th2 response through IL-4 expression (Nadif et al. 2013).

1.3. Monocytes and Macrophages

1.3.1. Myeloid origin

Human blood monocytes originate from haematopoietic precursors in the bone marrow (Ammon et al. 2000). They develop through a series of sequential differentiation stages: the common myeloid progenitor, followed by the granulocyte-macrophage progenitor (Akashi et al. 2000) which lets the common macrophage and DC precursor (Fogg 2006) and finally the committed monocyte progenitor, which gate the same properties than macrophage and dendritic cell precursor, but with the lack CD135 expression (Hettinger et al. 2013). Although some macrophages originate in bone marrow, such as the blood macrophages, and intestinal macrophages, the major part of tissue-resident macrophages

originate from embryonic stem cells **(Bain and Mowat 2014; Epelman, Lavine, and Randolph 2015)**.

1.3.2. Distribution

Monocytes is the main group of myeloid cells that circulates in the blood, however they are also present in the bone marrow and spleen, constituting ~10% of the total of leukocytes in humans **(Italiani and Boraschi 2014)**. Human blood monocytes are characterized by a high size in comparison with lymphocytes, measured by the forward and side scatter (FSC), as well as the expression of the lipopolysaccharide (LPS) co-receptor CD14 and the CD16. Monocytes can be subdivided depending on CD14 and CD16 relative expression as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$) monocytes **(Hijdra et al. 2013; Ziegler-Heitbrock and Hofer 2013)**. Monocytes have several specific morphological features, such as irregular cell shape, ovalor kidney-shaped nucleus, cytoplasmic vesicles, and high ratio cytoplasm:nucleus. In normal conditions, monocytes survive in the circulation for 1 or 2 days until senescence and clearance. However, they extravase and are recruited into tissues as a consecuence of a danger or infection **(Italiani and Boraschi 2014).** In fact, peripheral blood monocytes can emigrate from the blood through the endothelial barrier into various tissues under both non-inflammatory conditions or in response to inflammation. After extravasation, monocytes changes phenotype and differentiate into macrophages and DCs **(Zaslona et al. 2009)**. However, these monocyte-derived macrophages can continue in blood vessels **(Epelman, Lavine, and Randolph 2015)**.

On the contrary, tissue-resident macrophages are scattered over different tissues of the organism, and involves macrophages from heart, kidney, Langerhans cells, microglia, alveolar macrophages, Kuppfer cells peritoneal macrophages, and intestinal macrophages (the only tissue-resident macrophages with hematopoyetical origin) **(Epelman, Lavine, and Randolph 2015)**. Tissue-resident macrophages can represent in adults mammals from 10 to 15% of the total cell number in quiescent conditions. This number can increase further in response to inflammatory stimuli **(Italiani and Boraschi 2014)**. Tissue-resident macrophages are highly heterogeneous in terms of their functions and phenotypes as a consequence of adaptation to different tissue environments **(Okabe and Medzhitov 2014)**. A clear example of macrophages variety is in the gut. The gut is populated with multiple types of macrophages

and DCs, which have distinct phenotypes and functions, but work together to maintain tolerance of the gut flora and food (Murray and Wynn 2012).

Macrophages constitute the front line defenders who recognize invading pathogens, and kill pathogens by phagocytosis. Moreover, Macrophages also initiate inflammatory response by maturation and release of pro-inflammatory cytokines (Kim, Yoon, and Ryu 2016). Macrophages take different names according to their tissue location. The best studied tissue resident macrophages are osteoclasts (bone); alveolar macrophages (lung); microglial cells (Central Nervous system); histiocytes (connective tissue); Kupffer cells (liver); and Langerhans cells (skin), which present similar functions to DCs, but share a common ontogeny with macrophages (Italiani and Boraschi 2014; Kaplan 2017).

DCs originate from the common macrophage and DC precursor, which differentiates into the common DC precursors in bone marrow and generates the plasmacytoid DCs and the conventional DCs. Plasmacytoid DCs end its differentiation in bone marrow. However, conventional DCs are differentiated on lymphoid and non-lympohid organs (Schraml and Reis e Sousa 2015). Conventional DCs lineage are $CD11c^{+}$ cells while plasmacytoid DCs express CD123 marker (Maecker, McCoy, and Nussenblatt 2012). DCs and macrophages contribute to the innate immune response through PRRs inducing the production of proinflammatory molecules and the activation of adaptive immune response (Franchi, Eigenbrod, and Núñez 2009). However, tissue resident macrophages and DCs use to present different distribution in the tissue. For example, in the lung, while macrophages are generally present in both the lung interstitium and alveolar airspaces, DCs are mainly located within the interstitium (Landsman, Varol, and Jung 2007).

Bone marrow-derived macrophages (BMDMs) are primary macrophages derived from bone marrow cells *in vitro* in the presence of growth factors such as macrophage colony-stimulating factor (M-CSF). M-CSF is responsible for the proliferation and differentiation of the common myeloid precursor into macrophages. Bone marrow cells are grown in culture dishes in the presence of M-CSF, which is secreted by fibroblasts (as the cell line L929) (Weischenfeldt and Porse 2008). BMDMs can be identifiyed by its cd11b and F4/80 markers expression (Ying et al. 2013). Bone marrow precursos or blood monocytes can also differentiate into (Bone marrow differentiated dendritic cells) BMDCs when

cultured in the presence of granulocyte and macrophage (GM)-CSF and IL-4. These DCs are identifiyed by CD11b and CD18 expression **(Ammon et al. 2000)**.

1.3.3. Functionality

Myeloid cells play their roles in development, tissue homeostasis, and the resolution of the inflammation. Monocytes represent more than circulating precursors of macrophages and they have effector functions and share mutual roles together macrophages during the inflammatory response **(Italiani and Boraschi 2014)**. Monocytes, DCs and macrophages, along with neutrophils and mast cells, are considered as 'professional' phagocytic and APCs **(Murray and Wynn 2012; Kambayashi and Laufer 2014)**. During an infection, classical monocytes can migrate to the site of infection to phagocyte pathogens and recruit other immune cells, by the MHC-class II surface expression. Non-classical monocytes are the result of classical monocytes when they return to bone marrow and suffer another maturation **(Chiu and Bharat 2016)**.

everal macrophage subsets with distinct functions have been described. Classical activation of macrophages or pro-inflammatory macrophages (M1 macrophages) mediate host response against infections in response to pathogen recognition **(Murray and Wynn 2012).** These cells can be identifiyed by MHC-class II, CD86, and the macrophage receptor with collagenous structure (MARCO) **(Stöger et al. 2012)**. As monocytes, they also present antigen via MHC-class II, initiate the inflammation, recruit granulocytes to the infection site, and activate the Th1 response **(Chiu and Bharat 2016)**. After an infection, M1 macrophages secrete IL-12, an important pro-inflammatory cytokine that drives T cells toward a Th1 phenotype **(Martinez, Helming, and Gordon 2009).** Moreover other proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, are released by M1 macrophages **(Zanin et al. 2012)**. M1 stimulation also upregulates the production of a group of chemokines from human macrophages, including the chemokine ligand (CCL) 13, CCL14, CCL17, CCL18, CCL22, and CCL24 **(Martinez, Helming, and Gordon 2009)**. Th1 response allows the attraction of Th1 cells, together other cells such as granulocytes and NK cells to the infection site. These cells secret IFNγ, which induce the development of specificity and effector functions in the immune response (Figure 2a) **(Martinez, Helming, and Gordon 2009)**.

Alternative activation of macrophages, anti-inflammatory macrophages or regulatory macrophages (M2 macrophages) downregulate the initial inflammatory response and promote the resolution of inflammation **(Murray and Wynn 2012; Chiu and Bharat 2016)**. M2 macrophages are differentially identifiyed by CD206 and CD163 marker expression (Stöger et al. 2012; L. Zhang et al. 2012). Pathogen recognition induce the release of IL-10, which attracts Th2 cells. These cells release IL-4 and IL-13 that is the final signal of the anti-inflammatory macrophages to develop the effector function (Figure 2b) **(Martinez, Helming, and Gordon 2009; Murray and Wynn 2012)**.

M1 or M2 macrophages may be re-polarized by a secondary stimulation by Th1 or Th2 cytokines or by exogenous stimuli. M1 macrophages could respond to alternative activation by IL-4 from Th2 cells, and IL-10 that induces the Th2 activation **(Gratchev et al. 2006).** However, other studies indicates that M2 macrophages presents more plasticity to be repolarized to M1 cells than M1 macrophages **(Van den Bossche et al. 2016).** Other findings indicate that NF-κB plays an essential role in the orientation of macrophage polarization both in vitro and in vivo **(Porta et al. 2009).** In addition, macrophages also are involved in the removal of cellular debris that is generated during tissue remodelling, and cell clearance **(Mosser and Edwards 2008)**.

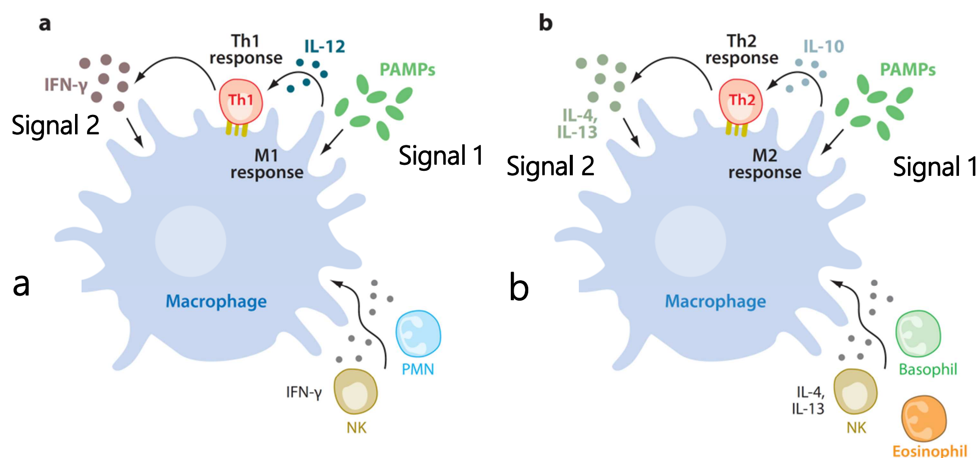


Figure 2. Two-signal macrophage activation model (Martinez, Helming, and Gordon 2009). First signal is the pathogen recognition by its specific receptors. As a consecuence, macrophages release IL-12 that induce the Th1 response. Th1 cell recognition is accompanied by the IFNγ secretion, which acts as a second signal upon macrophages to develop its function. On the contrary, after first signal, M2 macrophages secrete IL-10 that activates the Th2 response. Th2 cells recognition enables the release of anti-inflammatory cytokines, which act as a second signal on M2 macrophages to develop the resolution of inflammation.

DCs are a heterogeneous family of cells, subsets of which may in fact function to suppress rather than stimulate immune responses. In the innate immune response, DCs are characterised by their ability to migrate to secondary lymphoid tissues and activate T cells to induce the adaptive response. (Schraml and Reis e Sousa 2015).

1.3.4. Macrophages adhesion

Cell adhesion is an important feature of macrophages that allow migration, invasion and proliferation, processe that are controlled by receptor tyrosine kinases, G-protein coupled receptors and integrins (Dowling and Kiely 2015). Specifically, integrins play a role in these cellular processes linking the extracellular matrix (ECM) and the actin cytoskeleton (Taherian et al. 2011). Findings in recognition of integrin receptor by ECMs were 30 years ago (Hynes 1987). Integrins are heterodimers formed by two transmembrane proteins, one α subunit and one β subunit (Hynes 2002). In higher vertebrates, the integrin family is composed of 18 α subunits and 8 β subunits bond by non-covalent bindings between extracellular, transmembrane and cytoplasmic domains (McCall-Cullbreath and Zutter 2008).

α1-α6 and αv can associate with β1, forming the very late activation antigens (VLA) subfamilies. They are involved in both cell-cell and cell-substrate adhesion. αL, αM and αX are capable to associate with β2, and are important to attach to the endothelium during cell migration. These integrins are recognised as adhesion molecules of the leukocytes (LEU-CAMs) due to interaction with leukocytes receptors such as lymphocyte function associated antigen-1 (LFA-1) and macrophage antigen-1 (MAC-1). αv and αIIb can bind with β3 forming the cytoadhesins, wich is the vitronectin receptor (αvβ3) and platelets receptors (αIIbβ3) (Albelda and Buck 1990). Moreover, it is known that shedding of β integrin promotes macrophage migration out from inflammatory sites, and the release of soluble integrin heterodimers may also limit local inflammation (Gomez et al. 2012). For example, β3 integrin has an important role in macrophage polarization to M1 (L. Zhang et al. 2012). Some reports indicate that αVβ3 activation can maintain chronic inflammatory processes in pathological conditions. On the contrary, loss of αVβ3 binding allow macrophages to change the inflammatory state to normal state (Antonov et al. 2011).

Binding specificity of integrins is determined by the extracellular domain that recognize diverse ECMs. Integrin formed by α5β1 or α4β1 recognise fibronectin, the one formed by α1β1 or α2β1 bind collagen, and heterodimers α2β1, α3β1 or α6β1 recognise laminin **(Sroka et al. 2011; Huttenlocher and Horwitz 2011).** αV associated with β5, recognise fibronectin. β4 is involved specially on laminin recognition **(Albelda and Buck 1990).**

One importan subgroup of the integrin family, are the collagen receptors. Most studied members of this subgroup are α1β1 and α2β1 integrins. α1β1 integrin is related with cell spreading and binds to the monomeric form of type I collagen. However, α2β1 integrin facilitates cell spreading on fibrillary type I collagen matrix **(Jokinen et al. 2004).** Type IV collagen forms a nonfibrillar complex network that both α1β1 and α2β1 integrins can recognise **(Vandenberg et al. 1991).** Evidences suggest that the collagen IV–binding integrin α1β1 inhibits macrophages to leave a peripheral inflammatory lesion **(Becker et al. 2015).**

The plasma protein fibronectin is an important opsonin in wound repair and host defense. αvβ3 block phagocytosis of fibronectin and α5β1 mediates adhesion to fibronectin-coated surfaces. Neither αvβ5 nor αMβ2 ligation affects α5β1 phagocytic function. However, αvβ3 binding suppresses the phagocytic competence of high affinity α5β1. These mechanisms are important for macrophage migration **(Blystone et al. 1994)**.

2. Inflammatory response

2.1. Introduction

The inflammatory response is the physiologic process that protects the organism against infections, tissue injury, or stress **(Medzhitov 2008)**. Initially, immunologists thought that the physiological significance of the inflammatory response only resides in the host defence against infections **(Medzhitov 2008)**. This vision was based in the self/non-self model. Later, the danger model proposed by Dr Matzinger included the view that APCs present the ability to discriminate and be activated by signals released from injured cells. This concept changed the concept of immune system recognising antigens in microorganisms and the "foreignness" of a pathogen is not the important feature that triggers an immune response **(Matzinger 2002)**. Currently, we know that the inflammatory response is involved in many different physiological and pathophysiological processes such as tissue-reparation, adaptation to stress and restoration of the homeostatic state among others **(Medzhitov 2008)**. Indeed, inducers of the inflammatory response can present an endogenous origin or an exogenous origin **(Medzhitov 2008)**.

2.2. Exogenous origin-inducers of the inflammatory response

Exogenous signals of the inflammatory response involves pathogenic, also named pathogens associated molecular patterns (PAMPs), and non-pathogenic signals such as allergens, irritants, foreign bodies and toxic compounds **(Medzhitov 2008)**.

Focusing on PAMPs, one of the most studied is LPS from *Escherichia coli*, that not only is able to activate macrophages but also DCs and other cells of the innate and adaptive immune system such as neutrophils and B cells **(Willart and Lambrecht 2009).** Similar to LPS, other structural components of microorganisms that are different from eukaryote are also PAMPs, such as bacterial DNA, RNA from virus, flagellin, peptidoglycan, zymosan, H5N1 peptide from influenza virus **(H. Kumar, Kawai, and Akira 2011).** Other PAMPs are toxins produced by microorganisms such as the pore-forming toxins listeriolysin O (LLO) from *Listeria monocytogenes*, the crystal toxin of *Bacillus thuringiensis* or streptolysin O (SLO) from group A *Streptococci* **(Freche, Reig, and van der Goot 2007)**.

2.3. Endogenous origin-inducers of the inflammatory response: danger signals

Danger signals are also termed as “danger associated molecular patterns” (DAMPs) **(Scheibner et al. 2006; Martinon, Mayor, and Tschopp 2009; Pedra, Cassel, and Sutterwala 2009).**The production and release of DAMPs allows the sensing of damage by immune cells and initiates the repair of the dangerous situation, as the damage of a tissue **(Martinon, Mayor, and Tschopp 2009; Saïd-Sadier and Ojcius 2012; Pedra, Cassel, and Sutterwala 2009; Scheibner et al. 2006)**. DAMPs are capable of activate APCs, playing an Important role in regulating innate and adaptive immunity (B. D. Brown and Lillicrap 2002; Aswad and Dennert 2007). DAMPs can be constitutive or inducible, intracellular or secreted, or being a part of the extracellular matrix **(Matzinger 2002)**. DAMPs are signals released from stressed, dead cells or damaged tissues such as extracellular adenosine 5’-triphosphate (ATP), or extracellular nuclear proteins (high-mobility group box 1, HMGB1), endogenous molecules that crystalize in different situations such as uric acid crystals, degradation products from extracellular matrix such as hyaluronan or heat shock proteins (Medzhitov 2008; Pelegrın 2011).

The term DAMPs presents some controversy. In this Thesis, it is considered that the term DAMPs cover a wide range of endogenous extracellular and intracellular signals not always produced as a consequence of cell death, as MSU crystals, and therefore is defined as “danger associated molecular patterns” as a group of signals produced in dangerous situations **(Medzhitov 2008; Scheibner et al. 2006; Martinon, Mayor, and Tschopp 2009; Pedra, Cassel, and Sutterwala 2009)**. However, other authors explain the term DAMPs as “*damage* associated molecular patterns” **(Foell, Wittkowski, and Roth 2007; Saïd-Sadier and Ojcius 2012)**. In this case, the term DAMPs is more strictly associated to the concept of “alarmins” or “endokines” as molecules released by dying cells **(Foell, Wittkowski, and Roth 2007)**. Intracellular molecules such as DNA, high-mobility group nucleosome-binding protein 1 (HMGN1) has been considered as alarmins **(McDonald et al. 2010; Xiao and Fu 2011; Rajamäki et al. 2013; Saïd-Sadier and Ojcius 2012).** In the following part, we are going to review the most important DAMPs for monocytes and macrophages activation.

2.3.1. Extracellular ATP

It is known that extracellular nucleotides like adenosine 5'-diphosphate (ADP), ATP and uridine 5'-triphosphate (UTP) are released from secretory cells such as nerve terminals, mast cells or platelets. In particular, the ability of these immune effector cells has generated increasing interest in the development of novel anti-inflammatory therapeutics targets **(Stokes and Surprenant 2007)**. The importance of extracellular ATP has been thoroughly studied for years in communication between cells in the nervous and vascular systems. However, its role in the immune system is less well known. Grassi and colleagues proposed that ATP released by T cells is able to activate TCR signalling by autocrine recognition **(Schenk et al. 2008)**. This effect drives to Th17 cells differentiation in the gut **(Trautmann 2009).** ATP can be released by several agents in different environments: formyl-methionyl-leucyl-phenylalanine (fMLP) triggers ATP release from neutrophils; bacterial endotoxin LPS potentiates ATP release from macrophages; enteropathogenic *E. coli*, *Salmonella enterica* and *Shigella flexneri* trigger ATP release from intestinal epithelial cells **(Virgilio 2007).** Low concentrations of extracellular ATP that exists around cells in a resting state are enough to keep a tonic signal. Transient increases of extracellular ATP are used for different physiological signaling in the nervous and vascular systems. High concentration of extracellular ATP are associated with cell death and tissue injury, bein considered as a danger signal during the inflammatory processes. **(Trautmann 2009).** ATP mediates its effects through binding to purinergic receptors named P2Y (G protein-coupled receptors) and P2X (ligand-gated cation channels) **(Saïd-Sadier and Ojcius 2012)**. Purinergic signalling will be described with detail in the following chapter.

Additionally, extracellular ATP levels are controlled by ectonucleotidases that degrade extracellular ATP, keeping its concentration low **(Pelegrın 2011)**. This is followed by extracellular degradation to adenosine by a cascade of ectonucleotidases, which include CD39 that is a nucleoside triphosphate diphosphohydrolase (NTPDase) and CD73, that is a 5'-ectonucleotidase **(Haskó et al. 2007)**.

2.3.2. HMBG-1

HMGB1 is a member of the high-mobility group (HMG) protein superfamily, that are nuclear proteins founded in all mammalian tissues and is highly conserved

among various species. HMGB1 is a single polypeptide chain of 215 amino acids in length and is organized into DNA **(Pisetsky, Erlandsson-Harris, and Andersson 2008)**. HMGB1 also participates in innate immune system through activation of the receptor for advanced glycation end products (RAGE) and/or TLR2 and TLR4 **(Tang et al. 2011; Saïd-Sadier and Ojcius 2012)**. The alarmin activity of HMGB1 is predominant in acute and chronic inflammatory conditions, including rheumatic diseases or sepsis. **(Pisetsky, Erlandsson-Harris, and Andersson 2008)**. Several articles have described a role for HMGB1 as a pro-inflammatory agent whose secretion is regulated by nucleotide binding domain leucine-rich repeat containing receptors (NLRs) and its adaptor proteins **(Saïd-Sadier and Ojcius 2012)**.

2.3.3. Hyaluronan

Hyaluronan (HA) is a negatively charged high molecular weigh glycosaminoglycan that form part of the extracellular matrix, and is present in the membrane of normal lungs, joints, and vitreous fluid. In the inflammatory environment, HA is depolymerized to lower molecular weigh fragments via oxygen radicals and enzymatic degradation by hyaluronidase, β-glucuronidase, and hexosaminidase. Although HA is considered an homeostatic protein. In the inflammation site HA is depolymerized to lower molecular weigh fragments hyaluronidases and hexosaminidases, and these HA fragments activate the inflammatory response in epithelial cells, endothelial cells, fibroblasts, dendritic cells (DCs), and macrophages **(Scheibner et al. 2006)**.

2.3.4. Crystals

Uric acid is the end product of purine metabolism in humans and higher primates. However, it is known that is released from injured cells and acts as a danger signal **(Eleftheriadis et al. 2013).** Extracellular uric acid reacts with free Na^{+} to form monosodium urate (MSU) crystals. Uric acid crystals induce the activation of the NLRP3 inflammasome and the release of IL-1β and IL-18 cytokines, being this explained in the following chapter. This mechanism explains the pathophysiological mechanism of gout (Pelegrın 2011; Martinon et al. 2006b). Moreover , extracellular uric acid stimulates dendritic cell maturation, enhances the generation of responses from $CD8^{+}$ T cells during infection, increase Th17 responses in adaptive immunity and activates NF-κB driving the expression of

different pro-inflammatory cytokines (**Shi, Evans, and Rock 2003; Conforti-Andreoni et al. 2011).**

Calcium pyrophosphate dehydrate (CPP) crystals commonly are found in the synovial fluids and tissue joints during the osteoarthritis process. They are the cause of the chronic CPP arthritis, one of them the acute monoarthritis or pseudogut, mediated by the inflammasome activation **(Conway and McCarthy 2018)**.

Excessive amounts of cholesterol induces the development and progression of atherosclerosis. Accumulation of cholesterol in early atherosclerotic lesions results in the formation of foam cells, macrophage with the accumulation of lipid droplets. Extracellular cholesterol accumulation lead to crystals formation in the atherosclerotic plaques. Cholesterol crystals activate the inflammasome and are proposed to initiates the inflammatory response in atherosclerosis **(Grebe and Latz 2013).**

2.4. Inflammatory cytokines

During an infection, the production and release of cytokines and soluble factors by DCs, monocytes, granulocytes and tissue resident macrophages includes among others TNFα, IFNγ, interleukins (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18), chemokines (CCL2, CCL4, CCL13, CCL14, CCL17, CCL18, CCL22, and CCL24) and TGFβ. These cytokines and chemoquines are able to recruit more immune cells and activate them (B. D. Brown and Lillicrap 2002; Martinez, Helming, and Gordon 2009; Lacy and Stow 2011). The most important cytokines in the inflammatory response that has the major relevance in this Thesis will be described: IL-1 family (including IL-1β and IL-18), TNFα, IL-6, and IL-8.

Some cytokines belong to families, as the IL-1 family (IL-1F) that is composed by 11 members which include IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18 among others. IL-1β is the most studied cytokine of this family. It is a pro-inflammatory cytokine that participates in the generation of systemic and local inflammatory responses to infection and tissue injury. It perform its biological actions after binding to the IL-1 receptor 1 and induces fever, pain, and activation and infiltration leukocyte **(Lamkanfi and Dixit 2009).** IL-1β signalling is tighltly regulated and for example its binging to the type II IL-1 decoy receptor results in lack of signalling, as well as the soluble type II IL-1 receptor quench soluble IL-1β

(C. a Dinarello 2010). Importantly, IL-1Ra is also produced during inflammation and binds to IL-1RI and impide IL-1α and IL-1β signalling. The recombinant human IL-1Ra is used in clinics as anti-inflammatory drug for the treatment of certain autoinflammatory syndromes, but is also able to decrease blood vessels in inflammed joints in patients with rheumatoid arthritis (C. a Dinarello 2010). IL-18 mainly induces IFN-γ in T and NK cells and therefore contributes to establish a Th1 response after binding to the IL-18R. IL-18 activity is also controlled by the L-18 binding protein, that quench biological activity of IL-18 (Lamkanfi and Dixit 2009). In addition, IL-1β and IL-18 present another control level, they both are initially produced in the cell as inactive preursors that are not able to bind to their respective receptors. These cytokines needs proteolytic claveage caspase-1 to produce the mature for of IL-1β and IL-18 (Lamkanfi and Dixit 2009; C. a Dinarello 2005). Upon cleavage, the release of these cytokines from the cell is then regulated by different unconventional protein release mechanism that involves pyroptotic cell death, microvesicle shedding, lysosomal exocytosis and autophagy (MacKenzie et al. 2001; Bergsbaken et al. 2011; Dupont et al. 2011; Nakahira et al. 2011). IL-33 is the most recent adition to the IL-1 family. IL-33 activates mast cell functions and drives allergic reactions and Th2 responses. (C. a Dinarello 2009; Cayrol and Girard 2009). Although IL-33 is also released from the cell via unconventional protein release mechanisms (Kouzaki et al. 2011), caspase-1 activation inhibits this cytokine (Cayrol and Girard 2009).

TNF is another superfaily of cytokines. The most studied member of the TNF superfamily is TNFα, a pleiotropic pro-inflammatory cytokine produced by several immune cells during acute inflammation (Ordás et al. 2007). TNFs were first isolated as cytotoxic factors to tumour cells that induce tumour regression in mice. Currently, TNFs is a superfamily of 19 members that signal through 29 receptors. These cytokines regulate different aspects of the immune response, being TNFα a pro-inflammatory cytokine, however the different TNF members present a wide range of actions including cellular proliferation, survival, differentiation or apoptosis (Aggarwal 2003). Neutralizing anti-TNFα antibodies are used as treatment of reumathoid arthritis, inflammatory bowel disease or psoriasis (Feldmann and Maini 2001; De Simone et al. 2013; Khan, Asim, and Lichtenstein 2014).

IL-6 is a systemic pro-inflammatory cytokine mainly released by macrophages and monocytes. IL-6 production is regaulated by IL-1β and microbial products and is the major

inducer of acute phase proteins (APPs) synthesis from the liver (Charles A. Janeway 1992; Jain, Gautam, and Naseem 2011). IL-6 also favors Th17 differentiation over Treg, and IL-17 produced by these Th17 cells further decreases Treg activity. IL-6 is increased in the plasma in different pathologies, as sepsis, lupus nefritis, cirrhosis, and different autoimmune inflammatory diseases such as rheumatoid arthritis and has been proposed as biomarker for these diseases (Rincon 2012; Jekarl et al. 2013; Abdel Galil, Ezzeldin, and El-Boshy 2015; Lin et al. 2015). Humanized antibodies blocking IL-6 are used in clinics to treat different inflammatory diseases (Puchner and Blüml 2015).

IL-8 is a systemic proinflammatory and quimioattractant cytokine produced by a variety of tissue and blood cells, including monocytes, neutrophils, fibroblasts, endothelial cells, and several tumour cells (Utgaard et al. 1998; Thurau et al. 1996; Reeves et al. 2011; David et al. 2016). In the inflammatory response, this cytokine recruits leukocytes in the site of infection (David et al. 2016). IL-8 participates in cancer and is a potent angiogenesis stimulator due to the capacity to induce the endothelial cell-proliferation, to initiate the formation of new blood vessels (Cao et al. 2016; David et al. 2016). However, IL-18 posses a dual role in tumor. Tumor cells express IL-8 and recruit a significant amount of neutrophils, and myeloid supresor cells, including M2 macrophages (Cao et al. 2016; David et al. 2016).

2.5. Acute phase proteins

APPs are elevated in plasma at high concentrations during inflammatory processes and modulates the immune response by activating the complement system. APPs bind cellular remnants, neutralize enzymes, and scavenge free hemoglobin and radicals. The variety of processes which APP are involved is termed as acute phase response (APR) (Jain, Gautam, and Naseem 2011; Gruys et al. 2005). Pro-inflammatory cytokines as IL-6 activates a janus-kinase (JAK) in target cells iducing the synthesis of APP (Heinrich 1998). The three most important APPs are C-Reactive protein (CRP), serum amyloid P (SAP), serum amyloid A (SAA), and Procalcitonin (PCT) (Jain, Gautam, and Naseem 2011; Panico and Nylen 2013). However Sistemic CRP levels increase due to infection-induced pro-inflammatory cytokine production and CRP is an important clinical marker for infection, in fact high levels of CRP are found in the blood of septic patients (Jain, Gautam, and Naseem 2011; Pepys and Hirschfield 2003). Elevation of CRP is also associated with non-infectious disease where pro-inflammatory cytokines are produces, as in the case of insulin resistance

and metabolic syndrome, and some authors have proposed CRP as a biomarker for type 2 diabetes (Pepys and Hirschfield 2003).

2.6. Pattern Recognition Receptors

2.6.1. Introduction to PRR

PAMPs are recognized by PRRs that are highly specific and conserved markers to microbes. These receptors not only are expressed in APC professional cells (DCs and macrophages). Various non-proffesional immune cells such as lymphocytes. Except NLRs, PRRs signaling upregulates the gene transcription of a large variety of pro-inflammatory cytokines, chemokines and antimicrobial proteins (Osamu Takeuchi and Akira 2010). NLRs, RIG-I-like RNA helicases (RLHs), and C-type lectin receptors (CLRs) are included between PRRs (Pedra, Cassel, and Sutterwala 2009).

2.6.2. Toll-like receptors

The most intensely studied are the Toll-like receptors (TLRs) that are expressed by many cells from immune system (Martinon, Mayor, and Tschopp 2009). TLRs are PRRs that recognize microbial PAMPs or DAMPs (C A Janeway 1989; O'Neill, Golenbock, and Bowie 2013; Yeretssian, Labbé, and Saleh 2008). Once activated by PAMPs, the TLRs initiate different signaling cascades that finish in activation of the transcription factors, for example, NF-κB, and interferon-regulatory factor (IRF)- 3 resulting in the initiation of inflammatory response. TLR activation results in the production of antimicrobial peptides, inflammatory cytokines and chemokines, and molecules for cell adhesion and migration (Martinon, Mayor, and Tschopp 2009; Saïd-Sadier and Ojcius 2012; Pedra, Cassel, and Sutterwala 2009; Zeiser et al. 2011; Scheibner et al. 2006). LPS is the major component of gram negative bacteria outer cell wall and probably is with difference the most studied PAMP in the activation of the innate immune system (Raetz 1990). LPS is specifically recognised by innate immune cells through TLR4 (Paul-Clark et al. 2012). TLR4 was the first TLR described (Medzhitov and Janeway 1997) as a human homologue of Drosophila Toll receptor, an invertebrate receptor induced by Gram-positive bacteria or fungi that activates the production of antimicrobial peptides (Gay and Keith 1991). LPS discovery helped to Baltimore and colleagues to identify the Nuclear Factor- kappa B (NF-κB) transcription

factor and its activation in B cells **(Sen and Baltimore 1986).** TLR share with the IL-1R1 200 common aminoacids in their cytoplasmic domain, both presenting a Toll–IL-1-resistence (TIR) domain that is important for NF-κB signalling **(Heguy et al. 1992; Ip et al. 1993; Akira and Takeda 2004; C. A. Dinarello 1991)**. By contrast, the extracellular region of the TLR and IL-1R1 is different, the TLR contains leucine-rich repeat (LRR) motifs of 24-25 aminoacids that are responsible to bind to different PAMPs and DAMPs, whereas the extracellular region of IL-1R1 contains three immunoglobulin-like domains that specifically interact with bioactive IL-1α and IL-1β. Structurally, TLR extracellular LRR domain is linked to the cytosolic TIR domain via a transmembrane domain. The cytosolic TIR domain contain a central structure composed of five-stranded parallel β-sheet, which is surrounded by five α-helices on each side **(Akira and Takeda 2004).** PAMPs and DAMPs binding to the LRR of TLR induces dimerization of the receptors and therefore an approximation of their cytoplasmic TIR domains that now interacts with the protein myeloid differentiation-primary response gene 88 (MyD88). **(Saïd-Sadier and Ojcius 2012; Kluwe, Mencin, and Schwabe 2009; Charles A. Janeway and Medzhitov 2002; Dunne and O'Neill 2003)**. MyD88 has a C-terminal TIR domain that mediates its homotypic interaction with the TIR domain of TLR and the N-terminal death domain of IL-1R-associated kinases (IRAKs). IRAK binds to TGF-β-activated kinase (TAK1) through TNF-receptor-associated factor 6 (TRAF6) and subsequently, TAK1 binds to TAK1-binding protein 1 (TAB1) and TAB2 **(Charles A. Janeway and Medzhitov 2002; Dunne and O'Neill 2003; Kluwe, Mencin, and Schwabe 2009; O'Neill, Golenbock, and Bowie 2013; Dong et al. 2016)**. Then, TAK1 is able to activate transcription factors TLRs can bind to mitogens and transcription factors to favors the proinflammatory gene (predominantly activates NF-κB, IRF7 and the activator protein 1 (AP-1). In the case of NF-κB activation, TAK1 activates the inhibitor of κB kinase alpha (IKKα), wich phosphorylates and inactivates the inhibitor of κB alpha (IκBα), allowing the NF-κB activation and internalization into the nucleous to induce the pro-inflammatory genes expression (Figure 3) **(Kluwe, Mencin, and Schwabe 2009; O'Neill, Golenbock, and Bowie 2013)**. However, TLR4 can internalize in endosomes together other TLRs **(O'Neill, Golenbock, and Bowie 2013)**. In this case, signalling pathway is mediated by TIR domain-containing adaptor protein inducing IFNβ (TRIF). TRIF can bind to the TRAF6-IRAK complex through the receptor interacting protein-1 (RIP1) and developes the classical signalling pathway to activate NF-κB **(Kluwe, Mencin, and Schwabe 2009; O'Neill, Golenbock, and Bowie 2013)**. However, TRIF can alternatively bind to TRAF3, which specifically activates the TRAF family member-associated NF-kappa-B activator-

binding kinase 1 (TBK1)-IKKε complex, and the following activation of IRF3 transcription factor (Figure 3) **(O'Neill, Golenbock, and Bowie 2013)**.

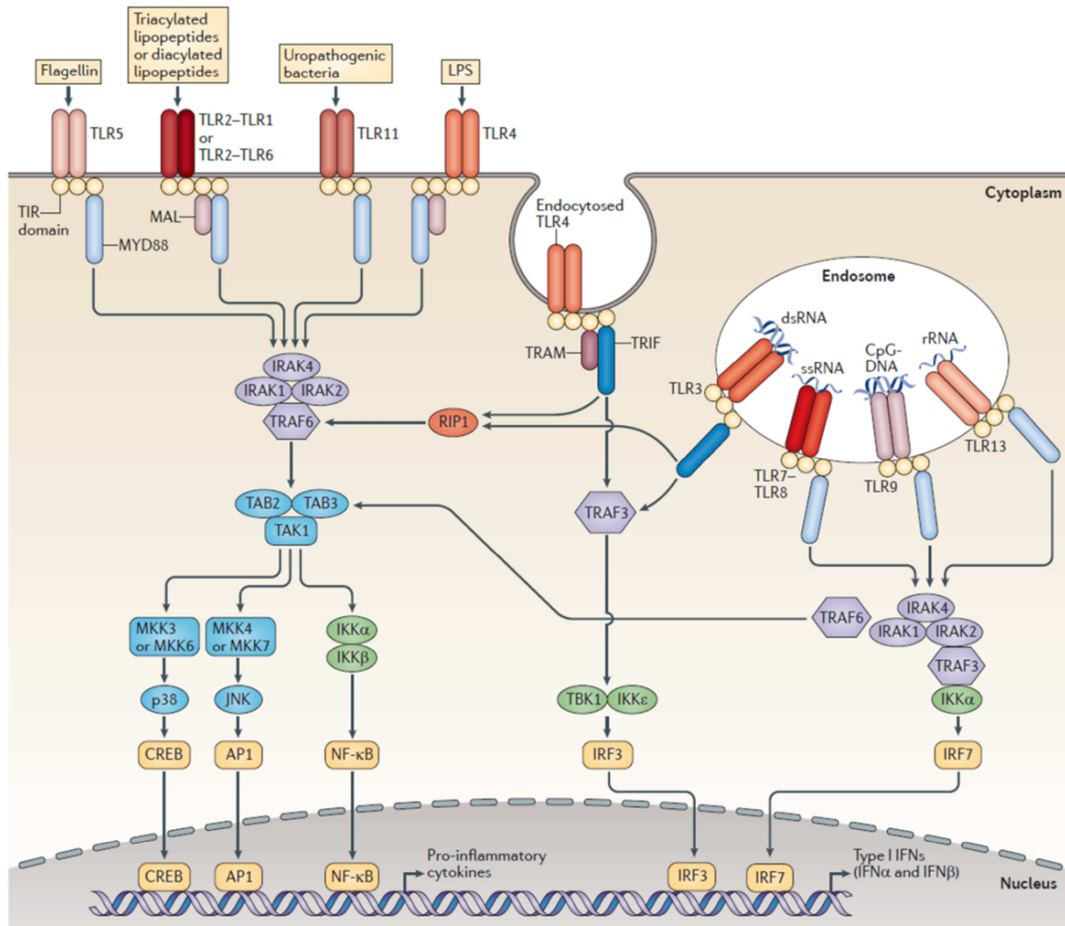


Figure 3. Mammalian TLR signalling pathways model performed (O'Neill, Golenbock, and Bowie 2013). It is shown a differential signalling pathway activated by endogenous (TLR3, TLR7, TLR8, TLR9 and TLR13) or cell surface TLRs (TLR4, TLR5, TLR2, or TLR11). On the one hand, cell surface TLR are recruited by phagosomes and endocytosis. On the other hand, endogenous TLRs are recruited by endosomes ER vesicles. The other difference is that endogenous TLR are specialized to recognise bacterial or viral DNA and RNA, meanwhile, cell surface receptors recognise different microbial proteins.

TLRs can be divided in two groups: TLR that are located on the cell surface and are recruited to phagosomes after activation, that includes TLR1, TLR2 and TLR4; and TLR that are located on the endosome and are involved in the recognition of nucleic acid structures, including TLR3, TLR7 and TLR9 (Figure 3) **(Akira and Takeda 2004; O'Neill, Golenbock, and Bowie 2013).**

TLRs recognise a large variety of ligands. For example, TLR4 has been implicated in the recognition of different PAMPs and DAMPs as LPS, lipotechoic acid (LTA), heat shock protein 60, S100 proteins and the fusion protein of the respiratory syncytial virus among others (Kurt-Jones et al. 2000). However, the binding of LPS to TLR4 is supported by accessory proteins as it recognise the complex of LPS with LPS-binding protein (LBP), an LPS transport protein present in serum (Ogata et al. 2000). Also TLR4 prsent co-receptors for LPS, as CD14, a predominant receptor in myeloid cells that loads LPS to TLR4, or the oligomerization with the myeloid differentiation protein 2 that also facilitate the binding of LPS to TLR4 (Miyake 2007).

Similar to TLR4, other TLR recognise specific ligands: TLR3 recognises double stranded (ds) DNA (O'Neill, Golenbock, and Bowie 2013), TLR5 recognise flagellin (Hayashi et al. 2001), TLR7 and TLR8 are involved in the recognition of single stranded (ss) DNA from virus (O'Neill, Golenbock, and Bowie 2013), TLR9 recognises CpG-motifs present in bacterial DNA (Akira and Takeda 2004; Krieg 2000), mouse TLR11 detect components from uropathogenic bacteria (D. Zhang et al. 2004), and mouse TLR13 recognize bacterial ribosomal RNA (Oldenburg et al. 2012).

The repertoire of ligands that TLR could recognie increase when some TLR form heterodimers with other TLR. For example, TLR2 oligomerize with TLR1 or TLR6 (Ozinsky et al. 2000) and this allow TLR2 to recognise numerous ligands, such as *Staphylococcus*, (Hajjar et al. 2001), LPS from *Prophyromonas gingivitis* (Hirschfeld et al. 2001; Paul-Clark et al. 2012), bacterial lipoproteins (O Takeuchi et al. 2000), gram positive peptidoglycan (Takeuchi O Hoshino K 1999), *Leptospira interrogans* (Werts et al. 2001), *Trypanosoma cruzi* (Campos et al. 2001), HA (Scheibner et al. 2006), or glycosylphosphotidylinositol lipid and zymosan from yeast cell walls (Underhill et al. 1999). Similarly, although the ligands for TLR10 have not been identified, its similarity to TLR1 allow to heterodimerize with TLR2 (O'Neill, Golenbock, and Bowie 2013). Another example of TLR cooperation is TLR11, that is involved with TLR12 to recognise *Toxoplasma gondii* profilin protein (Andrade et al. 2013).
Therefore, TLR offer a series of receptors that allow immune cells to ideintify patterns associated to different situations of infecton and tissue injury and activate a transcriptional pro-inflammatory programme aimed to resolve the infection or the injury.

2.7. Nucleotide-binding domain and Leucine-rich repeat containing Receptor

NLR is a family of PRR that includes proteins with the presence of a nucleotide-binding domain and LRRs. In 2008 a consensus suggested that NLR family stands for "Nucleotide-binding domain and Leucine-rich repeat containing Receptor" referring to the domain composition of their members and this abbreviation was then used by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) (J. P.-Y. Ting et al. 2008). Differently to TLRs, NLRs localize to the cytoplasm of the cell and activate in response to intracellular PAMPs and DAMPs **(Kufer 2008).** NLRs compose a family of 23 members in humans that share three common structural domains: a C-terminal region characterized by LRRs of 20-30 amino acid in length; a central nucleotide-binding and oligomerization domain (NOD), also referred as NACHT domain; and an N-terminal effector domain. Alternatively, this N-terminal domain can be a caspase recruitment domain (CARD) denoting the NLRC subfamily, or a pyrin domain (PYD) constituting the NLRP subfamily **(Bertin and DiStefano 2000; Tschopp, Martinon, and Burns 2003).** In some NLR a baculovirus inhibitor of apoptosis repeat (BIR) is alternatively present in the N-terminal and is called NLRB or NLR family apoptosis inhibitory protein (NAIP); or the presence of an acidic activation domain between the CARD and NBD domain is present in the case of NLRA that is also known as MHC-class II transactivator (CIITA) complex **(Martinon, Mayor, and Tschopp 2009; Saïd-Sadier and Ojcius 2012; J. P. Y. Ting et al. 2008; Pedra, Cassel, and Sutterwala 2009);** Finally, this NLR family also includes NLRX as a receptor with an N-terminal with no homology to other NLR subfamily members **(J. P. Y. Ting et al. 2008)**. Of note, NOD1, NOD2, CIITA and NAIP are the only NLR receptors that have not been renamed to the standardized NLR nomenclature, this is to avoidel confusion in the field because these gene names are associated to a vast amount of literature **(J. P. Y. Ting et al. 2008)**.

In the last decade, it has been found that the NLR family plays a central role in innate immunity and a high interest on understanding the regulation of these receptors has been extended among laboratories. The different NLRs singnal in two different ways, forming complexes termed NOD-signalosomes (NOD1/2) or inflammasomes (NLRP1/3/6, NLRC4) **(Martinon 2009).**

These signalosomes are associated with the receptor interacting protein (RIP) 2 and TAK1, and the subsequent activation of NF-kB and mitogen activated protein kinases (MAPK) in a

similar way than TLR signalling **(Saïd-Sadier and Ojcius 2012; Yeretssian et al. 2011)**. NOD-signalosomes activates the classical MAPK extracellular signal regulated kinase (ERK)1/2, p38 and JNK kinases, inducing the expression of a wide plethora of pro-inflammatory genes. However, depending the accessory proteins recruited to NOD-signalosomes will differentially activate NF-kB and ERK1/2 for NOD1 or JNK and p38 for NOD2. If NOD receptors binds CARD9 they will preferentially signal to activate JNK and p38. On the contrary, if NOD receptors binds the pro-apoptotic BH3-only BCL2 family member BID, they will preferentelly activate NF-κB and ERK1/2 (Figure 4) **(Yeretssian et al. 2011).** This signalling depends of the PAMPs that is recognised. Further studies indicate that NOD1 recognises D-glutamyl-meso-diaminopimelicacid from gram negative bacteria, meanwhile NOD2 recognises muramyl-dipeptide (MDP) from gram positive bacteria **(Girardin et al. 2003; Chamaillard et al. 2003; Moreira and Zamboni 2012)**. NOD-signalosomes does not result in the activation of caspases **(Le Blanc et al. 2008).**

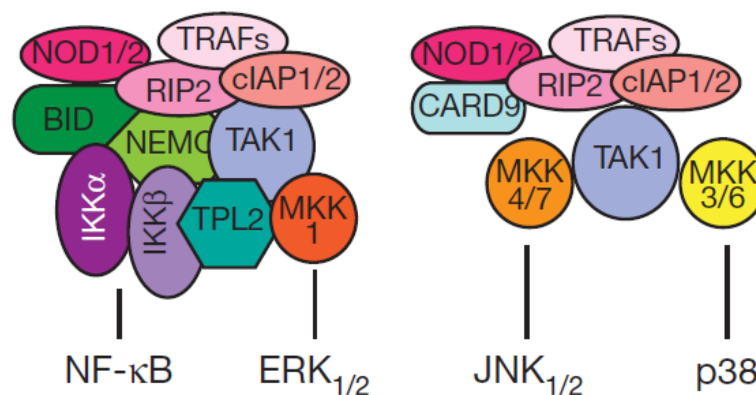


Figure 4. Representation of NOD1 and NOD2-signalosomes (Yeretssian et al. 2011). NOD signalosomes are differentially associated to BID or a CARD9 to activate transcription factors as represented.

The NLRs NLRP1/3/6 and NLRC4 are able to signal by forming inflammasomes **(Franchi, Eigenbrod, and Núñez 2009)**. The term inflammasome was first described by Tschopp and collaborators in 2002 **(Martinon, Burns, and Tschopp 2002)** to describe a large (>700 kDa) multiprotein complex that triggers the activation of caspase-1 **(Lamkanfi and Dixit 2009)**. By activating this caspase, inflammasomes control the maturation of pro-inflammatory cytokines IL-1β and IL-18, being therefore a central modulator of the inflammatory response **(Tzeng et al. 2016).** NLR receptors that form inflammasomes are mainly expressed in innate immune cells and their expression is modulated by PAMPs or

cytokines, however, they also express in non-immune cells where they could form inflammasomes and shape the immune response to specific characteristics of the tissue, as the case of keratinocytes that are able to form active inflammasomes in response to UV light (Watanabe et al. 2007).

Not all NLR are pro-inflammatory molecules, there are NLR that repress the inflammatory response, as NLRP10 that present important anti-inflammatory functions. NLRP10 contains a PYD and a NOD domain, with the absence of LRRs (Y. Wang et al. 2004) and is able to bind and inhibit ASC, reducinging caspase-1-mediated IL-1β maturation induced by inflammasomes (Y. Wang et al. 2004). However, recently it was discovered that NLRP10 positively regulates NOD1 upon *Shigella flexneri* infection (Damm et al. 2016). NLRP10 is also important for DCs migration through dedicator of cytokinesis 8 (DOCK8) signalling (Damm et al. 2016), and contributes to elicit a Th1 response (Vacca et al., 2017). Therefore, the regulatory role of NLRP10 comprises different pro- and anti-inflammatory roles.

NLRP11 is another negative regulator NLR that inhibits TLR signalling by targeting TRAF6 for degradation (C. Wu et al. 2017). Other NLRs such as NLRP2, NLRP5 and NLRP7 could have similar functions to NLRP11 (Ellwanger et al. 2018).

2.8. Types of inflammasomes

The best characterized inflammasome, and the one most important as a sensor of PAMPs and DAMPs is the NLRP3 inflammasome (Savage et al. 2012; Schroder and Tschopp 2010). The NLRP3 inflammasome is a key component of the innate immune response to pathogenic infection and tissue damage (Juliana et al. 2012). Structure and activation of NLRP3 inflammasome will be described with more detail in the next chapter.

Although less studied than NLRP3, the NLRP1 inflammasome was the first inflammasome described and besides a N-terminal PYD domain, it also contains a C-terminal CARD domain after the LRR domain (Martinon, Burns, and Tschopp 2002; J. P. Y. Ting et al. 2008) (Figure 5). The human NLRP1 inflammasome is stimulated by the presence of cytosolic MDP and Bacillus anthracis letal toxin (Faustin et al. 2007; Pedra, Cassel, and Sutterwala 2009; Saïd-Sadier and Ojcius 2012; Broz and Dixit 2016).

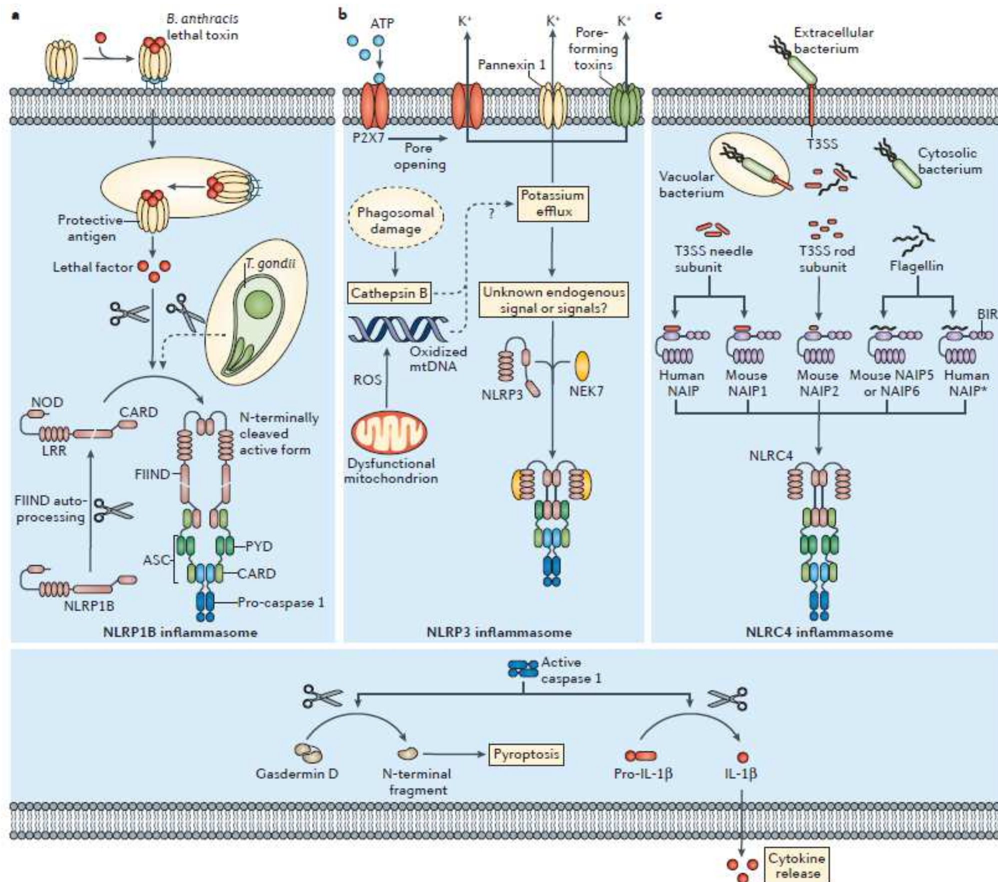


Figure 5. Escheme of NLR activation inflammasomes (Broz and Dixit 2016). Inflammasomes activation depend on different mechanism: NLRP1 inflammasome is activated by *Bacillus anthracis* recognition, NLRP3 inflammasome is activated through a K^{+} depletion, induced by different extracellular signals, NLRC4 inflammasome respond against different extracellular bacteria. Each inflammasome present its own assembly mechanism. However, the activation of caspase-1 is the common step for all inflammasomes.

NLRC4 receptor (also termed as IPAF) is another NLR that form active inflammasome upon activation by some Gram-negative bacteria as *Salmonella typhimurium*, *S. flexneri*, *Pseudomonas aeruginosa* and *Legionella pneumophila* **(Saïd-Sadier and Ojcius 2012; Pedra, Cassel, and Sutterwala 2009; Broz and Dixit 2016)** (Figure 5). Although NLRC4 is able to directly recruit caspase-1 via CARD-CARD interactions, ASC binding amplify caspase-1 activation and in response to *Salmonella* it might form inflammasome complexes togheter with NLRP3 **(Broz et al. 2010; Qu et al. 2012; Qu et al. 2016; Freeman et al. 2017)**. Although ASC is dispensable for caspase-1 activation by NLRC4 **(Saïd-Sadier and Ojcius 2012)**, it has been recently found that NLRC4 inflammasome could form a small activating hub for caspase-1 that result in a prolongued activation of caspase-1 **(Boucher et al. 2018)**. NLRC4 inflammasome is formed upon recognition of bacterial flagelin

or type III secretion system in the cytosol of the cell, however these PAMPs do not directly binds NLRC4, instead are directly recognised by NAIP and the conformation of the complex NAIP-PAMP is able to recruit inactive NLRC4 proteins to form an inflammasome **(Lage et al. 2014; Vance 2015; Hafner-Bratkovič 2017; Nichols, Von Moltke, and Vance 2017)**. NLRC4 gene encoding mutations induce the autoinflammation with infantile enterocolitis (AIFEC) **(Masters et al. 2009)**.

NLRP6 is another NLR that could form inflammasomes, however their molecular structure has not been identified, nor is known their direct activator. NLRP6 is present at high level in the intestine and it plays an important role in intestinal homeostasis. NLRP6 has been strongly studied in colitis models and colitis-associated colorectal cancer **(Broz and Dixit 2016)**. Its deficiency in mice is associated with an altered gut microbiota that increases the susceptibility to colitis and colitis-associated colorectal cancer **(Saïd-Sadier and Ojcius 2012)**. This phenotype was linked to a decrease of IL-18 levels from intestinal epithelial cells **(Broz and Dixit 2016; Saïd-Sadier and Ojcius 2012).**

There are other proteins that contain a PYD domain but do not belong to the NLR family, but are able to form functional inflammasomes and activate caspase-1 (Figure 6) **(Broz and Dixit 2016)**. This is the case of absent in melanoma 2 (AIM2) protein, that contain a PYD domain together a HIN domain. The HIN domain binds dsDNA and induces oligomerization of AIM2, that now is able to recruit ASC via PYD-PYD interactions, activate caspase-1 and control the maturation and secretion of IL-1β and IL-18 **(Reinholz et al. 2013)**. AIM2 is able to bind dsDNA from microorganism as virus or bacteria, and also dsDNA from the own host cell, therefore AIM2 is a cytosolic sensor for dsDNA. However, it was recently described that in human monocytes, AIM2 was not able to form inflammasomes in response to cytoplasmic dsDNA, but in this cell type cytosolic dsDNA activates the NLRP3 inflammasome via stimulator of interferon genes protein (STING) induced cell death **(Gaidt et al. 2017)**. AIM2 was the first non-NLR protein discovered to interact with ASC by PYD-PYD binding **(Saïd-Sadier and Ojcius 2012; Broz and Dixit 2016)**.

Pyrin protein (not to be confused with pyrin domain, PYD) is also a non-NLR protein able to form inflammasomes (Figure 6). Pyrin is encoded by the *MEFV* gene and is composed of a PYD domain, a B-Box, a coiled-coil domain and a B30.2/SPRY domain **(Mariathasan and Monack 2007; López-Castejón and Pelegrín 2012; Mariathasan et al. 2006)**.

At basal conditions, pyrin protein is phosphorylated at Ser242 and binds the 14-3-3 protein that maintain pyrin in an inactive conformation. Pyrin dephosphorilation occurs upon inactivation of the Rho family of GTPases, as RhoA, by bacterial toxins and then 14-3-3 protein dissociate from pyrin allowing oligomerization of pyrin and the recruitment of ASC via PYD-PYD interactions to form a inflammasome that activate caspase-1 and induces IL-1β and IL-18 release **(Masters et al. 2016; Moghaddas et al. 2017).** Mutations in the *MEFV* gene are associated to different autoinflammatory syndromes, while mutations in the B30.2/SPRY domian associates wtih familial Mediterranean fever (FMF) **(Broz and Dixit 2016)**, mutations in Ser242 or adyacent residues result in pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) **(Masters et al. 2016; Moghaddas et al. 2017).** Surprisingly, although both mutations result in caspase-1 activation, FMF and PAAND patients display a different cytokine driver. FMF patients present elevated concentration of IL-1β and are successfully treated with recombinant IL-1Ra, PAAND patients present elevated concentrations of IL-18 in plasma **(Latz, Ts, and Stutz 2013; Moghaddas et al. 2017).** It is not known what mechanisms gouvern this differential profile of cytokines in these diseases. FMF symptoms are fever, localized inflammation, which affects to serosa, joints and skin, and the presence of amyloid deposits in the kidneys and digestive system **(López-Castejón and Pelegrín 2012; Erdem et al. 2018)**.

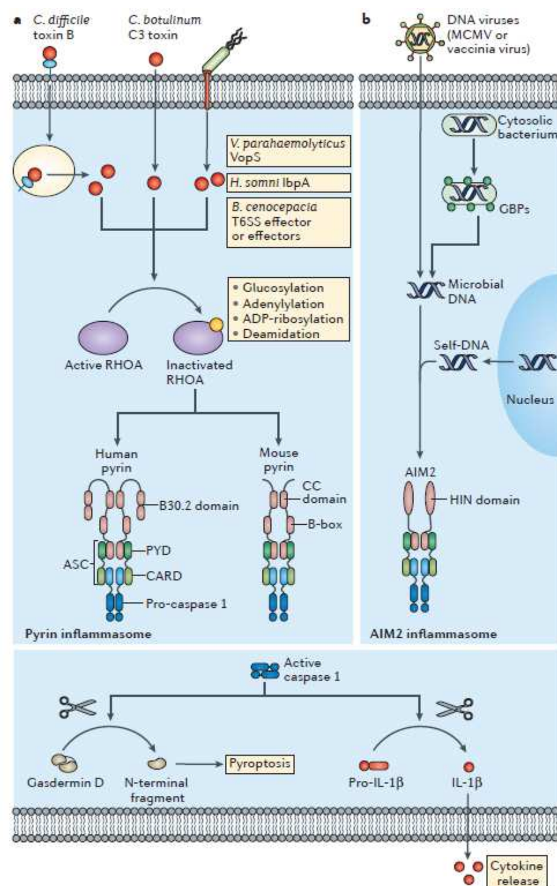


Figure 6. Non-NLR inflammasomes (Broz and Dixit 2016). Pyrin inflammasome recognises bacterial toxins such as CdtB from *C. difficile*. AIM2 recognises the double stranded DNA from Virus. Both inflammasomes activates caspase-1 as an effector mechanism.

The inflammasomes amplify caspase-1 activation by the assembly of ASC to oligomers of NLR or other effector proteins (AIM2 or Pyrin) **(Vajjhala, Mirams, and Hill 2012)**. ASC is a protein consisting of two domains, a PYD and a CARD **(Vajjhala, Mirams, and Hill 2012)** ASC is recruited to inflammasomes via PYD–PYD homotypic interacitions forming a structure that allow the recruitment of new ASC molecules via PYD-PYD interactions and forming helical filaments **(Dick et al. 2016)**. For some inflammasomes, as NLRC4, the initial recruitment of ASC is via CARD-CARD interactions, however, the structure of this interaction is not known. ASC filaments leave the CARD domain of ASC in the outside of the filament and now CARD–CARD interactions among different molecules of ASC in the filaments induce the compactation of these filaments into a single macromolecular aggregate that is commonly called ASC speck or pyroptosome **(Cai et al. 2014; Lu et al. 2014; Dick et al. 2016; Sborgi et al. 2015)**. Pro-caspase-1 present a p10, p20 and a CARD domain that allow pro-caspase-1 recruitment into the ASC complex and this close interaction of pro-caspase-1 subunits allow activation of caspase-1. Caspase-1 activity reside in heteroteramers including p10 and p20 subunits, and within the ASC oligomer the two main forms of active caspase-1 are one containing two pro-caspase-1 subunits, and other that contains the p10 subunit and the CARD-p20 subunit **(Broz and Dixit 2016; Boucher et al. 2018).** The p10 and p20 subunitis normally detected by Western blot experiments do not present caspase-1 activity as they are very unstable and dissociate each other **(Boucher et al. 2018)**.

2.9. Effector mechanisms of inflammasomes

As it was mentioned, the inflammasomes controls the processing and release of different pro-inflammatory cytokines as IL-1β or IL-18 via caspase-1 activation. **(Yeretssian, Labbé, and Saleh 2008; Pétrilli et al. 2007; Netea et al. 2009; Jitprasertwong et al. 2014; Faustin et al. 2009)**. Therefore, the main effector mechanism of the inflammasome is caspase-1 activation (Figure 7). This enzyme has multiple substrates in the cell that induces the unconventional protein secretion **(Keller et al. 2008; Agard, Maltby, and Wells 2010; de Torre-Minguela et al. 2016)**, and for example it blocks glycolysis and induce a specific type of cell death termed pyroptosis **(Labbe 2011; Dick et al. 2016)**. Pyroptosis is frequent during the inflammatory response against pathogens, but it could also happened in sterile conditions in the absence of infections **(Faustin et al. 2009)**. The term ''pyroptosis'' has been proposed to describe this specific programmed cell death that is highly pro-inflammatory: ''pyro'' is

related to fire or fever and ''ptosis" denotes failing **(Yeretssian, Labbé, and Saleh 2008)**.

Pyroptosis was first described in macrophages infected with *S. typhimurium* **(Kroemer et al. 2008)**, and considered as a mechanism to stop bacterial infections that proliferate in intracellular phagosomes of the macrophage **(Järveläinen, Galmiche, and Zychlinsky 2003)**. Pyroptosis is executed by the protein gasdermin D (GSDMD) that is a substrate of caspase-1 and caspase-4/5 in humans or caspase-11 in mice. Upon cleavage of GSDMD by caspase-1 the N-terminal fragment inserts into the plasma membrane, forming a pore with an inner diameter of 10-15 nm and an outer diameter of 32nm, which facilitates the release of caspase-1 substrates, as IL-1β, and other intracellular content that are not direct substrates of caspse-1, as HMBG1 (de Torre-Minguela et al. 2016; X. Liu et al. 2016; Sborgi et al. 2016; Evavold et al. 2017). These pores also disturb the ionic balance of the cell, leading to cell swelling and osmotic lysis. Upon pyroptotic cell death the ASC oligomers are also released to the extracellular space, where they amplify the inflammatory response **(Franklin et al. 2014; Baroja-Mazo et al. 2014)**. Some cells, as neutrophils or monocytes, could release IL-1β withouth pyroptotic cell death **(Diamond et al. 2017)**, however in this situation caspase-1 activation occurs in low levels, but the release is also dependent on GSDMD **(Boucher et al. 2018)**. Therefore, some unknown mechanisms could control the pore formed by GSDMD. GSDMD belongs to the gasdermin family, where in humans is compoused by four members: GSDMA, GSDMB, GSDMC, and GSDMD. It has been shown that caspase-1 is also able to cleave GSDMB and that caspase-3 could cleaveage GSDME, generating in both cases a lytic N-terminus domain (Aglietti et al. 2016; X. Liu et al. 2016; Evavold et al. 2017). Similarly, neutrophils elastase is able to cleavage GSDMD and induce pyroptosis in neutrophils independent of caspase-1 **(Kambara et al. 2018)**. Therefore, the concept of pyroptosis will need to be redefined as gasdermin-mediated cell lysis independently of the processed gasdermin and the protease that is involved in this processing.

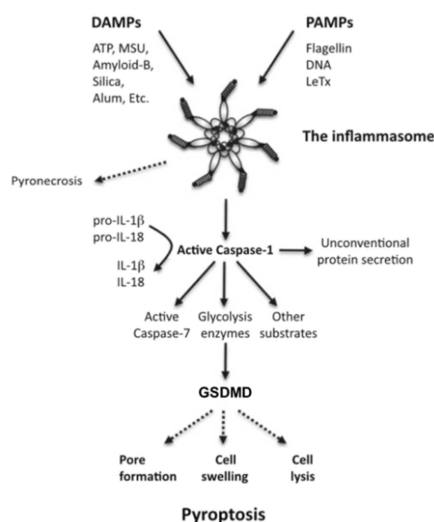


Figure 7. Effector mechanism of inflammasomes after caspase-1 activation (original figure from Labbé; (Labbe 2011)). Once Caspase-1 is activated, caspase-1 process IL-1β and IL-18. However, caspase-1 is able to activate other substrates, induces an unconcentional protein secretion, and inhibit the glycolysis by inactivation of metabolic enzymes. These effecto mechanisms drives to pyroptosis through the pore-forming GSDMD.

2.10. Non-canonical inflammasome

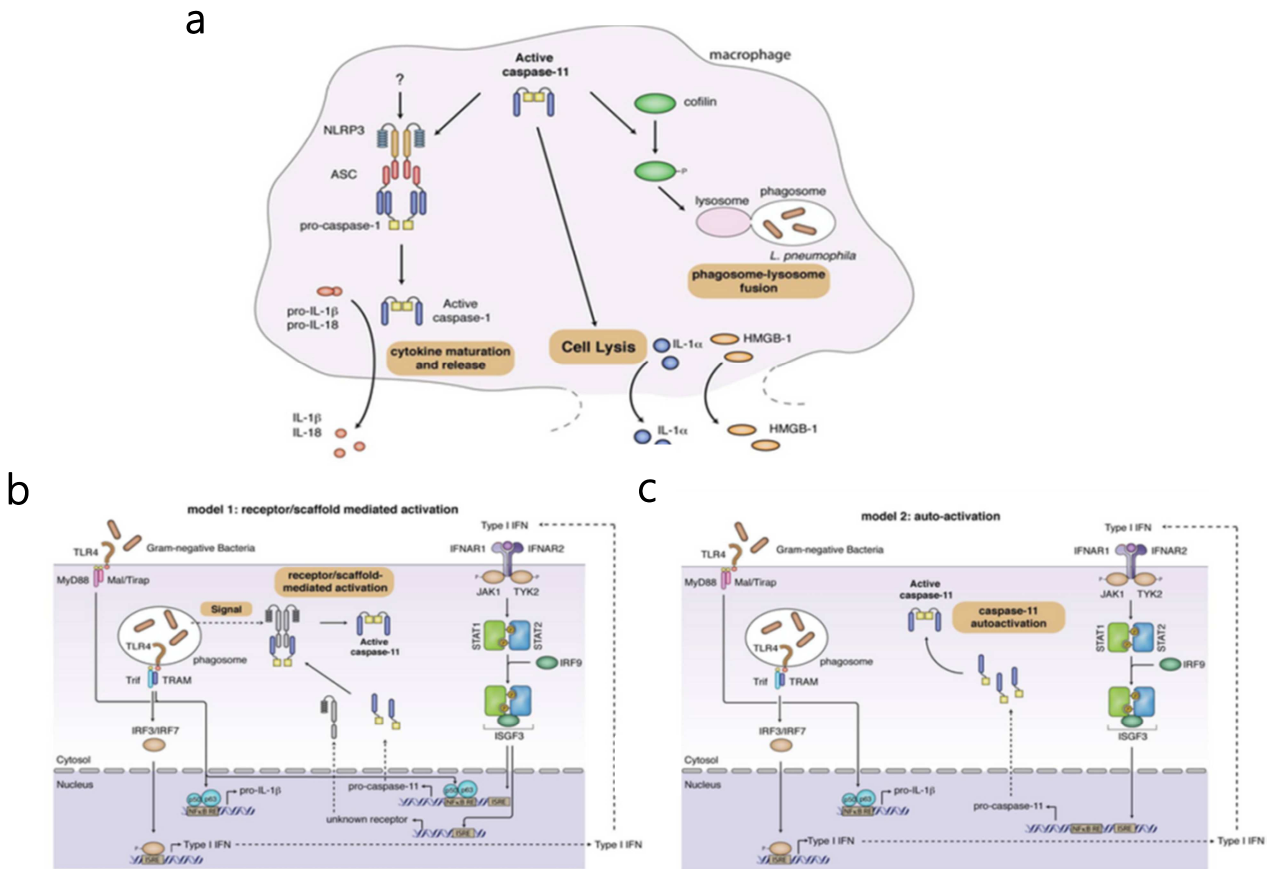


Figure 8. Caspase-11 activation during the non-canonical inflammasome pathway (Broz and Monack 2013). a) Caspase-11 is able to induce HMBG-1 and IL-1α without NLRP3 inflammasome participation. **b)** Caspase-11 activation by receptor-scaffold model. TLR4 cell surface sensing induces the expression of pro-IL-1β, meanwhile phogocyted bacteria induces type I IFN signaling through IRF3 and IRF7. This type I IFN can induce the expression of pro-caspase 11 together assotiated receptors that recognises LPS and form scaffolds to activate caspase-11. **c)** Caspase-11 autoactivation by direct recoginsion of LPS.

The term non-canonical inflammasome was established by Dixit to define the activation of caspase-11 independently of NLRP3 inflammasome as an important immune effector mechanism to control Gram-negative bacteria infections **(Kayagaki et al. 2011; Kayagaki et al. 2015)**. Caspase-11 in mouse or caspase-4/5 in humans are actiated upon intracellular LPS sensing and drive a GSDMD-dependent pyroptotic cell-death associated with intracellular content release, including IL-1α and HMGB1 **(Bodnar and Petrilli 2014).**

However, caspase-11 cannot cleave pro-IL-1β or pro-IL-18, therefore during the initial pyroptotic events drived by caspase-11 there is an actiation of NLRP3 inflammasome leading to the activation of caspase-1, resulting in IL-1β and IL-18 maturation and release (Bodnar and Petrilli 2014; Latz, Ts, and Stutz 2013).

To explain the activation of caspase-11 by intracellular LPS, there are two different models (Figure 8). The first depends on the expression of an unknown intracellular LPS receptors, that upon LPS binding oligomerise and serve as a scaffold for pro-caspase-11 recruitment favoring its autoprocessing. The second model supports that caspase-11 is autoactivated after LPS bining to pro-caspase-1 (Rathinam et al. 2012; Broz and Monack 2013). Caspase-11 could be also activated by different oxydized lipids, as he oxidized phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAC) (Zanoni et al. 2016; Chu et al. 2018), therefore it could be involved in the inflammation associated to metabolic diseases. In humans caspase-4 and -5 are analogues of caspase-11, and meanwhile some studies support that both human caspases could be activated in response to intracellular LPS (Broz and Dixit 2016), other studies suggest that caspase-4 and not caspase-5 is the caspase able to respond to LPS (Kajiwara et al. 2014). These differences could be due because the different cellular models used in both studies.

Other alternative pathways for NLRP3 inflammasome activation have been indicated in monocytes, where LPS recognition is enough to activate NLRP3 inflammasome through the TRIF–RIPK1–FADD pathway, wich ends in the activation of caspase-8 (Gaidt et al. 2016).

3. NLRP3 inflammasome

The NLRP3 inflammasome links the innate and adaptive immune responses in several diseases, including cancer (Ghiringhelli et al. 2009), obesity (Stienstra et al. 2011; Vandanmagsar et al. 2011), gout (Martinon et al. 2006), atherosclerosis (Duewell, Kono, Rayner, Sirois, Vladimer, et al. 2010), arthritis (Muruve et al. 2008), silicosis (Hornung et al. 2008), type 2 diabetes (Juliana et al. 2012) and Alzheimer (Saresella et al. 2016; Heneka et al. 2013). For this reason, NLRP3 is activated by a wide range of signalling and is not only present in cells from innate immune system, but also is present in different cells such as keratinocytes from connective tissue, synovial cells or astrocytes (Yazdi, Drexler, and Tschopp 2010). Mutations in NLRP3 also result in deregulated inflammasome activity

present in several autoinflammatory diseases, including the different types of Cryopirin Associated Periodic Syndromes (CAPS) **(Hoffman et al. 2001)**.

3.1. Structure and components

NLRP3 is a protein of 1016 amino acids encoded by the gene *CIAS1*, which is located on human chromosome 1q44 and consists of 9 coding exons **(Lamkanfi and Dixit 2009).** NLRP3 contains the central NOD domain and a C-terminal LRR similarly to all other NLR family members, and an effector PYD domain at its N-terminal sequence (Figure 9) **(Kanneganti, Lamkanfi, and Núñez 2007)**. The NLRP3 NOD domain has not been crystalized, but due to homology with the NOD domain of NOD2 protein, it has been shown that contain 12 conserved motifs (Figure 9) **(Proell et al. 2008)**. Some of them are similar to ATPases associated with various cellular activities, in particular to the AAA+ superfamily. Therefore, the NLRP3 NOD domain present an ATPase activity that has been shown as important for its activation **(J. P. Y. Ting et al. 2008)**. This ATPase activity resides on the Walker A and P loop defined by the consensus sequence GXXXXGKT/S (being X any amino acid). Walker A domain binds to a sensor 1 motif that recognises the γ-phosphate from ATP. Adjacent to sensor 1, is the sensor 2 motif, that connects with the Walker B hydrophobic motif, that is defined by the consensus sequence HHHHDD/E (being H any hydrophobic amino acid **(Proell et al. 2008)**. Walker A and B motifs take their name in honor after John E. Walker discover them in 1982 **(Walker et al. 1982)**. These motifs are responsible for the hydrolysis of ATP, a process that has been suggested as important for a conformational change after NLRP3 activation **(Proell et al. 2008)**.

The central NOD domain engages with the 12 LRR motifs domain in the C-terminal. LRR domain is characterized by several repetitions with Leucine predominance (from two to 40 repetitions) of L-X-X-L-X-L sequence being X any amino acid **(Virgilio 2007)**. On the other hand, NOD domain is bond at the N-terminus to a PYD domain, to interact with ASC protein by PYD-PYD assotiation (Lamkanfi and Dixit 2009; Bae and Park 2011b).

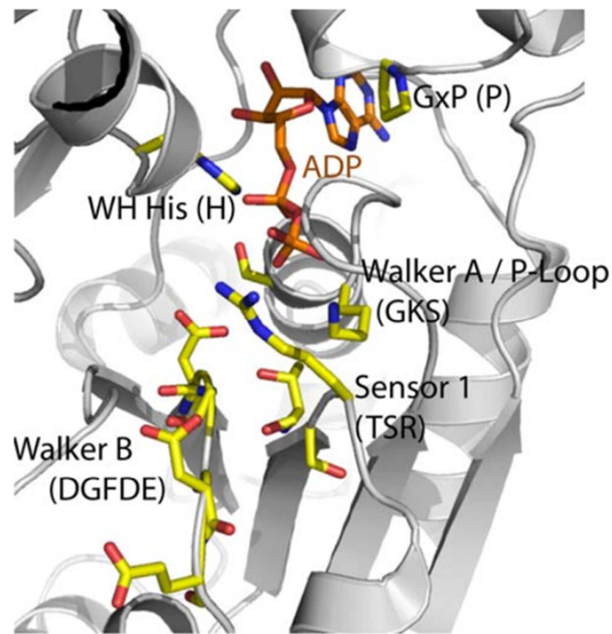


Figure 9. Model of NOD domain in a nucleotide-binding of NOD2 receptor (Proell et al. 2008). ADP is is bond to Walker A motif, allowing a conformational change from inactive to an active conformation.

NLRP3 inflammasome requires a priming process to its following activation an contain multiple phosphorylation sites **(Stutz et al. 2017)**. It is known that JNK is able to phosphorylate the NLR3 inflammasome at S194 (PYD-NOD interdomain), being this inflammasome in an actove conformation that allows its assembly **(Song et al. 2017)**. On the contrary, NLRP3 inflammasome phosphorylation at S5 (PYD), S291 (NOD) or Y861 (LRR) facilitates its ubiquitination and proteasome-mediated degradation, and this process blocks the NLRP3 inflammasome activity **(Spalinger et al. 2016; Stutz et al. 2017)**. It is well known that deubiquitinase enzymes regulate the release of IL-1β secretion and P2X7R-dependent inflammasome activitiy, being as a potential antitherepautic strategy against inflammatory diseases **(López-Castejón and Pelegrín 2012)**.

Upon NLRP3 oligomerization, it recruit ASC by PYD-PYD interactions to form filaments and pro-caspase-1 is then oligomerized by CARD-CARD assotiation **(Creagh, Conroy, and Martin 2003).** NLRP3 active complexes are stabilized by interaction with NEK7 protein **(He et al. 2016).**

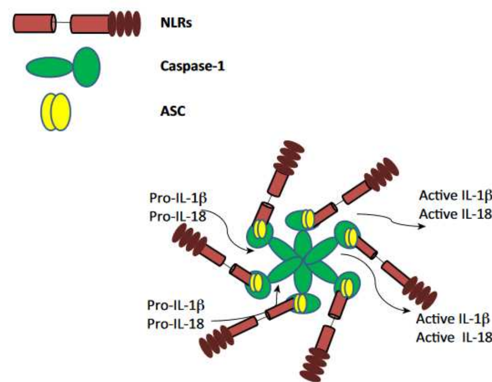


Figure 10. Basal structure of NLRP3 inflammasome formation (Cordero, Williams, and Ryffel 2018).

3.2. NLRP3 activation

Different triggers (PAMPs and DAMPs) activate the NLRP3 inflammasome by largely unknown mechanisms (Broderick et al. 2014), what is known that these activators do not directly binds NLRP3 and they induce different intracellular signalling that is sensed by NLRP3. The most studied danger signal able to activate NLRP3 inflammasome is extracellular ATP, followed by some signals derived from microorganisms such as nigericin the ionophore (Compan et al. 2012). However, it has been described a great variety of activators of the NLRP3 inflammasome, including DAMPs and PAMPs. Between DAMPs and particles than mimic DAMPs that activate the NLRP3 inflammasome we find extracellular ATP; extracellular pH; extracellular increase of Ca^{2+} concentration, that triggers NLRP3 through a G coupled protein; the presence of some ionic particles such as Co^{2+}, Cr^{3+}, Mo^{5+}, Ni^{2+}; hyaluronan; glucose; MSU crystals; amyloid-β peptide; skin irritants; imidazoquinoline compounds (such as imiquimod); hypotonicity; asbestos; silica and alum particles (Kanneganti, Özören, et al. 2006; Mariathasan et al. 2006; Martinon et al. 2006a; Yazdi, Ghoreschi, and Röcken 2007; Caicedo et al. 2009; Cassel et al. 2008; Halle et al. 2008; Hornung et al. 2008; Yamasaki et al. 2009; Zhou et al. 2010; Rossol et al. 2012; Grebe and Latz 2013; Rajamäki et al. 2013). Between PAMPs that activate NLRP3 inflammasome, we find adenovirus, sendai virus, influenza, encephalomyelocarditis virus, japanese encephalitis virus, *Candida albicans*, *Saccharomyces cerevisiae*, *L. monocystogenes*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, bacterial pore-forming toxins, and the nigericin ionophore (Kanneganti, Body-Malapel, et al. 2006; Mariathasan et al. 2006; Duncan et al.

2009; O. Gross et al. 2009; Poeck et al. 2010; Compan et al. 2012; Kaushik et al. 2012). Up next, we are going to describe with detail the most important DAMPs for NLRP3 activation.

3.2.1. P2X7 receptor in NLRP3 inflammasome activation.

P2X7 is a purinergic receptor that is activated by high concentrations of ATP (North et al. 2002), such high concentrations of extracellular ATP are considered as a DAMP as has been previously described. P2X7 is an ion channel with different functions on the immune system (revised in the next section), but importantly it is a strong activator of the NLRP3 inflammasome in different myeloid cells (Di Virgilio et al. 2017). Its activation produce a fast efflux of K^+ from the cell, and this is one of the main mechanisms that links to NLRP3 activation (D. Perregaux and Gabels 1994; Wewers and Sarkar 2009). The pathway that involves extracellular ATP, P2X7 receptor and NLRP3 activation is depicting the mechanism of inflammation induction in different pathologies as cancer, neurodegenerative diseases, osteoporosis, and myocardial ischemia-reperfusion injury, atherosclerosis, rheumathoid arthritis or autoinflammatory diseases such as majeed syndrome (where lipid metabolism is alterated through gain-of-function mutation of the Lipin-2 gene-encoding) (Baroja-Mazo, Barberà-Cremades, and Pelegrín 2013; Herlin et al. 2013; Lordén et al. 2017; Salaro et al. 2016; Albalawi et al. 2017; Kvist et al. 2017; Z. Chen et al. 2018). Of note, although ATP has been extensively used to activate NLRP3, it only function on LPS-primed or TNFa-treated myeloid cells (Wewers and Sarkar 2009; Franchi, Eigenbrod, and Núñez 2009), therefore ATP alone is not enough to activate NLRP3 *in vitro*.

3.2.2. NLRP3 inflammasome activation by crystals and particles

Crystalline molecules are able to activate the NLRP3 inflammasome. Uric acid crystals and calcium pyrophosphate dihydrate cause gout and pseudogout, respectively. No less importantly, crystalline silica and asbestos are responsible for causing the fibrotic lung disorders, silicosis and asbestosis (Pedra, Cassel, and Sutterwala 2009). The first studied crystals as a danger signal were uric acid crystals, which forms MSU (Shi, Evans, and Rock 2003). At high local concentration, uric acid precipitates and forms crystals. This effect induces the release of IL-1β after caspase-1 and NLRP3 inflammasome activation (Eleftheriadis et al. 2013; Gasse et al. 2009), and causes inflammation resulting in gout disease (Gasse et al. 2009), and osteoarthritis (Denoble et al. 2011).

LDL is accumulated in atherosclerotic plaques in form of intracellular cholesterol esters or as extracellular crystals. Macrophages phagocytate these cholesterol crystals, causins a lisosomal damage and resulting in DAMP that activates NLRP3 inflammasome by nuclear factor erythroid 2-related factor 2 (Nrf2) **(Duewell, Kono, Rayner, Sirois, Bauernfeind, et al. 2010; Freigang et al. 2011; Grebe and Latz 2013).**

Particles as silica, alum or CPP crystals are also activators of the NLRP3 inflammasome, therefore the use of alum as adjuvant could explain its effect by activating this pathway **(Hornung et al. 2008; Conway and McCarthy 2018)**. Macrophages treated with silica have been shown to secrete IL-1β **(Cassel et al. 2008)**. Indeed, It has been discovered additional polymorphisms in the IL-1Ra wich have been linked with sensitization to silicosis in humans **(Yucesoy et al. 2001)**. Asbestos is associated with the development of lung cancer and malignant mesothelioma in humans **(Chow et al. 2012)**. As silica, Tschopp and collaborators discovered the Asbestos induce the NLRP3 inflammasome activation **(Dostert et al. 2008).**

Crystals and particles activate the NLRP3 inflammasome after being phagocytosed by myeloid cells **(Grebe and Latz 2013)**. Phagosomes containing crsytals and particles fuse with lysosomes, however the particulate content cannot be degraded by phagolysosomes and this desestabilize their membrane, releasing the content of phagolysosomes to the cytosol. Cathepsin B was thought to be that responsible for NLRP3 activaton, since inhibitors of cathepsin B were able to abolish NLRP3 activation in response to crystals **(Orlowski; Gregory et al. 2016)**. However, later experiments with macrophages from cathepsin B deficient mice demonstrated that cathepsin B was not involved in NLRP3 activation and was found disruption of lysosomes induces an efflux of K^+ from the cytosol that was responsible to activate the NLRP3 **(Muñoz-Planillo et al. 2013).** However, it is not known the mechanism on how K^+ escape from the cell.

3.2.3. NLRP3 activation by antimicrobial peptides

Antimicrobial peptides (AMPs) are effector molecules of the innate immune response of all pluricellular organisms, needed for the response against bacterial infections (Pfalzgraff et al. 2016; M L Mangoni 2006). AMPs could be produced as a consecuence of

cytokine activation mediated by bacterial LPS **(Maria Luisa Mangoni et al. 2008)** or are constituyents of venoms **(M L Mangoni 2006)**. Usually AMPs are small peptides of 12–50 amino acids that affect cells by disrupting the barrier function of lipid membranes, although AMPs disrupt prokaryotic walls, they could also lysate eukaryotic plasma membrane **(Zasloff 2002; Ginsburg, van Heerden, and Koren 2017)**.

In mammals, AMPs are present in granulocytes, skin and in the mucous membranes of the gastrointestinal, genitourinary, and respiratory tracts, where they can lisate invading bacteria and also trigger an inflammatory response **(M L Mangoni 2006)**. The two main anti-microbial peptide families in mammals are the defensins and cathelicidins, which are produced by phaothgocytes and epithelial cells **(Yeretssian, Labbé, and Saleh 2008)**. Between cathelicidins, we found LL-37 **(K. L. Brown et al. 2011)**. Further studies has been demonstrated that LL-37 is able to activate the NLRP3 inflammasome **(Elssner et al. 2004; Kahlenberg and Kaplan 2013)** or indolicidin, which was purified from the cytoplasmic granules of bovine neutrophils **(Halevy et al. 2003)**. In amphibians there is a large variety of different AMPs, including temporins from *Rana temporaria*, magainins from *Xenopus laevis*, buforins from *Bufo bufo gargarizans*, bombinins from *Bombina* genus or dermaseptins from *Phyllomedusa sauvagii* **(Q. Chen et al. 2004)**.

AMPs contain a cluster of cationic and hydrophobic aminoacids of different structural classes **(Zasloff 2002)**. Most of them reveals a similar organization, with a cationic ring structure and a hydrophobic domain **(Clark et al. 1994)**. This structure with an hydrophobic tail is the responsible for peptide-membrane attraction and pore formation **(Hsu and Yip 2007)**

Melittin, an anti-microbial peptide, is the major constituent of the European honeybee *Apis mellifera* venom **(van den Bogaart et al. 2008)**. This AMP is a 26-residue linear peptide: GIGAVLKVLTTGLPALISWIKRKRQQ-NH2 **(Habermann and Kowallek 1967)**. Melittin is composed structurally by a bent α-helical rod. Both the primary and secondary structures are similar to many other antimicrobial peptides **(Yang et al. 2001)**. However, Melittin pore forming was discussed between two differents forms: the barrel slive described by Vogel and Jahnig in 1986 **(Vogel and Jähnig 1986)** and the younger toroidal model **(Yang et al. 2001)**. Nowadays, it is believed that melittin adapts the membrane comformation depending on peptide concentration: a low concentration of melittin binds

to the membrane and forms an amphipatic α-helix oriented in parallel to the membrane. Increase of melittin concentration, α-helix inserted in perpendicular, which cause the pore formation **(van den Bogaart et al. 2008)**. Melittin is able to insert into the plasma membrane of eukaryote cells and form pores. In macrophages these pores have been related with K^+ efflux and NLRP3 inflammasome and caspase-1 activation. Therefore, it is postulated that the NLRP3 inflammasome could be a player on the inflammatory response to venoms **(Palm and Medzhitov 2013)**.

3.2.4. Infections and microbial products activating the NLRP3 inflammasome

NLRP3 inflammasome activation could also be induced by different microbial components that will affect plasma membrane, either by permeabilization (similar to melittin effect) or by inducing a specific flux of K^+ ions. Among them we could found the pore-forming toxins maitotoxin from the marine dinoflagellate *Gambierdiscus toxicus*, listeriolysin O from *L. monocytogenes*, aerolysin from *Aeromonas hydrophila*, *Mycobacterium tuberculosis* pore forming toxin and *S. aureus* α-hemolysins **(Pedra, Cassel, and Sutterwala 2009)**. Some of these poreforming toxins are able to induce necrosis together with NLRP3 inflammasome activation **(Craven et al. 2009)**, and their primary mode of action is the induction of K^+ efflux.

There are other microbial specific K^+ ionophores that specifically induces a efflux of K^+, these includes the antibiotics nigericin or valinomycin from different *Streptomyces* strains **(Próchnicki, Mangan, and Latz 2016).** Nigericin is widely used as NLRP3 activator in *in vitro* assays. Nigericin is a K^+/H^+ ionophore that at high extracellular Na^+ conditions is also able to transport Na^+ **(Rodríguez and Sitges 1996)** Nigericin activates the NLRP3 inflammasome by decreasing intracellular K^+.

3.2.5. NLRP3 activating cascade

A decrease in the intracellular concentration of K^+ seems the key common step for all activators for NLRP3 in LPS-primed macrophages (D. Perregaux and Gabels 1994; Warny et al. 1999; Vyleta, Wong, and Magun 2012; Muñoz-Planillo et al. 2013). In fact, this was already described before NLRP3 inflammasome discovery by Chritopher Gabel group from Pfizer in 1994, where they described that an efflux of K^+ was necessary for IL-1β processing

and release (D. Perregaux and Gabels 1994). However, up to date is not known how K^+ regulates NLRP3.

Besides K^+ efflux, there is another common mechanism for NLRP3 activation mitochondrial damage (Schroder and Tschopp 2010; S.-B. Liu, Mi, and Wang 2013). In fact, imiquimod, is able to activate NLRP3 in the absence of K^+ efflux but inducing mitochondrial damage (C. J. Gross et al. 2016). Intracellular Ca^{2+} increase damage the mitochondria, and Ca^{2+} has been suggested as second messenger in the pathway of NLRP3 activation (Lee et al. 2012; Rossol et al. 2012; Murakami et al. 2012), however recently there are controversies if Ca^{2+} is important for NLRP3 activation (Katsnelson et al. 2015). Mitochondrial damage could also be induced by K^+ efflux and is related with mitochondrial ROS production (Saïd-Sadier and Ojcius 2012). ROS are highly reactive molecules formed from molecular oxygen, such as H_2O_2, superoxide radical ($O^{2\bullet-}$), hydroxyl radical ($OH^\bullet$), and some reactive nitrogen species, such as peroxynitrite ($ONOO^-$) (Azad and Tomar 2014). ROS and more specific, mitochondrial ROS, have been also described to activate the NLRP3 inflammasome (Zhou et al. 2011) via specific interaction of NLRP3 with the thioredoxin-interacting protein (TXNIP) (S.-B. Liu, Mi, and Wang 2013). Mitochondrial ROS have been also involved in NLRP3 activation by oxydizing mitochondrial DNA, and this DNA was suggested to activate NLRP3 (Shimada et al. 2012). In support that mitochondrial ROS production, as well as the release of oxidized mitochondrial DNA, could be responsible to activate the NLRP3, NLRP3 activation has been found to be regulated by mitophagy (Kim, Yoon, and Ryu 2016). However, defects on the glycolysis have been also linked to NLRP3 (Moon et al. 2015). In summary, depending the laboratory, different intracellular pathways have been proposed to activate NLRP3, however no consensus have been reached and we still do not understand how NLRP3 is activated.

3.3. Development of NLRP3 inhibitors

Different inhibitors of the NLRP3 inflammasome pathway have been described to date. Some of them acting downstream the inflammasome and for example biologic blocking IL-1 signaling are actually used in clinics to treat auto-inflammatory syndromes. However, direct NLRP3 inhibitors are been developed with the aim to use them as potent anti-inflammatory compounds to treat chronic inflammation, metabolic disases and degenerative diseases (Perera, Kunde, and Eri 2017; Jiang et al. 2017). Among them we could

cite glyburide (glibenclamide), b-hydroxybutyrate, MCC950, Cy9, NBC series and INF series **(D. G. Perregaux et al. 2001; Latz, Ts, and Stutz 2013; Coll et al. 2015; Haitao Guo, Justin B. Callaway 2015; Baldwin, Brough, and Freeman 2015; Baldwin et al. 2017; Jiang et al. 2017).** Glyburide was described at first **(Cocco et al. 2014)**; me as a IL-1β maturation blocking agent, but it has been shown that inhibit specificly upstream the NLRP3 inflammasome pathway by blocking NLRP3 deubiquitination **(López-Castejón and Pelegrín 2012)**. From the newly developed compounds, INF39E and Cy9 block NLRP3 ATPase activity, therefore their specificity blocking other ATPases must be confirmed before traslating them into clinics. Of note, there are also some compounds that block execution of pyroptosis without inhibiting NLRP3 inflammasome activation and maturation of IL-1β, such as the complex polyphenolic compound punicalagin **(Martín-sánchez et al. 2016)**.

3.4. NLRP3 mutations and auto inflammatory syndromes

Dysregulation of the NLRP3 inflammasome has been linked to a variety of autoinflammatory disorders known as CAPS **(Pedra, Cassel, and Sutterwala 2009)**. CAPS present different clinical manifestations depending on their severity, the most severe chronic infantile, neurological, cutaneous, and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID); the intermediate Muckle–Wells syndrome (MWS); and the low severe familial cold autoinflammatory syndrome (FCAS). CAPS are associated to single mutations in different amino acid of the NOD domain of NLRP3 **(C. a Dinarello 2009; Bae and Park 2011a)**. There are described over 200 gain-of-function mutations in NLRP3 associated to CAPS **(de Menthière et al. 2003; Lamkanfi and Kanneganti 2010; Rowczenio et al. 2017).**

There are other auto-inflammatory syndromes that do not present mutations in NLRP3, but in other genes that could regulate NLRP3. $Ca2^{+}$ and cyclic adenosine 5´ phosphate (cAMP) are two key molecular regulators of the NLRP3 inflammasome that have critical roles in the molecular pathogenesis of CAPS **(Lee et al. 2012)**. There is also mutations in the gene encoding for phospholipase Cγ2 (PLCG2) that cause an auto-inflammatory syndrome with PLCG2-associated antibody deficiency and immune dysregulation (APLAID). This mutation enhances PLCG2 activity and causes an increase in intracellular Ca^{2+} released from the endoplasmic reticulum that activates NLRP3 inflammasome **(Chae et al. 2015)**.

4. Purinergic signaling

The concept of purinergic signalling emerge in the early ‘80s by Geoff Bursntok as a system that use purine nucleotides and nucleosides as extracellular signals. This concept was then reinforced with the cloning of purinergic receptors in the early ‘90s. In a steady state, intracellular ATP concentrations oscillates from 3 to 10 mM, whereas extracellular ATP is tightly controlled and low concentrations are found. Extracellular ATP and other nucleotides are sensed by purinoreceptors **(Mortaz et al. 2009; Burnstock 2006)**. ATP and other nucleotides modulate the function of inflammatory cells, such as neutrophils, macrophages, T cells, and DCs **(Cicko et al. 2010; Zanin et al. 2012)**.

Purinoreceptors are classified as P1 or adenosine receptors and P2 receptors (P2R). Between P2Rs we can separate P2X receptors (P2XRs) and P2YRs. P2YRs are seven-membrane-spanning G-protein-coupled receptors. Eight members of this subfamily are discrived in humans: P2YR P2Y1R, P2Y2R, P2Y4R, P2Y6R, P2Y11R, P2Y12R, P2Y13R and P2Y14R **(Myrtek and Idzko 2007; Stokes and Surprenant 2007; Cicko et al. 2010)**. Activation of some P2YRs, such as P2Y1R, P2Y2R, P2Y4R, P2Y6R, P2Y11R, and P2Y14R, induces PLC activation, inositol triphosphate (IP3) generation, and Ca^{2+} release from the endoplasmic reticulum through Gq protein, which could regulate among others cytokine release **(Burnstock 2006; Stokes and Surprenant 2007; Myrtek and Idzko 2007).** Alternatively, P2Y12R and P2Y13R bind to a G0 protein, and its activation inhibits the adenylate cyclase system **(Burnstock 2006).** P2XRs family has seven members from P2X1R to P2X7R. These receptors are ligand-gated ion channels assembled as homotrimers or heterotrimers **(Stokes and Surprenant 2007; Cicko et al. 2010; Marques-da-Silva et al. 2010)**.

Not only ATP but also other nucleotides such as ADP and UTP can be sensed by P2Rs to generate a fine-tuned response **(Riteau et al. 2012).** ATP and UTP induce actin polymerisation and chemotaxis in human neutrophils via the activation of P2Y2R **(Verghese, Kneisler, and Boucheron 1996).** In neuronal transmission, ATP is recognised by smooth muscle cells using P2X1R and in some vessels by P2X2R, P2X4R, and P2Y2R receptors, resulting in vasoconstriction. On the contrary, adenosine receptor activation mediate vasodilatation. P2X3R is present in some neuronal terminals and is important mediating pain sensation **(Cockayne et al. 2000; Burnstock 2006)**. Platelets express P2Y1R and P2Y12R that recognise ADP, as well as P2X1R **(Hollopeter et al. 2001)**, whereas immune cells

(including lymphoid and myeloid cells) express P2X7R, as well as P2X1R, P2X4R, P2Y1R, and P2X2R receptors **(Luttikhuizen et al. 2004; Burnstock 2006)**.

Due to the relevance of P2X7R in this Thesis, we will describe the known functions of this receptor. The P2X7R was first characterized in immune cells as the P2ZR **(Di Virgilio 1995)**, due to since upon stimulation it allow the passage of high molecular weigth dyes and this was different from the already described P2YR and P2XR. Later on, after cloining, it was found that was a member of the P2XR family **(Surprenant et al. 1996).** P2X7R plays a significant role in the immune innate response **(Roger et al. 2010)**. However, human monocytes express around four times more the levels of P2X7R present in lymphocytes when analyzed by flow cytometry **(Aswad and Dennert 2007; Wewers and Sarkar 2009).** P2X7R contains two transmembrane regions, a cysteine-rich extracellular loop and intracellular N-terminal and C-termianl regions. The C-terminal region is considerable larger than other P2XR, consisting of 200 aminoacids and is essential for the opening of large pores **(Tsukimoto et al. 2006).** This monomeric structure forms a trimeric channels with three binding sites for ATP **(Yan et al. 2010)**.

P2XR are cationic channels unspecific for Na^+, Ca^{2+} and K^+, as well as other cations. For this reason, after activation P2X7R induces membrane depolarization by large K^+, which activates the NLRP3 inflammasome in LPS-primed or M1 macrophages **(Pelegrín 2011; Roger, Pelegrin, and Surprenant 2008; Weber et al. 2010)**. However, in resting or M2 macrophages, P2X7 signaling uncoupled from NLRP3 inflammasome, but is associated to other signalling, as activation of metalloproteases or release of annexin A1, postulating that P2X7R it could play a role during the resolution of the inflammation **(de Torre-Minguela et al. 2016).**

Prolonged activation of P2X7R produce the opening of large conductances from the cell, this could be done upon association to accessory proteins (as pannexin-1) or by direct P2X7R pore dilation **(Pelegrín 2011)**. Pannexin (Panx) channels were originally identified in 2003 as a family of prteins with homology to the conexins gap junctions **(Bruzzone et al. 2003; Ma et al. 2009)**. There are three members termed Panx1–3. One of them, Panx1 is able to act as an hemichannel that allows the traffic of large molecules, as a consecuence of P2X7R activation **(Ma et al. 2009; Bryant and Fitzgerald 2009)**. Panx1 is also associated to the

rapid activation of the NLRP3 inflammasome **(Pelegrin and Surprenant 2006; Pelegrin and Surprenant 2007; Pelegrin and Surprenant 2009).**

P2XR are specificly adapted to sense determinated ATP levels depending of location. For example, P2X2 and P2X3 receptors are ion channels present on sensory neurons, and only require 1 µM extracellular ATP to activate, and respond against neuronal damage. However, P2X7Rs are located predominantly on immune cells where they need a higher extracellular ATP concentrations (up to 1 mM) to be activated **(Keating et al. 2011; Burnstock 2006)**

Extracellular ATP signalling depends on the enzymatic conversion of ATP to ADP due to action of different ectonucleotidases, as the ectonucleoside triphosphate diphosphohydrolase or CD39 that convert ATP or ADP to AMP, and the ecto-nucleotidase CD73 that converse AMP to adenosine. This conversions establish a balance between P2XR, P2YR and P1R signaling. Menawhile P2XR and P2YR activation has been linked to pro-inflammatory conditions, P1R signalling by adenosine has been shown to supresses the inflammatory response **(Idzko et al. 2007; Burnstock 2006)**. These conversions adapt extracellular ATP concentrations and turned different purinoceptors in controlling different aspects of the immune system, as T cell functions: 1 to 50 nM extracellular ATP (normal conditions) do not affect Th1 cells and Tregs. However, 250 nM ATP stimulates proliferation, cytokine release, and adhesion of Th cells. 1 mM or higher concentrations of extracelluar ATP activates P2X7R and induces apoptosis of Th1 cells, inhibiting the immunosuppressive activity of Treg cells **(Trabanelli et al. 2012).**

P2X7R actovation in naive T cells and NK cells is susceptible to ADP-ribosylation mediated by the enzyme ADP-ribosyltransferase 2 (ART-2). This enzyme uses NAD^+ as a cofactor and allows covalentely (and irreversible) gating of P2X7R (Adriouch et al. 2012; Kawamura et al. 2006; Björn Rissiek et al. 2015; Haag et al. 2007). However, the immunosuppressive role of Tregs is reduced by NAD^+ gating, that is accompanied of metalloprotease (ADAM) activation (Figure 11). ADAM induces cell death by shedding CD27 and CD62L from T cells **(Adriouch et al. 2012)**, which are important markers for adhesion to endothelium and migration to infection site **(Sengstake, Boneberg, and Illges 2006)**.

P2X7R is involved in other inflammasome-independent pathways, such as the release of prostaglandin E2 (PGE2) **(Barberà-Cremades et al. 2012)** and cathepsins from macrophages **(Lopez-Castejon et al. 2010)**, or impairment of APCs mediated by MHC-class I **(Baroja-Mazo, Barberà-Cremades, and Pelegrín 2013)**. P2X7R activity can be blocked by specifc inhibitors such as oxidized ATP (oATP) **(Wewers and Sarkar 2009),** however it is known that oATP is also able to block other P2Rs. Therefore, different pharmaceutical companies have developed more specific P2X7R antagonists, including AztraZeeneca (as AZ10606120), Abbott (as A438079 or A740003 or Pfizer (CE-224,535) **(Yan et al. 2010; Amoroso et al. 2015; STOCK et al. 2012)**. On the contrary, a high affinity P2X7R agonist was identifiyed with a higher activity than ATP, 2'(3')-O-(4-Benzoylbenzoyl)-ATP (BzATP) **(Donnelly-Roberts et al. 2009)**. P2X7R activation can be also modulated by specific-single domain antibodies fragments (VHHs) or nanobodies **(Danquah et al. 2016)**. Nanobodies were identifiyed from camelidae and are single heavy-chain antibodies from the IgG class **(Arbabi Ghahroudi et al. 1997; Maass et al. 2007).** These nanobodies also were discovered in sharks, (termed VNAR) **(Wesolowski et al. 2009)**. Nanobodies are formed by complementary determining regions (CDR 1,2,3) and CDR3 is the region that posseses the ability to recognises antigens due to its finger-like extensions **(Wesolowski et al. 2009; BjÃ¶rn Rissiek, Koch-Nolte, and Magnus 2014)**. The nanobodies that modulate P2X7R activity has been found to block or potentiate P2X7R function. This strategy opens new possibilities to use P2X7R as a therapeuthic target to treat different inflammatory diseases **(Danquah et al. 2016)**.

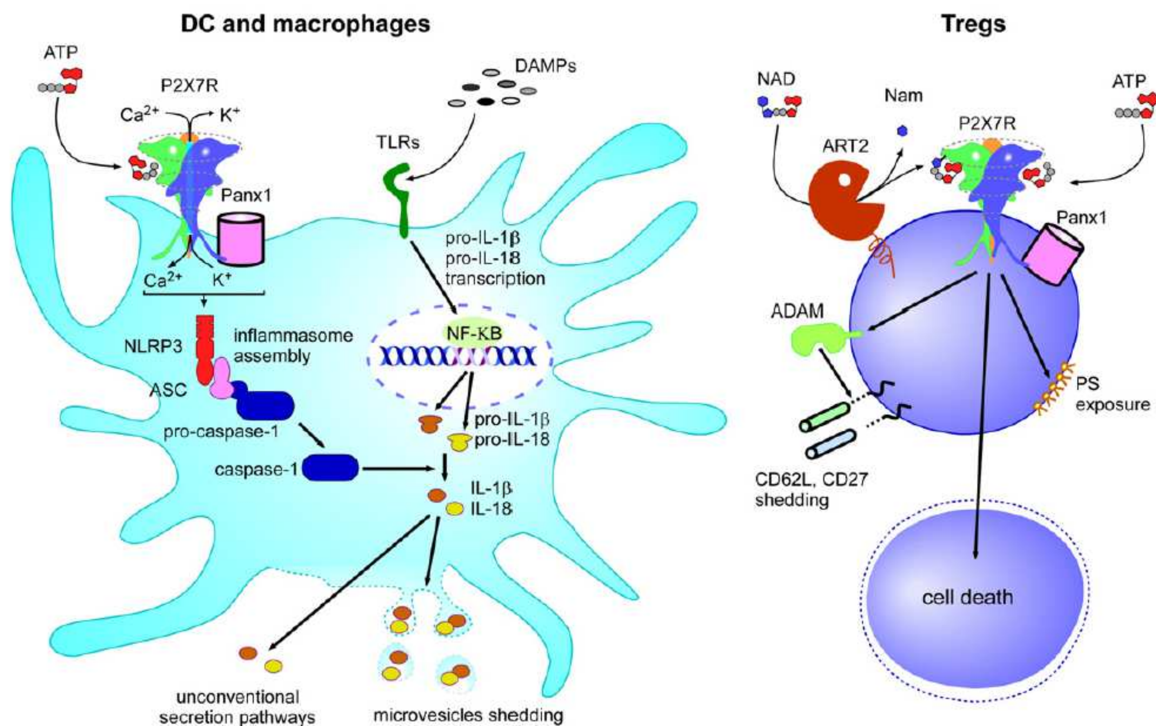


Figure 11. Differential role of P2X7R in inflammation in myeloid cells and Treg cells (Adriouch et al. 2012). In LPS-primed DCs and macrophages P2X7 is a pro-inflammatory factor, which activates NLRP3 inflammasome and induces the release of pro-inflammatory cytokines mediated by NLRPe inflammasome activation. On the contrary, in Treg cells, the pro-inflammatory role of P2X7R consists in the activation of induced cell death mediated by ADAM metalloprotease that cleavages CD62L and CD27 from cell surface.

5. Sepsis

5.1. Definition of Sepsis

Sepsis is the leading cause of mortality in the intensive care units of hospitals in both the U.S. and Europe **(Bar-Or et al. 2018).** However, there is no an effective treatment for sepsis because it induces a variety of abnormal changes in cells, tissues, circulatory, metabolic and immune system, which are not well understood (Y. Guo, Patil, et al. 2017).

The definition of sepsis changed in 2016 due to the increase of knowledge about disease **(Banerjee and Levy 2017)**. The word "sepsis" was first used over 2000 years ago [σηψις] in ancient Greek literature to describe death of organic material **(Geroulanos and Douka 2006)**. The concept of "sepsis syndrome" came from the systemic inflammatory response syndrome (SIRS). SIRS was first described by Bone in 1989. Bone defined it as "a systemic response to a suspected or documented infection and at least one organ dysfunction", whose symptoms are hypothermia or hyperthermia, tachycardia, tachypnea, infection, and organ dysfunction due to tissue hypoperfusion **(Bone et al. 1989)**. In 1991, the International Consensus Conference defined the concept of severe sepsis including two of the following criteria for SIRS: increase of respiratory rate, low arterial pressure of CO_2, increased heart rate, abnormal temperature, and lower or higher leucocytes in blood than normal rate **(ACCP 1992)**. Between 1991 and 2001 SIRS was acknowledged as a "systemic activation of the innate immune response, regardless of the cause". The change of definition was due to systemic chronic inflammatory response occurs in a large variety of disorders such as reumathoid arthritis, type 2 diabetes and cardiovascular diseases **(Medzhitov 2008)**. However, since 2001, SIRS meaning was adapted to sepsis symptoms: Sepsis was a SIRS that occurs during an infection, and organ dysfunction was included between criteria to explain severe sepsis **(Levy et al. 2003)**. In 2010, it was created a "public definition" and a "molecular definition":

-Public definition of sepsis: Sepsis is a life-threatening condition that arises when the body's response to an infection injures its own tissues and organs. Sepsis leads to shock, multiple organ failure, and death, especially if not recognized early and treated promptly.

-Molecular definition of sepsis: Host-derived molecules and foreign products of infection converge on molecular mechanisms that cause unbalanced activation of innate immunity. Foreign and endogenous molecules interact with pathogen recognition receptors expressed on cells of the immune system. Activation of pathogen recognition receptors culminates in the release of immune mediators that produce the clinical signs and symptoms of sepsis (Banerjee and Levy 2017; Czura 2011).

In 2016, sepsis definition was readapted by the Society for Critical Care Medicine and the European Society of Intensive Care Medicine from the severe sepsis concept. Sepsis is an infection with organ dysfunction, revealed by a Sequential Organ Failure Assessment (SOFA) score higher than 2, associated with an hospital mortality of 10%. Severe sepsis definition "per-sé" disappeared. Septic shock was then stablished when serum lactate was higher than 2 mmol/l, and a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg, wich envolves higher mortality than 40% in hospitals (Banerjee and Levy 2017; Brealey et al. 2002; Y. Guo, Patil, et al. 2017).

5.2. Physiopathology of Sepsis

Sepsis is physiologically induced by infections, but it can be induced by administration of a high amount of PAMPs that developes a systemic 'cytokine storm' as the release of different pro-inflammatory cytokines in the blood, as occurs in a SIRS (Ward 2012). One of the most studied inducers sepsis-like response is LPS from gram negative bacteria, in that case LPS induces an endotoxic shock (Maria Luisa Mangoni and Shai 2009).

Although systemic inflammation could be a common response in several pathologies including sepsis, trauma, burns, as well as during major surgery (Kiers et al. 2017), sepsis contains some specific particularities. Tradicionally, the host immune response in sepsis consists in an initial hyperinflammatory phase of few days, and then it continues with an immunosuppressive phase (Figure 10). Nevertheless, recent studies have shown that both pro-inflammatory and anti-inflammatory responses in sepsis can occur at the same time. However, we can observe that earlier hyper-inflammatory response is predominant upon anti-inflammatory response. In the late phase of sepsis this overlapping disappears and only remains the anti-inflammatory response, and a prolonged state of immunosuppression. This imbalance between pro-inflammatory and anti-inflammatory

response is the cause of tissue damage, multiple organ dysfunction and death (Glaros, Larsen, and Li 2009; Schulte, Bernhagen, and Bucala 2013; Richard S Hotchkiss, Monneret, and Payen 2013). Cytokines release induce endothelial dysfunction, vasodilatation and increase of capilar permeability assotiated with hypotension and edema **(Schulte, Bernhagen, and Bucala 2013)**. Hyponatremia is present in sepsis and is associated with morbidity and mortality **(Hannon and Boston 1990)**. Hypertonic solutions can regulate the immune system by favouring an anti-inflammatory effect that could decrease the early response to sepsis and reduce the multiple organ failure **(Wade 2002)**. In fact hypertonic solutions have been found to inhibit the NLRP3 inflammasome **(Compan et al. 2012)**. Other important factor in sepsis is oxygen deficiency that drives multiple organ dysfunction. (Y. Guo, Patil, et al. 2017).

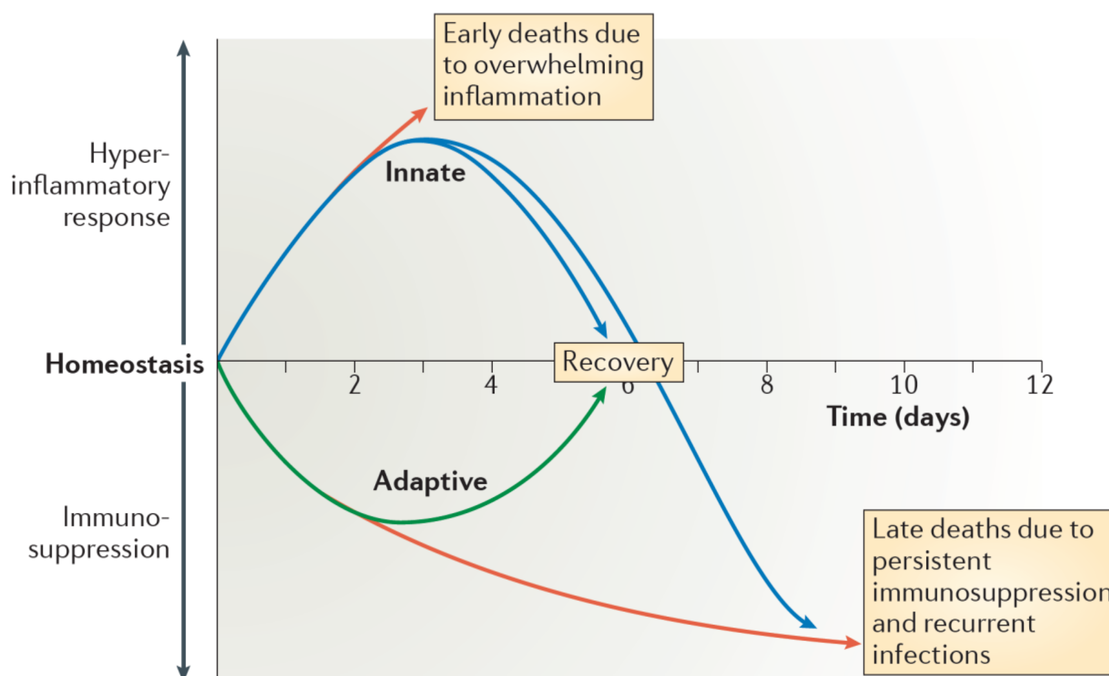


Figure 12. Most accepted course of sepsis (Richard S Hotchkiss, Monneret, and Payen 2013). Imbalance between pro-inflammatory and anti-inflammatory response is developed at early state of sepsis, giving to an hyper-inflammatory response, and causing death by "cytokine storm". At late state of sepsis, anti-inflammatory response is prolongued and the inflammatory factors disappear, causing death by immunosupresion.

The initial 'cytokine storm' in sepsis consists in an excessive production of pro-inflammatory cytokines including IL-6, TNF-α, IL-1β, IFN-γ, IL-8 and IL-12. This cytokine

storm induces an acute response by APPs and promotes leucocyte migration and a variety of pro-inflammatory factors including histamine, platelet-activating factor, bradykinin, nitric oxide, ROS, prostaglandins and complement components (Póvoa 2002; Y. Guo, Patil, et al. 2017; Schulte, Bernhagen, and Bucala 2013). In response to expression of these pro-inflammatory mediatos, sepsis also promotes changes in the expression of adhesion molecules on both endothelial cells and neutrophils surface (specially of β1 and β2 integrins levels) (Lerman et al. 2014). It is thought that this process in neutrophils is mediated by IL-33 production (Alves-Filho et al. 2010) as well as IL-33 is able to induce M2 polarization of macrophages (Nascimiento et al. 2017). The participation of neutrophils in sepsis decrease during the time, and levels of these cells decrease (Groeneveld et al. 2017).

Peripheral blood mononuclear cells (PBMCs) plays an important role during the different phases of sepsis. In sepsis, Th1 and M1 responses in T cells and DCs is characterized by the increase of increase of the MHC-class II expression and IL-12 release (Scumpia et al. 2005; Wen et al. 2006). MHC-class II signaling also is reducing at the same time with IL-10 release and Th2 response induction in T cells (Scumpia et al. 2005; Bhan et al. 2016b). During the late state of immunosuppression, T cell functions are arrested without production of pro-inflammatory neither anti-inflammatory factors causing anergy (Boomer et al. 2012; Richard S Hotchkiss and Karl 2003). Hotchkiss and colleagues revealed that Th cells decrease in sepsis, but Tc levels does not present any change (R S Hotchkiss et al. 2001), causing a T cell exhaustion state (Spec et al. 2016). Tc cells induce physiological dysfunction and systemic inflammation (C. Guo et al. 2016). Meanwhile in this state, myeloid and plasmacytoid DCs suffer apoptosis (Bhan et al. 2016b). The activity of NK cells against infection occurs in early phase of sepsis, activating the immune response by producing IFN-γ (Bhan et al. 2016b; Y. Guo, Patil, et al. 2017). Later, NK cells could initiate the immunosuppressive phase together Tc cells (Bhan et al. 2016b). It is known that levels of the anti-inflammatory cytokine IL-10 are increased in patients with sepsis and this increase has been proposed to predict mortality (Richard S Hotchkiss and Karl 2003). During sepsis, NKT cells participate modulating the host responses by producing IL-4 that polarizes the Th cells toward a Th2 response (Bhan et al. 2016b). However, there is a controversy in the role of NKT cells in sepsis. Other studies have been demonstrated that NKT cells contribute to organ injury by promoting neutrophil recruitment due to IFN-γ production (Li et al. 2007). Tregs also play an important role in sepsis due to its capacity to induce immunesupression and Treg increase during sepsis (Wisnoski et al. 2007). On the contrary, Th and B cells

decrease during immunosupresive phase of sepsis (R S Hotchkiss et al. 2001). During sepsis, monocytes are able to transit from a pro-inflammatory to an anti-inflammatory state, due to the increase of Th2 and Treg during immunosupression (Shalova et al. 2015; Cavaillon and Adib-Conquy 2005).

There is a cause-effect relationship between redox imbalance and SIRS responses during sepsis (Bosmann and Ward 2013). This is related to the observation that Otto Warburg made in cancer cells, where glycolysis was the main method to generate ATP (Warburg and Wind 1926; Bar-Or et al. 2018). This effect was then called the Warburg effect and indicates that cancer cells leads to an anaerobic metabolism, in comparison with non-cancer cells, that obtain ATP through the tricarboxylic acid cycle (TCA). Pyruvate dehydrogenase (PDC) is activated by dephosphorylation. Active PDC catalyse pyruvate into acetyl-coenzyme A reaction. Acetyl-CoA enters into the TCA cycle, but if PDC is inactive, pyruvate is converted to lactate by oxidative phosphorylation. This effect could activate the NLRP3 inflammasome (Bar-Or et al. 2018). In septic monocytes, there is a change to aerobic glycolisis due to the Warburg effect and also there are defects in the mitochondrial metabolism (Cheng et al. 2016). Lactate production is upregulated by hipoxia inducible factor-1α (HIF-1α) (Arts et al. 2016). Therefore, durign sepsis there is an impairment at the mitochondrial level, and organ dysfunction could partially be explained due to mitochondrial dysfunction (Cinel and Opal 2009).

Immunosuppresion during sepsis is also related to an extenuation to PAMPs, and Opal in 2002 defined LPS tolerance or endotoxin-tolerance as "the phenomenon where pre-exposure to LPS induces a reduced sensitivity to a subsequent challenge of LPS". (Opal and Huber 2002). This phenomenom is associated with a decrease of the TLR4 response (Opal and Huber 2002; O'Neill, Golenbock, and Bowie 2013). Cubillos-Zapata and colleagues indicated that NFκB and HIF-1α could also be a key regulator of endotoxin tolerance in monocytes (Cubillos-Zapata et al. 2014). As a result of this endotoxin tolerance, monocytes/macrophages polarize from M1 to M2 state (Bhan et al. 2016b).

To study the pathophysiology mechanisms of sepsis, different animal models have been developed, mainly in rodents, but also in other animals such as rabbits and non-human primates (Ward 2012). Some of these models include intravenous (iv) or intraperitoneal (ip) administration of LPS in mice, that develops similar symptoms than human sepsis, such as hemathological features and cytokine storm in serum (Rittirsch, Hoesel, and Ward 2006).

Nevertheless, there are some differences betweein these sepsis animal models and human sepsis **(Belikoff 2008)**. One of the initial models of sepsis used in mice was the fecal pellet peritonitis, which consist to administrate fecal content ip. Lately, this model was replaced by others such as ip bacterial inoculation **(Rittirsch, Hoesel, and Ward 2006)**. Host intestinal barrier disruption are more advanced models of sepsis. These models consist to induce a damage into the host, with the aim that the own microbiota cause infection **(Belikoff 2008)**. One of them is the colon ascendens stent peritonitis (CASP), which induces sepsis in mice due to microbiota invadig the peritoneal cavity. This model can cause in mice organ dysfunction and septic shock followed by death **(Rittirsch, Hoesel, and Ward 2006)**. Another highly used model in mice is the cecal ligation and puncture (CLP) model, which consist to induce an intestinal damage. CLP is the more similar model to human sepsis, but does not mimic it completely **(Ward 2012)**.

Inflammation developed by sepsis leads to an increase of oxidative stress because of the production of ROS. For this reason the current research strategies for sepsis treatment are antioxidants agents as recombinant thioredoxin-1 (TRX-1), that can regulates inflammation as well as apoptosis **(Brenner et al. 2010)**. Moreover, this antioxidant has been tested in CLP-sepsis model in mice, resulting in a reduction of initial inflammation and being considered as a potential therapeutic target against septic shock **(Hofer et al. 2009)**. In addition, N-acetylcysteine (NAC) has been studied to contrarrest clinically the inflammatory response in sepsis **(Paterson, Galley, and Webster 2003)**. NAC is able to increase antioxidant properties in the endothelium, reduce the cytotoxicity and inhibit pro-inflammatory cytokines expression as well as inducible Nitric oxide syntase (iNOS) through NF-κB blocking **(Pinsky 2003)**. Clinically approved therapies to treat septic patients are anthracyclines such as daunorubicin or doxorubicin, which are commonly used for chemotherapy a variety of cancers, due to these anthracyclines compounds induce DNA damage by intercalating between base pairs, and the following inhibition of the topoisomerase II **(Figueiredo et al. 2011)**.

5.3. Prognostic markers and severity scores of sepsis

Acute Physiology and Chronic Health Evaluation-II (APACHE-II) and SOFA scores are the used scores in critical care units for septic patients **(Meisel et al. 2009)**. As a consecuence of organ dysfunction inclusion in the clinical SIRS criteria, SOFA score was performed to

guide the organ functionality of patients **(Bone et al. 1992)**. SOFA score is not a predictor value. This score determines the severity degree of each organ and asses the multiorgan dysfunction. SOFA score includes values for 5 organ systems. Each one recovers a value from 0 (normal value) to 4 (most abnormal value). SOFA score gates: respiration index, measured by O_2 arterial pressure between oxygen volume respired by the patient coefficient in mmHg (PaO_2/FiO_2); coagulation measured by platelets amount ($x10^3/mm^3$); liver dysfunction measured by bilirubin (mg/dl); cardiovascular hypotension measured by amount of administrated adrenergic agents as dopamine, epinephrine or norepinephrine in the patient; and renal dysfunction measured by creatinine (mg/dl). **(Vincent, Moreno, and Takala 1996)**. Traditionally the Glasgow coma scale was included into SOFA score. Glasgow coma scale is a clinical scale for assessing the depth and duration of impaired consciousness and coma **(Vincent, Moreno, and Takala 1996)**. Alternatively, there is another quick score used in critical care units termed quick SOFA (qSOFA). However, qSOFA is a poorly sensitive predictive marker for mortality in hospitals **(Maitra, Som, and Bhattacharjee 2018)**. SOFA variation (ΔSOFA) also has been considered as an indicated prognostic value for sepsis-related mortality in hospitals **(Alan E. Jones 2009)**. Another common used score in hospitals is APACHE II score, which is an initial point score based on values of 12 routine physiologic measurements, such as age, health status, or Glasgow coma scale. The aim of this score is to predict a degree of severity of the disease based on physiological conditions of patients. The score range is from 0 to 71, being 71 the most severe prognostic **(Knaus et al. 1985)**.

In sepsis, also is used the concentration of acute-phase protein CRP, as reflects the intensity of acute phase and inflammation. CRP is produced by hepatocytes, predominantly under transcriptional control by the pro-inflammatory cytokine IL-6. CRP reaches the maximal amount in plasma after 48 hours of sepsis development **(Pepys and Hirschfield 2003)**. CRP protein is well studied and crystalized. It posseses a pentameric disc-like structure that gates Ca^{2+}, and a phosphocoline located in the ligand-binding site **(Thompson, Pepys, and Wood 1999)**. Similarly to CRP, that increases in SIRS and sepsis, PCT is also a marker used to diagnose sepsis. PCT is considered as a better biomarker than CRP due to its specificity **(de Jong et al. 2016)**. Indeed, in 2003, PCT was compared with CRP and other markers used in sepsis such as IL-1β, TNFα, IL-6 or IL-8, reflecting that PCT was the most accurated marker in sepsis **(Balci et al. 2003).** In opposite to PCT and CRP, the recombinant human activated protein C, was the first anti-inflammatory and anti-coagulant

agent used in sepsis treatment. Activated protein C inactivates factors Va and VIIIa, avoiding synthesis of thrombin, is anti-apoptotic and blocks cell adhesion induced by monocytes. Nowadays, activated protein C is approved only for use in very critical patients according with APACHE II score **(Richard S Hotchkiss and Karl 2003; Cinel and Opal 2009)**. Other molecular markers are used as prognostic markers of sepsis such as brain natriuretic peptide (BNP), a member of the natriuretic peptides (NPs) family. BNP posseses vasodilatory, diuretic and natriuretic properties and is increased in several cardiac diseases and in sepsis. BNP is able to modulate the production of several inflammatory factors, such as pro-inflammatory cytokines and ROS **(Chiurchiù et al. 2008)**. For this reason BNP and also its N-terminal propeptide NT-proBNP are important predictors of mortality in sepsis **(F. Wang et al. 2012)**

5.4. Role of P2X7 receptor and NLRP3 inflammasome in sepsis

The extracellular concentration of ATP released by circulating neutrophils increases in plasma during chronic inflammation, ischemia, and hypoxia **(Idzko, Ferrari, and Eltzschig 2014; Ledderose et al. 2016)**. There is a controversy of the role of P2X7 in sepsis, since sepsis induce either an increase of survival or mortality on P2X7 deficient mice receptor deficient mice **(Csoka et al. 2015; Hirayama et al. 2015; Greve et al. 2017)**. CD39 NTPDase improves survival in microbial sepsis due to decreases systemic inflammation induced by ATP. Indeed, Csoka and colleagues proposed CD39 as a novel therapeutic target **(Csóka et al. 2015)**. These evidences indicates that purinergic signaling and P2X7R could be involved in the SIRS induced by sepsis.

The NLRP3 inflammasome has also been implicated in sepsis and studies using an animal model of sepsis demonstrated that NLRP3 gene silencing results in reduced hepatic cytokines, neutrophil recruitment to damaged organs, and macrophage pyroptosis **(Y. Wu et al. 2015)**. Moreover, there are evidences of NLRP3 inflammasome activity in fibroblasts that suffered myocardial ischemia and reperfusion **(Kawaguchi et al. 2011)**, and IL-1β was released in cardiac dysfunction from polymicrobial sepsis **(Kalbitz et al. 2016; Wanderer 2008)**. In addition, studies in mice indicated that the initial inflammatory response in sepsis could be reduced by inhibiting IL-1β and IL-18 signaling, meaning that the inflammasome could be activated **(Mariathasan and Monack 2007; Vanden Berghe et al. 2014)**. However studies in septic patients and experimental model of endotoxemia revealed that caspase-1

is inhibited during the immunosupresive state of sepsis (Giamarellos-Bourboulis et al. 2011). Other studies in human PBMCs indicate that in sepsis, there is a large variety of altered genes for the upregulated NLRP3 and NLRC4 inflammasomes , and downregulated NOD1 and NLRP1 inflammasomes (Esquerdo et al. 2017). Moreover, it is known that HMGB1 is an important mediator in sepsis and it has been shown that its release was dependent on caspase-1 during endotoxemia (Lamkanfi et al. 2010). This observation is under controversy due to used mice used were caspase 1 deficient, but they expressed caspase 11, wich could be enough to release HMBG1 (Broz and Monack 2013). Evidences suggest that anti-HMGB1 administration in mice reduce the hyperinflammatory response in sepsis, inducing an immunosuppression state (Stevens et al. 2017). All these evidences indicate the participation of the NLRP3 inflammasome and caspase-1 during SIRS induced by an infection.

Nervertheless, although it has been shown by several studies the participation of ATP/P2X7R and NLRP3 inflammasome in sepsis, the concrete mechanisms of this pathways in the disese course of sepsis is not well understood, specially in a clinical relevant scenario of human sepsis and therefore further studies are necessary to understand the pathophysiological mechanism in sepsis.

Objectives

1- Elucidate the activation of the NLRP3 inflammasome by antimicrobial peptides.

2- Characterize the inflammatory response and the inflammasome function in human monocytes from septic patients.

3- Determine the expression and function of P2X7 receptor in peripheral blood mononuclear cells from septic patients.

4- Characterize the role of P2X7 receptor in macrophage adhesion to extracellular matrix.

Material and Methods

1. Reagents

Name	Concentration/dilution	Company (supported by)
Cell activators and inhibitors		
12-O-Tetradecanoylphorbol 13-acetate (PMA)	50µM	Sigma-Aldrich
3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079)	20-100µM	Tocris Bioscience
Adenosine 5'-triphosphate (ATP)	3mM	Sigma-Aldrich
Antimycin A	5µM	Sigma-Aldrich
Clostridium difficile toxin B	1µg/ml	Enzo Biosciences
FLPLIGRVLSGIL-NH_2 (temporin A)	10µM	A gift from Dr L. Rivas from the Physico-Chemical Biology Department of C.S.I.C. (Madrid)
GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2 (melittin)	0,1-10µM	Luis Rivas from Physico-Chemical Biology department of C.S.I.C. (Madrid)
ILPWKWPWWPWRR-NH_2 (indolicidin)	10µM	Luis Rivas from Physico-Chemical Biology department of C.S.I.C. (Madrid)

Ionomycin	500ng/ml	Sigma-Aldrich
LLGDFFRKSKEKIGKEFKRIVQRIKDF LRNLVPRTES-NH_2 (LL37 cathelicidin)	10μM	Luis Rivas from Physico-Chemical Biology department of C.S.I.C. (Madrid)
Lypopolysaccharide (O55:B5)	1μg/ml	Sigma-Aldrich
N-(1-{[(cyanoimino)(5-quinolinylamino) methyl] amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide (A740003)	20μM	Tocris Bioscience
Nigericin	10-25μM	Sigma-Aldrich
Pokeweed mitogen (PWM)	1μg/ml	Sigma-Aldrich
Protease inhibitor	(1:10)	Calbiochem
Pyrrolidindythiocarbamate (PDTC)	40μM	Sigma-Aldrich
Recombinant human gamma interferon (IFN-γ)	20ng/ml	Preprotech
Recombinant human interleukin-2 (IL-2)	20ng/ml	Preprotech
Recombinant human interleukin-6 (IL-6)	20ng/ml	Preprotech
Recombinant human tumoral necrosis factor alpha (TNF-α)	20ng/ml	Preprotech
Recombinant mouse interleukin-4 (IL-4)	20ng/ml	BD Pharmigen
Reagents for culture media, gel and buffer		
4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid (HEPES)	10mM	Sigma-Aldrich
Amonium Persulfate	20%	Sigma-Aldrich
Bis-acrylamide	8-12%	Sigma-Aldrich
Bovine serum albumin (BSA)	2%	Sigma-Aldrich
$CaCl_2$	2mM	Sigma-Aldrich
D-Glucose	13mM	Panreac
Ethylene glycol-bis (2-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA)	2mM	Sigma-Aldrich
Glutamax	2mM	Fisher scientific
High Glucose Dulbecco's modified Eagle's medium media	-	Lonza

(DMEM) (Lonza)		
KCl	2-147mM	Sigma-Aldrich
Methanol	3:10	Química Clínica Aplicada SA
$MgCl_2$	2mM	Sigma-Aldrich
NaCl	147mM - 1,5M	Merk
NaN_3 (Sodium azide)	0,09%	Sigma-Aldrich
N-Methyl-D-glucamin (NMDG)	147mM	Sigma-Aldrich
Penicillin/Streptomicin	1%	Life Technologies
Roswell Park Memorial Institute (RPMI) 1640	-	Lonza
Skim Milk	5%	Difco
Sodium Dodecyl Sulphate (SDS)	10%	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	4µl in a gel	GE Healthcare
Tris-HCl	0.5-3M in acrylamide gel 0.3M in TBS buffer	Sigma-Aldrich
Tris-NaOH	0.5M in lysis buffer 0.2M in TBS buffer	Sigma-Aldrich
Triton X-100	2%	Sigma-Aldrich
Tween	0,2%	Sigma-Aldrich
ECMs to coat in plates		
Collagen I	10µg/ml	Sigma-Aldrich
Collagen IV	3µg/m	Sigma-Aldrich
Fibronectin	4µg/ml	Sigma-Aldrich
Laminin	6µg/ml	Sigma-Aldrich
Reagents for assay detection		
3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)	250µg/ml	Sigma-Aldrich
3,3,5,5 - tetramethylbenzidine (TMB ELISA detection reagent)	Each well at 50%	Cusabio/eBiosciences/EBL /MBL /R&D Systems
4-[(3-Methyl-1,3-benzoxazol-2(3H)-ylidene)methyl]-1-[3-(trimethylammonio)propyl]quinolinium di-iodide (Yo-Pro)	2-2,5µM	Life Technologies
enhanced chemiluminescence (ECL)-Prime detection reagent	1ml per membrane	GEHealthcare
Fluorochrome-labelled inhibitor of caspase-1 (FLICA)-660	1:150	Immunochemistry Technologies
JC-10	1:250	Abcam
Lactate Dehydrogenase (LDH) cytotoxicity assay kit	Compound A : Compound B (1:45)	Roche
Sulfuric acid	0.16M	Cusabio/eBiosciences/EBL /MBL /R&D Systems

Table 1. List of reagents purchased and used in the PhD thesis.

In table 1, the different reagents used in this thesis are summarized together its concentration of use and the company where they were purchased. Table differentiates in four groups: Reagents to cell treatments, reagents and buffers to add in cell culture, ECMs to coat on plates in cell adhesion experiments, and finally different assay detection kits.

2. Abs

Name	Clone	Dilution	Application	Company
Donkey polyclonal anti-mouse IgG-alexa fluor-647		1:1000	Flow cytometry	Life Technologies
Human Fc-Block		1:1000	Flow cytometry	BD biosciences
Llama DimAlb anti-human P2X7R single blocking Ab	13A7	1:500	Cell culture/Flow cytometry	A gift from Dr. F. Koch-Nolte, Institute for immunology, University Medical Centre (Hamburg, Germany) **(Danquah et al. 2016)**
Llama DimAlb anti-human P2X7R single potentiating Ab	14D5	1:500	Cell culture/Flow cytometry	A gift from Dr. F. Koch-Nolte lab, Institute for immunology, University Medical Centre (Hamburg, Germany) **(Danquah et al. 2016)**
Mouse monoclonal anti-human CD127-FITC	HIL-7R M21	1:200	Flow cytometry	BD biosciences
Mouse monoclonal anti-human CD16-PE/Cy7	3G8	1:500	Flow cytometry	BD biosciences
Mouse monoclonal anti-human CD69-FITC	FN50	1:500	Flow cytometry	BD biosciences
Mouse monoclonal anti-human CD19-PerCP/Cy5.5	HIB19	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal	OKT4	1:500	Flow cytometry	Tonbo Biosciences

anti-human CD4-PerCP/Cy5.5				
Mouse monoclonal anti-human CD25-PE	BC96	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal anti-human CD3-FITC	Hit3a	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal anti-human CD3-APC	Hit3a	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal anti-human CD8-PE	OKT8	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal anti-human CD14-APCH7	G46-6	1:500	Flow cytometry	Tonbo Biosciences
Mouse monoclonal anti-human P2X7R	L4	1:5000	Flow cytometry	Glaxo SmithKline
Mouse monoclonal anti-human ASC-ATTO647	AL177	1:50	Flow cytometry	Adipogen
Mouse tetrameric anti-human cd3/cd28		1:50	Cell culture	Stem Cell
Mouse polyclonal anti-rabbit IgG horseradish peroxidase conjugated		1:2000	Western-Blot	GEHealthcare
Rabbit polyclonal anti-mouse IL1β		1:1000	Western-Blot	Santa Cruz Biotecnology
Rabbit polyclonal anti-mouse β1 integrin		1:1000	Western-Blot	Santa Cruz Biotecnology

Table 2. List of Abs used in all experiments from this PhD thesis.

In table 2, different Abs used in the performed experiments to the thesis were described together their used clone, dilution, techniques where they were employed and the company where they were purchased.

3. Buffers and cell culture media

The essential buffers were phosphate-buffered saline 1x (PBS) (Sigma-Aldrich) and dulbecco's phosphate-buffered saline (DPBS) (Life Technologies). Cell freezing buffer was composed by Foetal Calf Serum (FCS) (Life Technologies), and 10% Dimetilsulphoxide (DMSO) (Sigma-Aldrich).

Macrophage differentiation medium was composed by High Glucose DMEM, 15% FCS, 25% L cell culture supernatants containing M-CSF, 1% penicillin and streptomycin. Media to synchronize macrophage cultures contained High Glucose DMEM, 20% FCS and 50u/ml penicillin and streptomycin. RPMI medium was used to culture THP-1 cell line. DMEM:F12 (1:1) supplemented with 10% FCS, 2mM Glutamax and 1% penicillin-streptomycin was used to culture HEK 293 cell line.

E total (ET) buffer was composed by: HEPES, 13mM D-Glucose, 2mM $CaCl_2$, 2mM $MgCl_2$, 2mM KCl and 147mM NaCl. ET high potassium contained 149mM KCl and no NaCl. ET without Na^+ contained 147mM NMDG. ET without Ca^{2+} contained 2mM (EGTA).

Lysis buffer was composed by 60% distilled H_2O, 10% protease inhibitor, 0.15M NaCl, 10% Tris-NaOH (pH =8), 2% Triton X-100

Buffer used for flow cytometry (SB) contained PBS with 1% FBS, and 0,09% Sodium azide. Western-Blot washing solution was composed by 0.2M Tris-NaOH, 0.3M Tris-HCl, 1,37M NaCl and 0,2% Tween.

Tris-glycine buffer with and without SDS to Western-Blot assay were purchased directly from BioRad.

4. Human samples and clinical determinations

4.1. Human samples

Septic patient enrolled in this study developed abdominal origin severe sepsis at least with one dysfunctional organ according with Levy definition **(Levy et al. 2003)**. The inclusion criteria for septic patients used in this study were patients diagnosed of

intraabdominal origin sepsis confirmed by exploratory laparotomy (where were confirmed after fecal or infected material discovery in the abdominal cavity), with at least two diagnostic criteria for sepsis (fever or hypothermia; heart rate greater than 90 beats per minute; tachypnea, leukocytosis or leukopenia) and multiple organ dysfunction defined as the physiologic dysfunction in two or more organs or organ systems **(Levy et al. 2003)**. We excluded patients who were immune compromised or presented immunodeficiency (including antineoplastic treatments during the previous month to the septic episode), we also excluded terminal oncologic and hematologic neoplastic patients, as well as patients that had a delay of >24 h from intra-abdominal sepsis diagnosis to surgery, patients that stay less than 24 h in the Surgical Critical Unit, those that the infection was not cleared by the surgery or patients that presented another septic focus different to the abdominal focus. Age and gender were statistically analysed in septic patients in comparison with healthy and surgery controls.

Blood from 35 septic patients was recovered after a colorectal surgery within the first 24h of sepsis development and after 3, 5 or 120 days of sepsis development. Clinical, biochemical and microbiology data from the patients was recovered from the clinical history of the patient. Blood from 11 septic patients undergoing abdominal surgery without sepsis development and blood from 16 healthy donors was used as control. Blood from autoinflammatory syndromes patients, such as 3 CAPS patients, a FMF patient, a PAAND patient, and 2 APLAID patients was analysed to compare with controls and septic patients.

The clinical ethics committee from the *Clinical University Hospital Virgen de la Arrixaca* (Murcia, Spain) approve the procedures of this study **(World Medical Association et al. 2013)** and samples and data from patients included in this study were stored in the BioBank *Biobanco en Red de la Región de Murcia* (PT13/0010/0018), integrated in the Spanish National Biobanks Network (B.000859) and they were processed following standard operating procedures with the appropriate signed informed consent from each patient.

4.2. Clinical determinations

Within the first 24 h of sepsis development several markers for sepsis were determined: CRP, PCT, and the NT-ProBNP were detected as molecular markers of acute phase of sepsis. Other cellular and biochemical markers were analyzed in blood samples,

including the number of circulating neutrophils, monocytes and platelets, as well as the concentration of lactate, bicarbonate, creatinine, urea, bilirubin and hemoglobin. Respiratory parameters as central venous CO_2 saturation and partial O_2 pressure were also determined. Dara regarding coagulation parameters, as fibrinogen, Clinical scores for sepsis severity were calculated as SOFA (five days tracing), ∆SOFA between day 1 and 5, and APACHE II (first 24h).

4.3. Sample reception

Samples were collected after written informed consent from patients from the University Hospital *Virgen de la Arrixaca* Surgery Reanimation unit at days 1, 3, and 5, after sepsis development. Blood from four septic patients were recovered 120 days after sepsis recovery. Each sample constituted:

- Three 3ml heparin tubes, used to Peripheral Blood Mononuclear Cells (PBMCs) extraction (9ml in total).
- Two 3ml EDTA tubes, used to plasma and genomic ADN extraction (6ml in total).

Samples form autoinflammatory syndromes were collected after written informed consent from patients or legal representative from *Hospital Clinic* and *Vall d'Hebron* (Barcelona, for CAPS and APLAID), Hospital *Virgen de la Arrixaca* (Murcia, FMF), *Hospital Universitario 12 de octubre* (Madrid, PAAND).

4.4. PBMCs isolation

To separate the PBMCs from total blood, a Ficoll-based gradient separation method was used with a density of 1,077g/ml (Histopaque® 1077; Sigma-Aldrich). SepMate™-50; (STEMCELL) special Falcon type tubes were used to the gradient separation as well. These Falcon type tubes are physically separated in two chambers, providing the advantage of an easier separation. 15ml Ficoll were added in the bottom chamber until fill it in. 18ml PBS diluted blood (1:1) is added on the top chamber. Both chambers connect themselves, allowing a higher deceleration during the centrifugation process to the gradient separation, optimizing the cell separation time.

Then, 18ml of PBS diluted blood (1:1) was added, with the aim of decrease the blood density to avoid the loss of the phase change. Once prepared, blood was centrifuged at 1200xg

during 15 minutes (min). After the centrifugation, four phases are obtained (Figure X), based on their density **(Munteanu and Dinu 2005; Mosca and Forte 2016)**:

- Erythrocytes and PMNs phase are in the highest density layer.
- The ficoll phase that is mostly acellular layer.
- The PBMCs phase, forming a white ring and also called buffy coat. This was the layer used for experiments in this Thesis.
- The plasma phase.

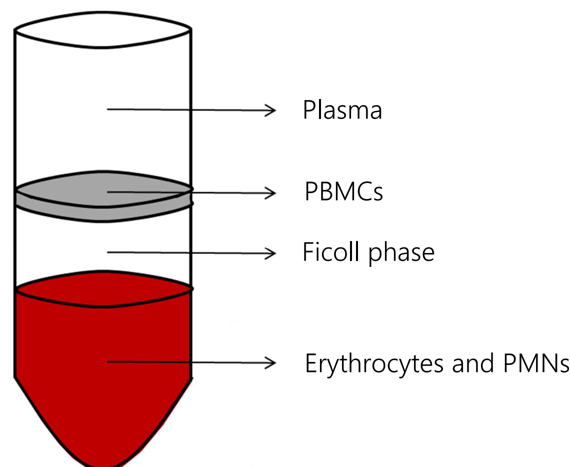


Figure 13. Scheme of the different phases of a ficoll separation system based on density gradients. Red cells deposits on the bottom of the tube. PBMCs ring is located between the plasma phase and ficoll phase.

PBMCs collection was carefully absorbed with a Pasteur pippete and the containing cells were deposited into a 50 ml tube. PBMCs were washed with 20ml PBS-twice at 300 x g during 10 min. Supernatants were discarded in all the washes. Then, PBMCs were resuspended on 3 ml of PBS, and were counted in a Bürker chamber (Marienfeld) diluting 1:10 in trypan blue (Sigma-Aldrich) to calculate the cell viability and confirmate the optimal state of the cells after separation. Cells were counted by an AE 2000 microscope (Motic).

Isolated PBMCs were used preferably the same day of the obtaining, in some cases PBMCs were frozen at -80ºC. For that PBMCs were centrifuged for 10 min at 300 x g and 10^6 cells were resuspended in 1ml of freezing buffer in 2ml cryotubes (Greiner Bio-one) and stored at -80ºC using an isopropanol-containing recipient (Nalgen) for 24 to 48 h. After this period, cells were stored in liquid nitrogen.

Plasma and frozen PBMCs was stored in the BioBank *Biobanco en Red de la Región de Murcia* (PT13/0010/0018), where PBMCs genomic DNA extraction was also performed.

4.5. PBMCs defrosting

Cells stored in liquid N_2 were defrosted in a 37ºC bath and viable cells were enriched by a negative magnetic selection process using the OctoMacs kit together separation columns of medium size (both from Miltenyi Biotec) according to manufacturer's recommendations. This procedure will retain phosphatidylserine positive cells (death cells) by using Annexin V conjugated to magnetic beads. Briefly, defrosted PBMCs were diluted in 9ml of PBS and centrifuged at 300 x g for 10 min. 10^6 cells were resuspended in 100µl of Annexin V conjugated with magnetic beads and 400µl of binding buffer (Miltenyi Biotec) and incubated for 15 min at room temperature (RT). Then the cells were added to calibrated columns (columns were calibrated with 500µl of binding buffer) and were washed 3 times with 500µl of binding buffer. The final volume of collected life-cells (phosphatidylserine negative population) was about 2.5ml; cells were then counted using with a Bürker chamber and trypan blue to exclude permeable non-viable cells. Chamber contains nine quadrants making a big square. Three quadrants were counted diagonally in the chamber. Total of counted cells were divided by 3 and multiplied by the chamber dimensions and trypan dilution, resulting in the number of cells in 1ml of the sample. To obtain the total number of cells, this volume per ml was multiplied by 2.5ml (total volume of each sample). Usually, trypan blue positive cells were less than 10%. Cells were then centrifuged at 300 x g during 10 min and resuspended in 100µl of SB buffer per 10^6 of cells.

5. Differentiation of mouse bone marrow-derived macrophages

5.1. Mice strains

Mice aged between 6 and 12 weeks were used under SPF conditions and according with the *Clinical University Hospital Virgen de la Arrixaca* animal experimentation guidelines and the Spanish national (RD 53/2013, Law 6/2013) and EU (86/609/EEC and 2010/63/EU) legislation.

Animal procedure was refined and approved by the *Clinical University Hospital Virgen de la Arrixaca* animal experimentation committee and approved by the *Animal Service, Murcia Fishing and Farming Council* (reference: C1310050308). All Used *Health* Mice were under C57BL/6 background and colonies were maintained under specific pathogen free (SPF) in ventilated racks, with free access to sterile water and food, with a temperature of 25ºC and a light-dark cycle of 12h.

- Wild-type mice were purchased from Harlan.
- P2X7R-deficient mice (*P2rx7*$^{-/-}$) were purchased from Jackson.
- NLRP3 deficient mice (*Nlrp3*$^{-/-}$) were originally created by Dr J Tschopp laboratory **(Martinon et al. 2006)** and ceded from Dr I Couillin (University of Orleans, France).
- Double caspase-1 and -11 deficient mice (*Casp1/11*$^{-/-}$) were originally created by Dr VM Dixit laboratory **(Kayagaki et al. 2011)** and ceded from Dr I Couillin (University of Orleans, France).

5.2. Bone Marrow extraction

Mice were euthanized by CO_2 inhalation, and femur and tibia were dissected. Bone marrow was flushed from bones and collected in 30ml of differentiation medium in sterile conditions.

The bone marrow suspension was seeded into three p150 petri dishes (Cellstar) and supplemented with 10ml of differentiation medium, reaching a total volume of 20ml per dish. Plates were incubated at 37ºC with a 5% CO_2 atmosphere in a Healforce incubator.

5.3. Bone marrow macrophage differentiation

Two days after bone marrow culture, 20ml of differentiation medium (containing macrophage-colony stimulating factor, M-CSF) was added per dish, and cells were incubated at 37ºC and 5% CO_2 atmosphere for further 4 days. After 6 days of culture, macrophages were completely differentiated. Supernatants were discarded and cells were washed with PBS and incubated at 4ºC during 5 min to let detachment of the cells. To help

detachment, cells were manually scraped and cell suspension was centrifuged at 400 x g for 5 min. Supernatants were discarded and cells were resuspended in 10ml of synchronization medium. BMDMs were counted and cultured in different densities as will be indicated later during 12 h at 37ºC and 5% CO_2. After this incubation macrophages cell cycle is synchronized and cells were ready for stimulation.

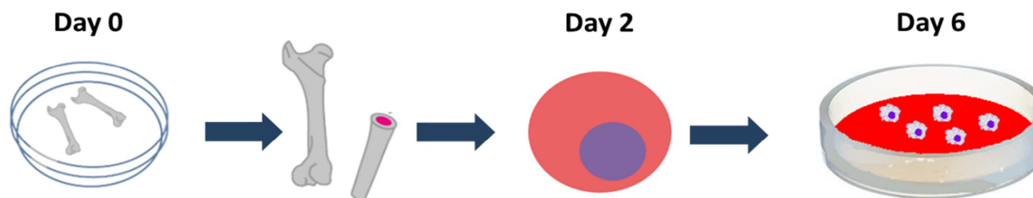


Figure 14. Bone marrow extraction and differentiation steps. Bones from mice are collected and cells are extracted from bone marrow. After six days in culture with M-CSF, cells are completely differentiated in BMDMs.

6. Cell lines

6.1. L929

L929 (CCL-1; American Type Culture Collection) is a mouse fibroblast cell line. L929 cells were cultured in DMEM supplemented with 5% FCS and 2mM GlutaMAX^{TM}. This cell line constitutively produces M-CSF and the media of 7 days cultures at 37ºC and 5% CO_2 was collected and filtered through 0.2μM pore (Millipore). Filtered media was frozen at -80ºC until macrophages differentiation procedure.

6.2. THP-1

THP-1 cells (TIB-202; American Type Culture Collection) were stored in RPMI medium and were supplemented with 10% FCS in functional assays. FCS was not supplemented on flow cytometry staining protocols. Cell lines were routinely checked to ensure they were mycoplasma-free with the Mycoplasma Detection Kit from Roche following manufacturer instructions.

6.3. HEK293T

HEK293T cells (CRL-11268; American Type Culture Collection) or HEK293T cells expressing P2X7 receptor were maintained DMEM:F12 (1:1) medium and were routinely tested for mycoplasma contamination with the Mycoplasma Detection Kit from Roche. Lipofectamine 2000 was used for the transfection of HEK293 cells according to the manufacturer's instructions (Life Technologies).

After cell culture medium removing, HEK cells were washed with PBS. 1ml trypsin at 0,25% with 0,02% EDTA (Sigma Aldrich) was added in the centre of the flask. And after 3 min at 4ºC, trypsin was removed from the flask to a Falcon tube, diluted in DMEM with 10% FCS. After 433 x g centrifugation during 5 min, supernatant was discarded, and new medium was added. Then cells were counted with a Bürker chamber and cells were cultured at different densities depending the experiment.

7. Cell stimulation

PBMCs from healthy controls were primed in complete THP-1 medium with 1µg/ml LPS during 2, 4, 24 or 48h at 37ºC and 5% of CO_2. ATP 3mM was added in ET buffer before or after LPS priming as indicated in the figures and was incubated during 30 min at 37ºC and 5% of CO_2. In a different set of experiments, recombinant human IFNγ, TNFα, IL-2 or IL-6 (each at 20ng/ml) or LPS (10ng/ml, 100ng/ml and 1µg/ml) or PMA (10ng/ml) alone or with ionomycin (500ng/ml), antiCD3/CD28 (20µl/ml), or pokeweed mitogen (PWM, 1µg/ml) were incubated with 10^5 PBMCs isolated from healthy donors for 2, 4, 24 and 48h in 96-wells plates at 37ºC and 5% of CO_2.

PBMCs isolated from auto-inflammatory patients were primed with 1µg/ml LPS during 2h at 37ºC and 5% of CO_2. After LPS-priming PBMCs from APLAID and PAAND patients were treated with 3mM ATP during 30 min at 37ºC and 5% of CO_2. After LPS-priming PBMCs from CAPS and FMF patients were treated with ATP (3mM) during 30min or with Clostridium difficile toxin B during 1h at 37ºC and 5% of CO_2.

Unless otherwise stated mouse BMDMs were treated with LPS (1µg/ml) during 4 h at 37ºC and 5% of CO_2 in uncoated tissue culture plates. In some experiments BMDMs were primed with IL-4 (20µg/ml) or left untreated for 4 h at 37ºC and 5% of CO_2. Alternatively, when indicated in the text BMDMs were plated in different extracellular matrixes. For that, collagen I (10µg/ml), collagen IV (3µg/ml), laminin (4µg/ml), or fibronectin (6µg/ml) solution was added to the wells of the tissue culture plate and incubated overnight at 4ºC. Next day excess was discarded and washed twice with PBS before BMDM seeding.

After BMDM priming with LPS, cells were treated with ATP (3 or 5 mM), antymicin A (5µM), nigericin (25µM), LL-37 (10µM), indolicidin (10µM), temporin-A (10µM), melittin (0.3, 0.5, 1, 5, 10 and 20µM) for 30 min in ET or ET high potassium at 37ºC and 5% of CO_2. In some experiments, before ATP stimulation cells were pre-treated for 10 min with the specific P2X7R inhibitor A438079 (10 or 100µM), the mitochondrial oxidation protector PDTC (40µM), P2X7R-specific modulating DimAlb nanobodies (as the inhibitor 13A7 or the potentiator 14D5 at 200nM).

HEK293T cells and HEK293T stabling expressing rat P2X7R were used as a model of P2X7R function in cell adhesion. Both cell types were incubated for 30 min with or without ATP (3mM) in ET, ET high potassium, ET without Na^+ or ET without Ca^{2+} at 37ºC and 5% of CO_2. In some experiments the cells were pre-treated for 10 min with the P2X7R antagonists A438079 or A740003 (10µM) at 37ºC and 5% of CO_2.

THP-1 cells were treated with PMA (50µM) during 30 min at 37ºC and 5% of CO_2 to attach and differentiate the cells into macrophage-like phenotype. Cells were then treated with LPS (1µg/ml) for 4 h at 37ºC and 5% CO_2. After LPS-priming, cells were stimulated with melittin (0.1, 0.3, 1, 5, 1 or 20µM) or nigericin (25µM) for 30 min in ET at 37ºC.

In some experiments, after stimulation, cells were used for flow cytometry or cell adhesion (detailed below). In other experiments, after cell stimulation, cell supernatants from 6 and 12 wells plates were collected from BMDM, THP-1 or HEK cells. In both cases, during priming cell density was at 10^6 cells/ml in 1ml of medium for 12 wells plates and 2ml for 6 wells plates. Stimulations were done in ET 400µl of final volume in 12-wells plates, and ET 800µL in 6 wells plates. Cells were lysed with 30µl of cold lysis buffer. Cell supernatants were centrifuged at 12856 x g during 30 seconds and the pellet was discarded. Cells were

lysed during 30 min at 4ºC and centrifuged during 10 min at 12856 x g. Supernatant from lysed cells were collected and stored together clarified cell-supernatants at -80ºC.

8. Cell adhesion protocol

HEK293, HEK293-P2X7R or BMDMs plated on 96 wells mainly at density of 75 x10^3 cells/well in the case of HEK cells and 150 x10^3 in the case of BMDMs in a final volume of 100µl of medium. Then, cells were stimulated or not with ATP (as explained above) for different times in a final volume of 100µl of ET. Then, culture plates were vigorously shaken for 30 sec at 1 x g. After that, cells were washed with PBS and attached cells were quantified either by Yo-Pro-1 staining or MTT assay (see below).

9. Flow cytometry

The majority of flow cytometry experiments were performed in a FACS Canto (BD Biosciences) together FACS Diva Analysis software (BD Biosciences). Alternatively, experiments using P2X7R specific nanobodies, a FACS Celesta flow cytometer (BD Biosciences) was used.

9.1. Determination of P2X7R surface expression

P2X7R expression was determined in different subsets of PMBCs in two different Ab combinations that allow us to determine P2X7R on regulatory T cells ($CD4^+CD25^+CD127^-$) and on T cells subset ($CD3^+CD4^+$ and $CD3^+CD8^+$), B cells ($CD3^-CD19^+$), natural killer cells ($CD3^-CD19^-CD14^-CD16^{++}$), and monocytes ($CD3^-$ $CD14^+$) (Figure 15).

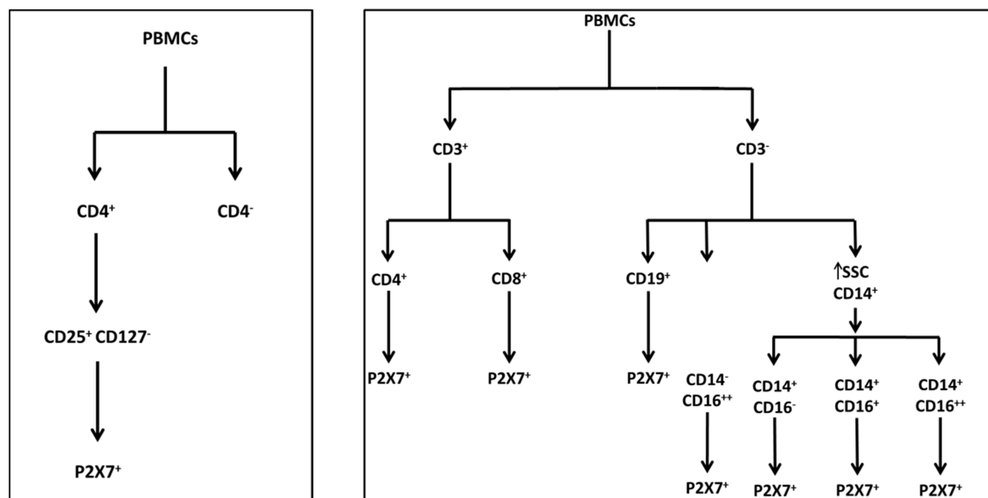


Figure 15. Overview of worksheet used to obtain the different cell populations examined for P2X7R expression. The left panel represents the worksheet used to obtain the P2X7R expression in T regulatory cells. The right panel represents the worksheet used to the P2X7R determination in different subsets: T cells, B cells, NK cells and monocytes.

$5x10^5$ µlPBMCs were resuspended in SB buffer (10 million/ml) in polystyrene flow cytometry tubes (Falcon) and incubated with an Ab to block the Fc receptor (0.5mg/ml) for 10 min at 4ºC. After blocking, PBMCs were stained for 30 min at 4ºC with: i) anti-CD4-PerCP.Cy5.5, anti-CD25-PE, anti-CD127-FITC and anti-P2X7R-APC (for dilutions used and clones see Table 2); or ii) anti-CD3-FITC, anti-CD4-PerCP.Cy5.5, anti-CD8-PE, anti-CD19-PerCP.Cy5.5, anti-CD14-APCH7, anti-CD16-PECy7 and anti-P2X7R-APC (for dilutions used and clones see Table X). CD25 isotype control was used to detect $CD4^{+}CD25^{+}CD127^{-}$ cells. CD14 isotype control was used for $CD3^{-}CD14^{+}$ monocytes. Isotype control for CD3, CD4 and CD8 were used to demonstrate T cells detection. B and NK cells were compared with a CD19 and CD16 isotype control respectively. To analyse the percentage of P2X7 on surface in PBMCs, or the P2X7 expression in monocytes, a P2X7 isotype control was used.

In some staining, non-conjugated anti-P2X7R Ab was incubated for 30 min at 4ºC and cells were washed before incubation with a secondary donkey anti-mouse IgG Ab conjugated with Alexa Fluor 647 (Life technologies) for another 30 min at 4ºC. This double staining protocol was done before the rest of surface staining to avoid the secondary Ab to recognise the conjugated Abs used to discriminate the different PBMCs populations.

In some experiments where PBMCs were stimulated in vitro anti-CD25-PE and anti-CD69-FITC were used to determine cell activation.

After Ab staining, cells were washed in SB buffer and centrifuged at 769 x g during 3 min. Supernatants were discarded and finally resuspended in 500µl of PBS before analysing by flow cytometry.

9.2. Detection of active caspase-1

To determine the presence of intracellular active caspase-1 the fluorescent caspase-1 inhibitor FLICA 660 (Immunochemistry Technologies) was used. This compound presents

the amino acid sequence YVAD that is able to bind to the active site of caspase-1 with relatively higher affinity than for other caspases (Earnshaw 2008) and a fluoromethylketone (FMK) group that will covalently react with the active site. The YVAD peptide is also linked to fluorophore that can be excited at 660 nm and emits at 690 nm. $5x10^5$ PBMCs were stained with anti-CD3-FITC, anti-CD4-PerCP.Cy5.5, anti-CD8-PE, anti-CD19-PerCP.Cy5.5, anti-CD14-APCH7 and anti-CD16-PECy7 as explained above. Then, cells were washed with PBS buffer and centrifuged at 769 x g during 3 min. Supernatants were discarded and cells were resuspended in 300µl of PBS for intracellular staining with FLICA 660 following manufacturer instructions. Briefly, a 30x FLICA 660 work solution was prepared by diluting 1:4 in PBS and 10µl of this dilution was added to the 300µl of cell suspension to achieve a 1x solution of FLICA 660. Each tube was then incubated for 20 min at 37ºC and then cells were washed twice with 2ml of washing buffer provided with FLICA 660 reagent. Cells were then centrifuged at 769 x g during 3 min and supernatants were discarded and finally resuspended in 500µl of PBS before analysing by flow cytometry.

Alternatively, FLICA 660 staining was in PBMCs during LPS, ATP or CdtB stimulation as indicated in the text. FLICA 660 was added to the ET buffer after 15 min stimulation and incubated for further 20 min together with stimuli at 37ºC in a 5% CO_2. During the last 5 min of FLICA 660 staining, PBMCs were stained with anti-CD3-FITC and anti-CD14-APCH7 as explained above, to select the $CD3^-$ $CD14^+$ monocytes. Then cells were washed with 2ml of wash buffer provided with FLICA 660 reagent. Then, cells were fixed in 500µl of fixation solution (provided with FLICA 660 reagent) for 15 min, then washed and finally resuspended in 500µl of PBS before analysing by flow cytometry.

9.3. Determination of mitochondrial membrane potential assay

To determinate the mitochondrial membrane potential the JC-10 dye was used (Ji et al. 2017). JC-10 increases its solubility in comparison with other markers for mitochondrial membrane potential as JC-1. JC-10 is capable of selectively load mitochondria with negative membrane potential, was it emits at a wavelength of 570nm and if mitochondria membrane potentials increase, JC-10 difuse to the cytosol and shift the emitted light to 520nm (Figure 16).

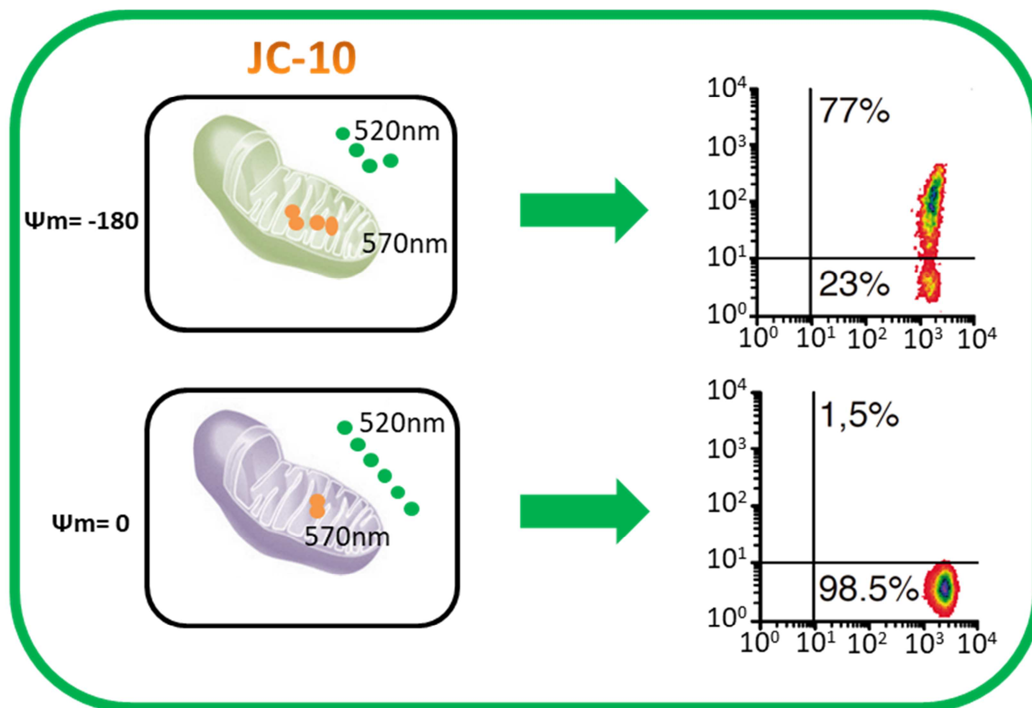


Figure 16. Mitochondrial membrane potential measurement with JC-10 staining. Functional mitochondria with a negative membrane potential are able to keep JC-10 inside, were it aggregates and emits at 570 nm. Major part of the cells will be double positives for green and red, since excess of JC-10 will stay on the cytosol. Meanwhile non-functional mitochondria with lack of membrane potential induces a leakage of JC-10 from the mitochondria, being the major part of the emission light in green due to its sole presence on the cytosol. Graphs were adapted from the JC-10 membrane potential assay protocol (Abcam).

In this Thesis, PBMCs were first stained with anti-CD3-APC, anti-CD14-APCH7 and anti-CD16-PECy7 (Figure X) as explained above and then were incubated with JC-10 following the manufacturer instructions. Briefly, JC-10 stock solution (250x concentrated) was diluted with the stained cells in 500µl of assay buffer (provided with JC-10 dye) during 30 min at RT before analysing by flow cytometry.

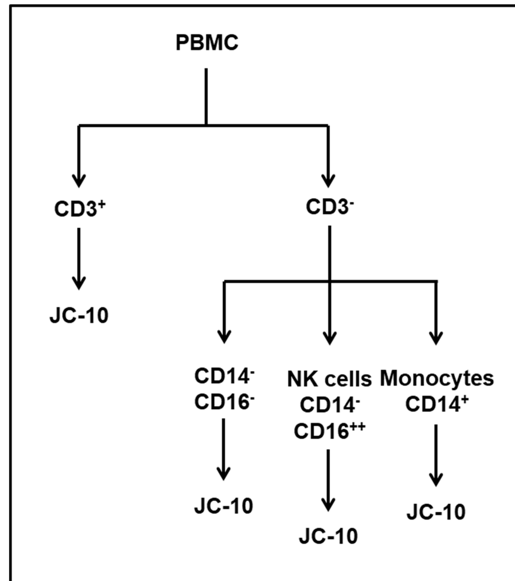


Figure 17. Overview of worksheet used to determine mitochondria membrane potential in different PBMCs population.

9.4. Multiplexing cytokine detection

Plasma from septic patients and control groups (healthy individual and abdominal surgery patients) was used to measure the concentration of several cytokines using the CBA Flex Set kit (BD bioscience). Parameters were compensated in a BD FACS Canto (BD Biosciences) flow cytometer following manufacturer instructions. Plasma samples were incubated with the CBA beads according with the manufacturer instructions. Standard cytokines were used at different dilutions (1:1, 1:3, 1:9, 1:27, 1:81, 1:243 and 1:729), and a blank tube without cytokines was prepared as a negative control. Each standard contains different known concentrations for: TNF-α, IFN-γ, IL-1β, IL-10, IL-2, IL-4, IL-8, IL-6, IL12p70, and IL17A. Standard and blanks were transferred into 96-well plate wells to make easier the procedure to manipulate a huge number of samples. Human plasma (50µl was diluted 1:3 on assay diluent buffer (provided with the BD Flet Set Kit) and added into the wells of the 96-well plate without duplicates. Then a mixture of capture beads specific for each cytokine was added to all samples and the plate was shaken at 42 x g for 5 min, and incubated during 2 h at RT. After incubation, samples were washed by centrifugation at 433 x g during 5 min with wash buffer (provided with the kit) and then the detection reagent was added and incubated for another 2h at RT. The last step was included to increase the sensitivity of detection. The Enhanced Sensitivity Detection Reagent (provided with the kit) was added to

all samples and standards and was incubated during 1h at RT. Samples were finally washed and transferred into flow cytometry tubes containing 300µl of wash buffer provided with the kit. Samples were analysed by flow cytometry using the FCAP Array Software (BD Biosciences).

9.5. Detection of ASC specks in plasma

ASC specks were detected following the protocol already published by our group **(Baroja-Mazo et al. 2014)**. Briefly, 500µl of plasma was centrifuged at 239 x g during 8 min, and supernatants were discarded. The precipitated fraction was resuspended in 50µl of SB buffer and incubated with 20µg/ml of anti-ASC Ab conjugated with ATTO647 for 1h at RT. After incubation, 1ml of SB buffer was added, samples were the centrifuged at 1844 x g during 4 min and supernatants were discarded. Pellets were resuspended in 500µl of SB buffer before analysing by flow cytometry.

10. Determination of mitochondrial membrane potential using a plate reader

BMDMs were plated at 10^5 cells per well in a 96-well black plate with flat transparent bottom (Costar), culture overnight and primed or not with LPS as stated above. After priming, cells were washed with ET and incubated with the dye JC-10 (1:400) for 15 min at 37ºC. After JC10 incubation, cells were washed at 769 x g during 3 min, and stimulated with ATP or antimycin A as stated above. A tracing for the mitochondrial membrane potential on ATP-treated or no treated cells was done by fluorescence measurement in a Synergy Mx plate reader (BioTek) at 490nm excitation and recording emission at 525nm and 590nm reading every 60 seconds during a total of 60 min. Data is represented as the ratio among the emissions at 525/590nm.

11. Enzyme-linked immunosorbent assays

The enzyme-linked immunosorbent assay (ELISA) is a biochemical technique that allows quantification of specific proteins in serum, plasma o cell supernatant samples based on the antigen-Ab recognition.

In this Thesis, a quantitative sandwich ELISA system was used (Figure X) to detect human HMGB1 (IBL International) in plasma, soluble human P2X7R (Cusabio) in plasma, human IL-18 (MBL) in plasma and THP-1 supernatants, human IL-1β (eBioscience) in THP-1 supernatants, and mouse IL-6, TNF-α, and IL-1β (R&D System) or mouse IL-1β and IL-18 (eBioscience) in BMDMs supernatants.

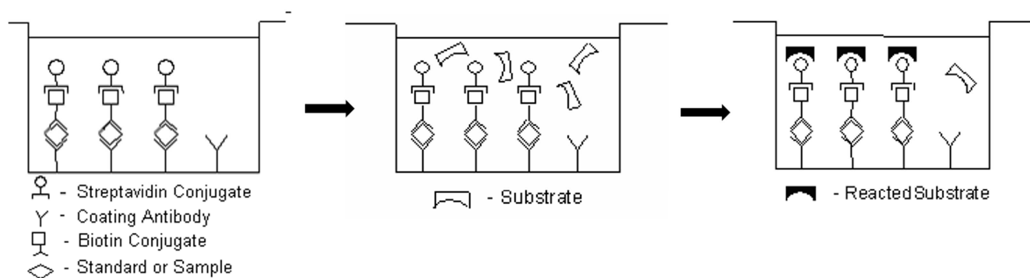


Figure 18. Graphic description of the sandwich system ELISA used in this Thesis. Images recovered from eBiosciences ELISA protocols.

ELISAs were done in 96-ELISA wells plate (Corning Costar). If applicable, first Ab coating into to the plate was done before Ag addition. Coating was done in coating buffer (provided with the kit). In other cases, plates are previously coated by the manufacturer. After coating, plates were washed by wash buffer (provided with the kit) to eliminate the coating excess. During each wash step, plates were manually shaked during 10 seconds and excess was discarded. After washing, standard cytokine and samples were added and incubated as describe each protocol to allow binding between first Ab and Ag. Samples dilutions were done with the corresponding sample diluent buffer provided with each assay kit (see table 3).

After samples incubation and washing (as described above) to eliminate excess and avoid unspecific binding, the secondary Ab or detection Ab was added. This Ab was conjugated with horseradish peroxidase (HRP)-avidin or HRP-streptavidin. This procedure is followed by an incubation (described by each kit) and washing as described above.

After secondary Ab incubation, samples were washed as described above. Then, 3,3',5,5'- Tetramethylbenzidine (TMB) as substrate reagent was added in all assays without light exposure. After TMB addition, blue colour developed in proportion to the amount of analyte presence in the sample. Colour development is stopped turning the colour in the

wells to yellow due to the Stop solution addition (acid solution). The absorbance of the colour at 450nm was measured read in a Synergy Mx plate reader (BioTek), using 570nm as reference.

ELISA Kit	Coating (first Ab incubation)	Sample dilution	Sample incubation	Secondary Ab incubation	TMB/ Stop solution
Human HMGB1 (IBL)	coated plate	without dilution	24h (37ºC)	conjugated Ab: 2h (RT)	30′ (RT)/ 100µl TMB + 100µl Stop (1:2)
Human IL-1β (eBiosciences)	coating Ab 1:250	1:2	2h (RT)	Ab: 1h (RT) HRP-Avidin: 40 min (RT)	10′ (RT) 100µl TMB + 50µl Stop (1:3)
Human IL-18 (MBL)	coated plate	without dilution	1h (RT)	conjugated Ab: 1h (RT)	30´ (RT)/ 100µl TMB + 100µl Stop (1:2)
Human P2X7R (Cusabio)	coated plate	without dilution	2h (37ºC)	Ab: 1h (37ºC) + HRP-avidin: 1h (37ºC)	15´ (37º)/ 90µl TMB + 50µl Stop (5:14)
Mouse IL-1β (eBiosciences)	coating Ab 1:250	1:5	2h (RT)	Ab: 1h (RT) HRP-avidin: 40 min (RT)	10′ (RT) 100µl TMB + 50µl Stop (1:3)

Mouse IL-18 (eBiosciences)	coated plate	without dilution	Together secondary Ab	Ab: 2h (RT) HRP-streptavidin : 1h (Rt)	30′ (RT)/100µl TMB + 100µl Stop (1:2)
Mouse IL-1β (R&D Systems)	coated plate	1:4	2h (RT)	conjugated Ab: 2h (RT)	5′ (RT)/100µl TMB + 100µl Stop (1:2)
Mouse IL-6 (R&D Systems)	coated plate	1:4	2h (RT)	conjugated Ab: 2h (RT)	5′ (RT)/100µl TMB + 100µl Stop (1:2)
Mouse TNFα (R&D Systems)	coated plate	without dilution	2h (RT)	conjugated Ab: 2h (RT)	15′ (RT)/100µl TMB + 100µl Stop (1:2)

Table 3. Details of ELISA kit protocols that has been used in this PhD thesis. Differences between first antibody, samples and second antibody incubation and substrate solution addition are described in this table.

12. Western-blot determination

12.1. Sample processing

Clarified cell supernatants obtained as described above were concentrated after centrifugation at 11000 x g for 30 min through a membrane with a 10kDa pore width (Millipore). The aim of this procedure was concentrate proteins from cells supernatants and increase the signal from cytokines in the Western blot. Both concentrated supernatants and cell extracts originally from $2x10^6$ cells were mixed with Laemmli buffer (Bio-Rad) at 1:2

proportions. Laemmli buffer contains 2-mercaptoethanol, capable to break disulphide bonds of proteins to avoid protein aggregation. Samples with Laemmli buffer were heated for 5 min at 95ºC, briefly spun down and then they were ready to load on the acrylamide gel.

12.2. Acrylamide gel preparation

SDS-polyacrylamide gel electrophoresis was done in denaturing conditions. Acrylamide/bis-acrylamide gels were freshly prepared before electrophoresis of proteins. 19:1 acrylamide/bis-acrylamide solution (Sigma Aldrich) was combined with distilled water in different concentrations on the separating gel: A two phases 8% and 15% acrylamide gel was used to determinate IL-1β. To determinate the presence of β1 integrin, a single phase 8% acrylamide/bis-acrylamide was done. Separating gel contained 3M Tris-HCl at pH 8.8 to trigger the protein movement through the gel according their size. By contrast, concentrating phase was added upon separating phase, and contained Tris-HCl at 0,5M and pH 6.8, which maintains concentration of proteins in a low conductivity state.

SDS was added in as an anionic detergent to facilitate denaturing conditions and skip the native charge of proteins. SDS addition allows the movement from cathode to anode independently of native charge of each protein and only dependent of protein size. Ammonium persulphate (APS) and TEMED are added into the gel to initiate polymerization reaction.

Polyacrylamide gels were made with a thickness of 1.5mm casted using the Mini-PROTEAN 3 system (Bio-Rad). Gels were physically separated from oxygen during polymerization by ethanol addition on the top and solidification occurred after 30-40 min depending on the percentage of acrylamide/bis-acrylamide. Once the separation gel is polymerized, ethanol is retired and washed with distilled water, the concentrating gel is poured and 10-wells Mini-PROTEAN electrophoresis cell combs of 1.5mm thickness (Bio-Rad) were used to make the wells. Once solidified, combs were retired and gel was placed into the Mini-PROTEAN electrophoresis cuvette.

12.3. Protein electrophoresis and transference

Samples were loaded into the different gel wells together with a pre-stained protein standard (BenchMark^{TM}, Life Technologies) that contains proteins of 6, 15, 19, 26, 37, 49, 64, 82, 115, 180kDa. Protein electrophoresis was done in a running buffer composed of 25mM Tris, 192mM glycine and 0,1% of SDS and pH 8.3 (Bio-Rad) at 200V for 40-50 min using a power supply from Bio-Rad. After protein separation, gels were placed over a nitrocellulose membrane (marca), and wrapped with Whatman® filter paper and foam pads, and casted into gel holders cassettes (Bio-Rad). The gel-membrane "sandwich" was introduced on the transference cuvette with the negative pole on the side of the gel and the positive pole on the side of the membrane. Transference cuvette was fill with transfer buffer (20% of methanol, 25mM Tris and 192mM glycine and pH 8.3) (Bio-Rad) and wet-transference was done at constant voltage (100V) during 1 h (stirring transfer buffer inside the cuvette to avoid over heating) or alternatively at constant amperage (50mA) overnight at 4ºC. In both cases using a power supply from Bio-Rad.

12.4. Ab blotting and bioluminescence detection

Once proteins were transferred to the nitrocellulose membranes, gels were discarded, and membranes were blocked for unspecific protein binding with skim milk (BD biosciences) diluted at 5% in TBS with 0.2% Tween (T-TBS) for 50 min with shaking at RT.

After blocking, primary Ab was added in T-TBS (for dilutions see table X) and was incubated during 1h at RT or overnight at 4ºC with shaking. Then membranes were washed in T-TBS to eliminate the excess of primary Ab and the secondary Ab conjugated with horseradish peroxidase (HRP) (GE Healthcare) was added at 1:2000 dilution in T-TBS with 5% of milk. Secondary Ab was incubated for 1 h of incubation at RT with shaking and then membranes were washed with T-TBS. Membranes were then incubated for 5 min in dark with 1ml of enhanced chemoluminescence prime detection reagent (GE Healthcare), excess was careful removed with a paper towel and light signal was detected using the ChemiDoc XRS (Bio-Rad).

13. Yo-Pro-1 cell staining

In this Thesis, Yo-Pro-1 staining was used with two main objectives: 1) determine plasma membrane permeabilization at real time; and 2) quantification of number of cells in cell adherence protocol. Yo-Pro-1 is a negative charge molecule, with XXX Da and cell-impermant, that strongly binds nucleic acids and emits at 525 nm upon excitation at 480 nm. Fluorescence of DNA-bound Yo-Pro-1 was measured in a Synergy Mx plate reader (BioTek) at 485±9 nm excitation and 515±9 nm emission.

13.1. Yo-Pro-1 to quantify number of cells

Yo-Pro-1 (Life Technologies) was used at 2μM and was added to the cells after cell adhesion protocol together with Triton X-100 (2%) detergent in ET buffer for 30 min with shaking, cells were then incubated during 5 min on ice and then were shaken again during another 30 min before fluorescence reading. Yo-Pro-1 fluorescent signal was proportional to the amount of DNA of adherent cells and therefore proportional to the total number of cells. This procedure allowed us to determine the relative number of cells that stay adherent after P2X7 receptor activation.

13.2. Yo-Pro-1 to study plasma membrane permeabilization

In this assay, the uptake of Yo-Pro-1 into the cells is monitored at real time during cell stimulation. Cells were incubated with 2.5 μM Yo-Pro-1 for 5 min, and then fluorescence was recorded every 10 s for 35 min at 37 ºC, before and after the addition of melittin or nigericin at different concentrations.

14. MTT assay

This assay is a colorimetric assay for assessing cell metabolic activity (Figure X) and therefore is an indirect measurement of cell viability and cell proliferation. In this Thesis, MTT was used as an indirect measurement of the number of cells adhered after P2X7R activation.

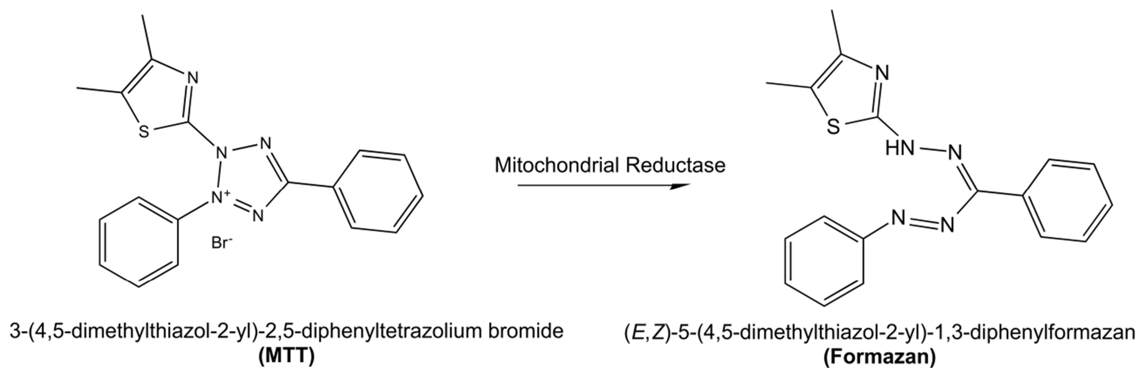


Figure 19. The reduction of tetrazolium salts, such as yellow colored salt 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is converted at mitochondria of living cells through dehydrogenase activity to the purple-colored dye formazan, which can be quantified easily using a plate reader at a certain wavelength (between 550 and 600nm).

MTT (Sigma Aldrich) was prepared as stock solution on DMSO at 5mg/ml stock solution. After cell adhesion treatment (see above), cells were washed with ET and MTT (250µg/ml) was added to the cells on a 150µl final volume of medium. Cells were incubated for 4 h at 37ºC and 5% CO_2h. Then, cell culture plate was centrifuged at 433 x g during 5 min, to aim the formazan crystal precipitation. Supernatants were discarded and 200µl DMSO was added to dissolve the crystals for 30 min with vigorous shaking. Finally, absorbance was read at 590nm with a reference absorbance at 620nm on a Sinergy MX plate reader (BioTek).

15. Lactate dehydrogenase detection assay

Extracellular lactate dehydrogenase (LDH) measurement was used to determine cell death due to plasma membrane permeabilization. The LDH Cytotoxicity assay kit (Roche) was used following the manufacturer indications. In brief, clarified supernatatns samples were dispensed into wells of a 96-wells plate and were diluted 1:4 in ET. Cell extracts from untreated cells were diluted 3:100 in ET and were used to obtain the total cellular LDH content. 100µl of LDH detection reagent was added to each sample and the reaction was incubated for 8 min at RT in the dark. Absorbance was then read at 492nm on a Sinergy MX plate reader (BioTek).

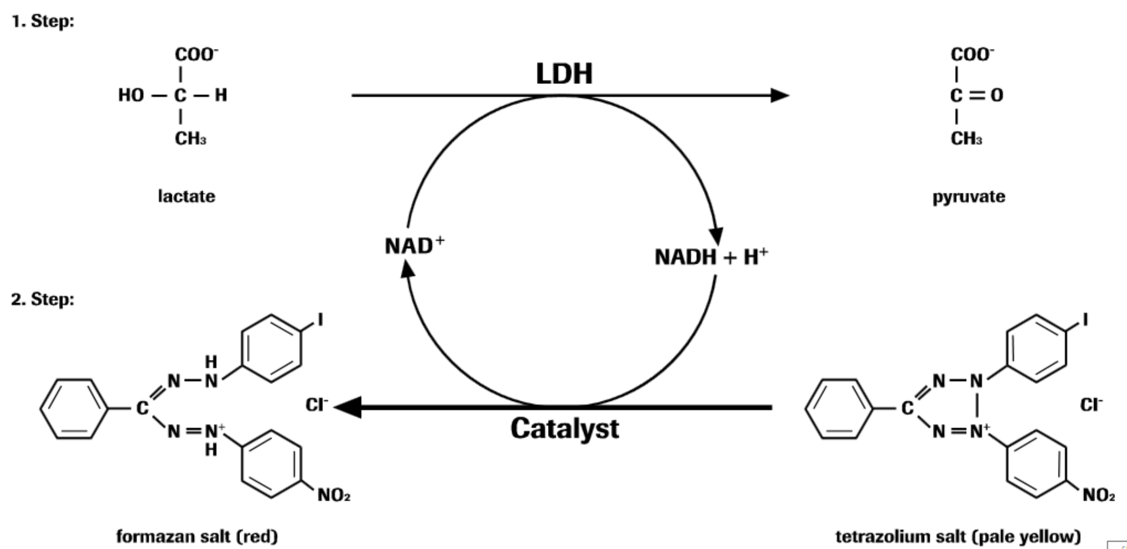


Figure 20. Representation of LDH extracellular release assay principle. NAD^+ is reduced to $NADH^+/H^+$ due to LDH by lactate oxidation to pyruvate. Subsequently, hydrogens are transferred from $NADH^+/H^+$ to the yellow tetrazolium salt 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) by a catalyst.

16. Real time cell analysis for cell adhesion

Real time cell analysis (RTCA) is a used method to initiate a dynamic monitoring of cell adhesion and spreading. This method is based essentially on the microelectronic E-plates. The functional unit of a cellular impedance assay is a set of gold microelectrodes fused to the bottom surface of a microtiter plate well. Once in an electrically conductive solution, there is an electric potential that allows the electron movement from the negative terminal, to the positive terminal. When cells are plated in to this system, there is impedance against the electronic movement, due to cell adhesion. This effect could be calculated by the RTCA Acea software using the cell index. In this measurement, cell adhesion is directly proportional to impedance to electron flow, and cell index.

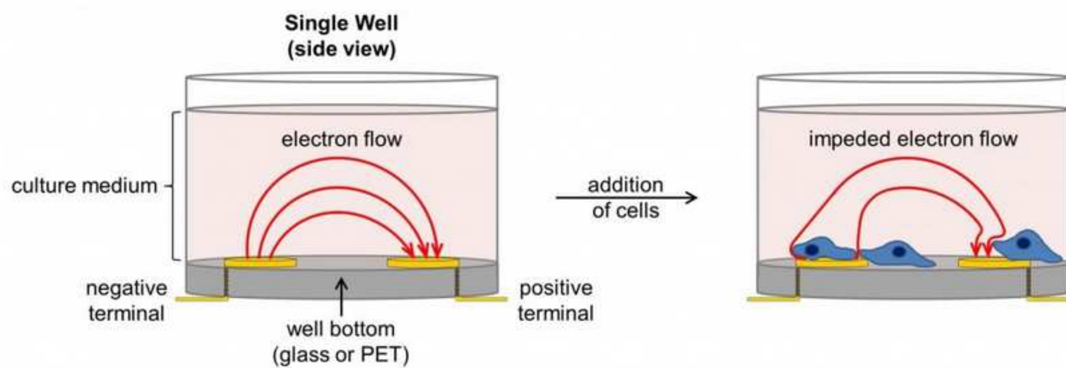


Figure 21. Overview of cellular impedance system used on RTCA analysis. Impeded electron flow is directly proportional to cell adhesion.

To assess the cell adhesion on BMDMs, different cells confluences were used at different confluences: 150000, 100000, 75000, and 50000 cells per well, as untreated or LPS primed cells at 90000 cells per well confluence. Cells were plated on 96-well microelectronic E-Plates (ACEA Biosciences) and instantaneously, baseline was calculated using the Real Time Cell Analyser (ACEA Biosciences). Cells were incubated at 37ºC and 5% CO_2 into the RTCA. Measurement was at 37ºC during 24 h.

17. Statistical analysis

Statistics were calculated with Prism software (GraphPad Software Inc.). Normality of the samples was determined with D'Agostino and Pearson omnibus K2 normality test and samples did not follow a Gaussian distribution, therefore non-parametric tests were performed. Mann-Whitney test was used to analyse differences between nonpaired two groups, Wilcoxon test was used to compare two paired groups, and Kruskal-Wallis test was used to analyse differences between three or more groups. The c2 test was used to determine whether there is a significant difference between different clinical variables among groups of septic patients. Pearson correlation was used to measure linear correlation between two variables. Kaplan-Meier was used to estimate the survival of septic patients and to compare the survival distributions of immunocompromised and non-immunocompromised samples the log-rank test was used. ROC analysis was used for sensitivity and specificity assessment of the release of different cytokines and formation of intracellular ASC specks to differentiate septic patients vs control groups, and inside septic patients to differentiate non immunocompromised vs immunocompromised individuals.

1.1. RESULTS

1. INFLAMMATORY RESPONSE INDUCED BY VENOMS AND INFECTIONS

1.1. Melittin activates NLRP3 inflammasome

1.1. Melittin activates the NLRP3 inflammasome

Cationic amphipathic antimicrobial peptides can permeabilize the plasma membrane of eukaryotic cells (Huang 2000) and are therefore candidates to activate the NLRP3 inflammasome. Different antimicrobial peptides were tested at 10 μM on LPS-primed BMDM for IL-1β release, with melittin (the major component of bee venom) being the only one able to induce robust release of IL-1β (Figure 22). In contrast, at this concentration, LL-37 induced only scant release of IL-1β (Figure 22). Temporin A and indolicidin failed to trigger significant secretion of IL-1β from macrophages (Figure 22), although indolicidin did have strong effects on the membrane of mammalian cells **(Subbalakshmi, Krishnakumari, and Nagaraj 1996)**.

Low intracellular K^+ concentration is a key step in NLRP3 activation, and it has been previously reported that bee venom-induced release of IL-1β was blocked using a high extracellular K^+ solution (Palm and Medzhitov 2013). In our study, we found that THP-1 macrophage incubation with melittin induced a significant decrease in intracellular K^+ concentration (Figure 23a). Furthermore, macrophage incubation with melittin in a buffer with a high K^+ concentration reduced IL-1β release from THP-1 macrophages (Figure 23b). BMDMs from NLRP3-deficient mice failed to release IL-1β after melittin treatment (Figure 23c). The release of IL-1β induced by melittin treatment was found to be the mature p17 form of the cytokine, as well as some pro-IL-1β (Figure 24a). IL-1β release induced by melittin was dependent on caspase-1 (Figure 24b). These data suggest that the decrease in intracellular K^+ in LPS-primed macrophages after melittin treatment triggers the NLRP3 inflammasome and activates caspase-1. Despite the induction of IL-1β release via caspase-1 activation, melittin failed to induce IL-18 release (Figure 25).

The NLRP3 inflammasome requires the adaptor protein ASC to activate caspase-1 (Schroder and Tschopp 2010; Compan et al. 2012). Accordingly, melittin-induced IL-1β release was reduced in ASC-deficient macrophages (Figure 26). This suggests that, melittin may activate caspase-1 via NLRP3 inflammasome using the ASC adaptor protein.

We then analysed if melittin was able to induce pyroptosis, which is a cell death process dependent on caspase-1 important for the release of IL-1β and mediated by GSDMD processing (Yeretssian, Labbé, and Saleh 2008; Dick et al. 2016). Cell death induced by melittin was dose-dependent reaching a plateau (Figures 27a and b), with a melittin LD_{50} of approximately 5 μM in both THP-1 and BMDMs. Similarly, the release of IL-1β was dose-dependent for small concentrations of melittin, but cytokine release decreased at high concentrations of melittin (≥ 10 μM) (Figures 27c and d), suggesting that an excessive rate of cell death by rapid cellular lysis induced by melittin leads to reduced NLRP3 inflammasome activation. While nigericin-induced cell death was dependent on NLRP3 and ASC (Figure 28a), melittin-induced cell death was independent on the NLRP3 inflammasome (Figure 28a). In line with this result, caspase-1 deficiency did not affect melittin cell death (Figure 28a). Furthermore, high extracellular K^+ reduced nigericin but not melittin-induced cell death (Figure 28b). This result confirms that nigericin was inducing pyroptosis dependent on inflammasome and caspase-1 activation, whereas melittin-induced cell death was independent of the inflammasome. Finally, addition of melittin to macrophages immediately induced rapid (≤1 min) plasma membrane permeabilization (Figures 28d). However, nigericin induced a delayed plasma membrane permeabilization (after 5 min) (Figure 28c), consistent with the time required time to activate caspase-1 and induce pyroptosis by GSDMD cleavage and insertion into the plasma membrane. In fact, caspase-1 deficiency impaired plasma membrane permeabilization induced by nigericin (Figure 7c), meanwhile melittin-induced plasma membrane permeabilization was independent on caspase-1 (Figure 28d). Altogether, our data show that melittin directly permeabilizes the plasma membrane of macrophages, inducing K^+ leakage and rapid activation of the NLRP3 inflammasome in order to activate caspase-1 via ASC and induce IL-1β maturation and release. Because macrophage viability and plasma membrane integrity were rapidly and directly compromised by melittin, this impaired the subsequent execution of pyroptosis mediated by caspase-1.

FIGURES:

Melittin activates the NLRP3 inflammasome

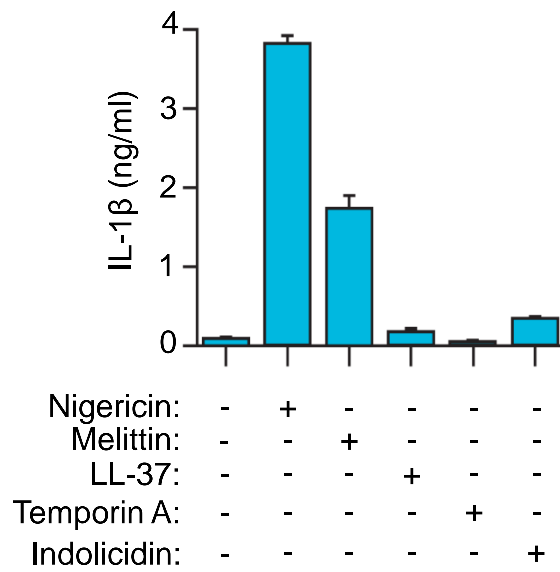
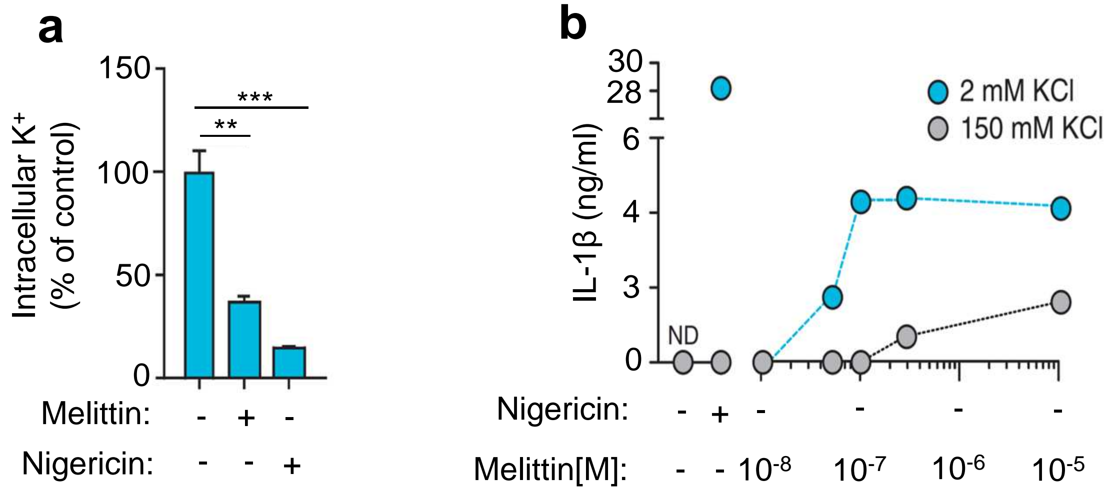

IL-1β (ng/ml)
0
1
2
3
4
Nigericin: - + - - - -
Melittin: - - + - - -
LL-37: - - - + - -
Temporin A: - - - - + -
Indolicidin: - - - - - +

Figure 22. Melittin, but no other antimicrobial peptides, induces release of IL-1β. ELISA for IL-1β release from BMDMs primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM), melittin, LL-37, temporin A or indolicidin (10 μM each).


a
Intracellular K+ (% of control)
150
100
50
0
***
**
Melittin: - + -
Nigericin: - - +
b
IL-1β (ng/ml)
30
28
6
4
3
0
ND
2 mM KCl
150 mM KCl
Nigericin: - + - - - -
Melittin[M]: - - 10-8 10-7 10-6 10-5

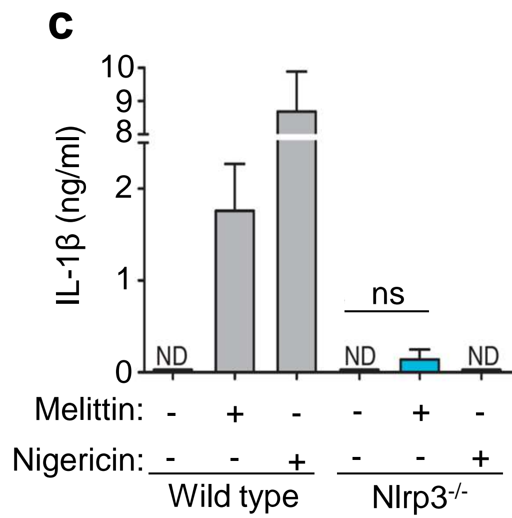
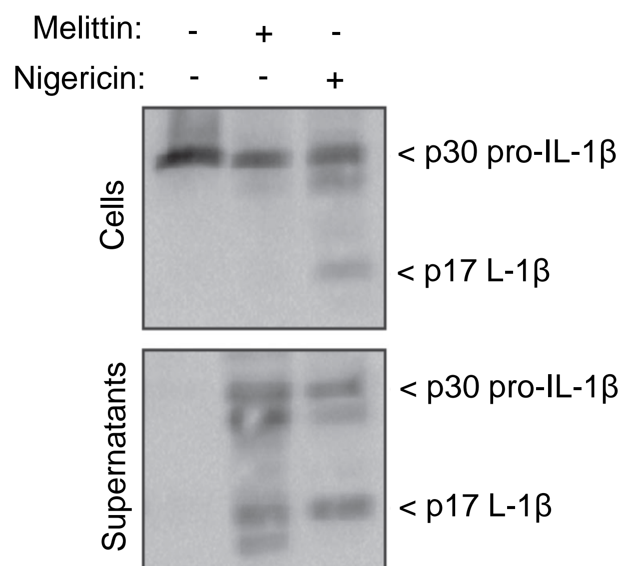

c
IL-1β (ng/ml)
10
9
8
2
1
0
ns
ND
ND
ND
Melittin: - + - - + -
Nigericin: - - + - - +
Wild type
Nlrp3-/-

Figure 23. Melittin induces K^+ depletion and NLRP3 activation. (a) Relative intracellular K^+ concentration from THP-1 primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM) or melittin (5 μM). **(b)** ELISA for IL-1β release from THP-1 primed as in panel (a), and then stimulated or not for 30 min with nigericin (25 μM) or melittin in normal K^+ buffer (2 mM KCl, blue circles) or high K^+ buffer (150 mM KCl, gray circles) at the doses shown. **(c)** ELISA for IL-1β release from wildtype or *Nlrp3*$^{-/-}$ BMDMs primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM) or melittin (0.5 μM); $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was done for a; ND, not detected.


a
Melittin: - + -
Nigericin: - - +
Cells
Supernatants
< p30 pro-IL-1β
< p17 L-1β
< p30 pro-IL-1β
< p17 L-1β

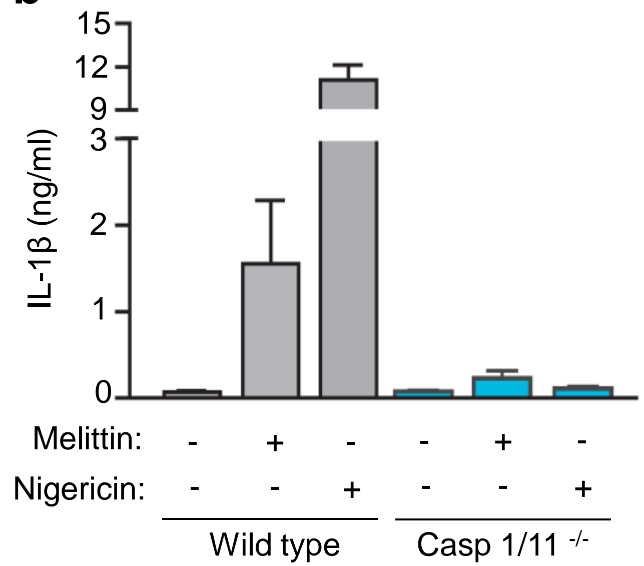
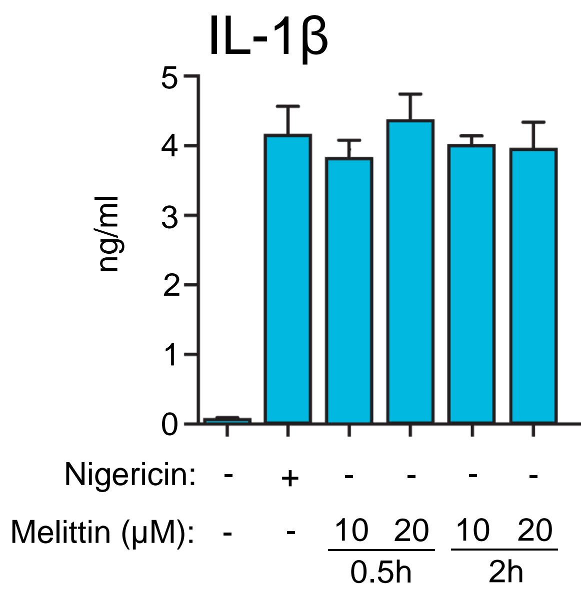

b
IL-1β (ng/ml)
15
12
9
3
2
1
0
Melittin: - + - - + -
Nigericin: - - + - - +
Wild type
Casp 1/11 -/-

Figure 24. Melittin activates caspase-1 in macrophages. (a) Western blot analysis of IL-1 β in cell extracts and supernatants from BMDMs primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM) or melittin (5 μM). **(b)** ELISA for IL-1β release from wild type or *Casp1/11*$^{-/-}$ BMDMs primed with LPS (1 μg/ml, 4 h), and activated or not for 30 min with nigericin (25 μM) or melittin (0.5 μM).


IL-1β
ng/ml
5
4
3
2
1
0
Nigericin: - + - - - -
Melittin (μM): - - 10 20 10 20
0.5h
2h

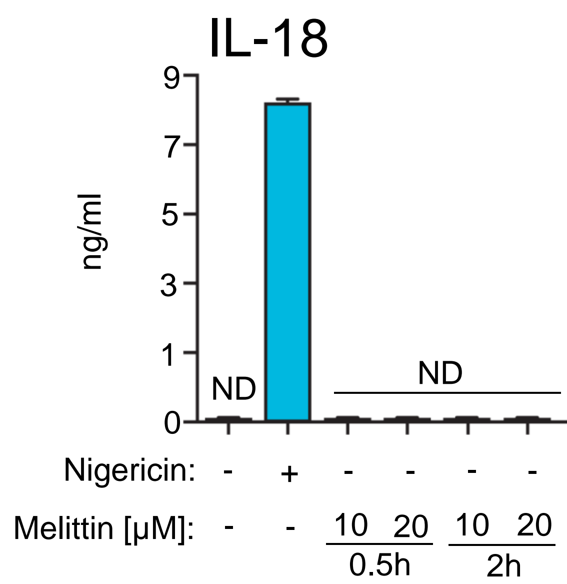

IL-18
ng/ml
9
7
5
3
1
0
ND
ND
Nigericin: - + - - - -
Melittin [μM]: - - 10 20 10 20
0.5h
2h

Figure 25. Melittin does not induce IL-18 release. ELISA for IL-1β or IL-18 release (as indicated in the graph) from BMDMs primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM) or the melittin concentrations and times shown; ND, not detected.

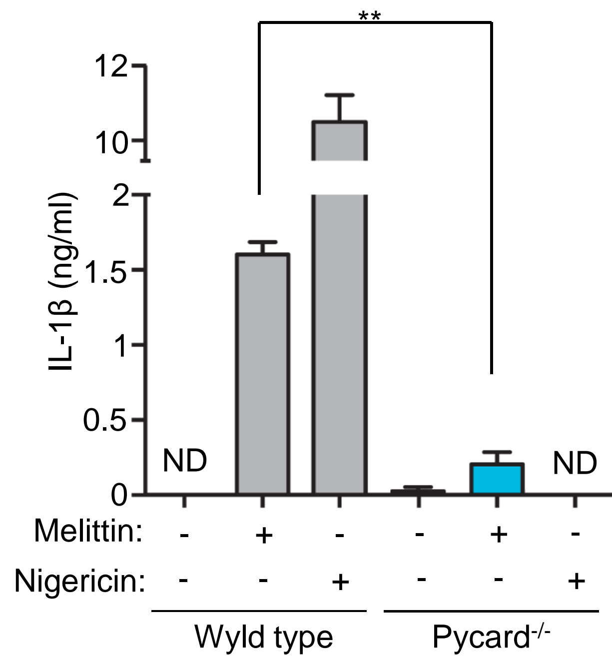

**
12
10
2
1.5
1
0.5
0
IL-1β (ng/ml)
ND
ND
Melittin: - + - - + -
Nigericin: - - + - - +
Wyld type
Pycard-/-

Figure 26. IL-1β release induce by melittin is dependent on ASC. ELISA for IL-1β release from wild type or *Pycard*$^{-/-}$ BMDMs primed with LPS (1 μg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 μM) or melittin (0.5 μM); $^{**}p < 0.01$; Kruskal-Wallis test was done; ND: not detected.

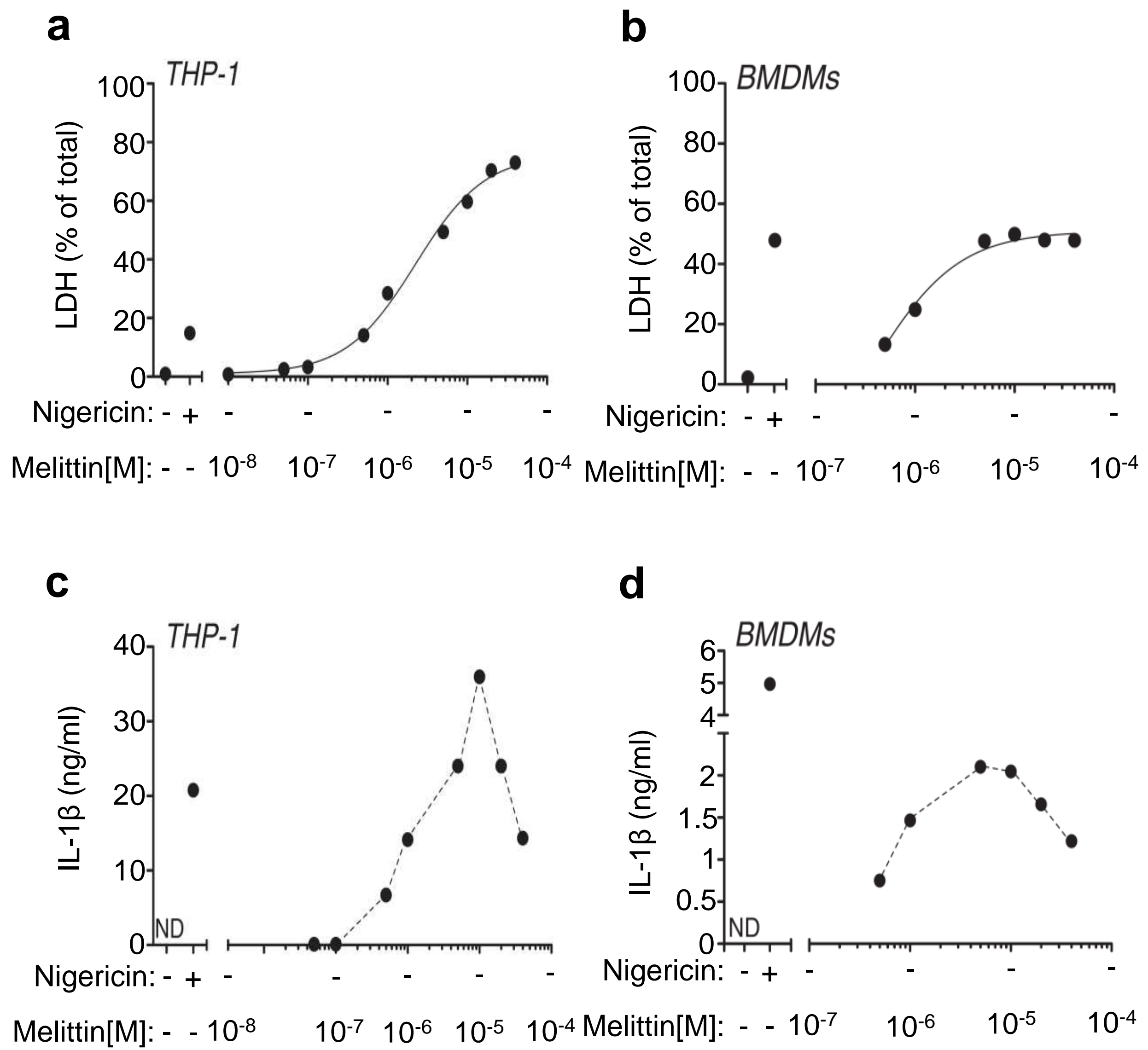

a
THP-1
LDH (% of total)
100
80
60
40
20
0
Nigericin: - + - - - - -
Melittin[M]: - - 10-8 10-7 10-6 10-5 10-4
b
BMDMs
LDH (% of total)
100
80
60
40
20
0
Nigericin: - + - - - -
Melittin[M]: - - 10-7 10-6 10-5 10-4
c
THP-1
IL-1β (ng/ml)
40
30
20
10
0
ND
Nigericin: - + - - - - -
Melittin[M]: - - 10-8 10-7 10-6 10-5 10-4
d
BMDMs
IL-1β (ng/ml)
6
5
4
2
1.5
1
0.5
0
ND
Nigericin: - + - - - -
Melittin[M]: - - 10-7 10-6 10-5 10-4

Figure 27. Melittin induces cell death in human and murine macrophages. (a,b) Dose–response curves for cell death in THP-1 **(a)** or BMDMs **(b)** primed with LPS (1 µg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 µM, as positive control) or with the indicated concentrations of melittin for 30 min. **(c,d)** Dose–response for IL-1β release detected by ELISA from THP-1 **(c)** or BMDMs **(d)** treated as in a and b. ND: not detected.

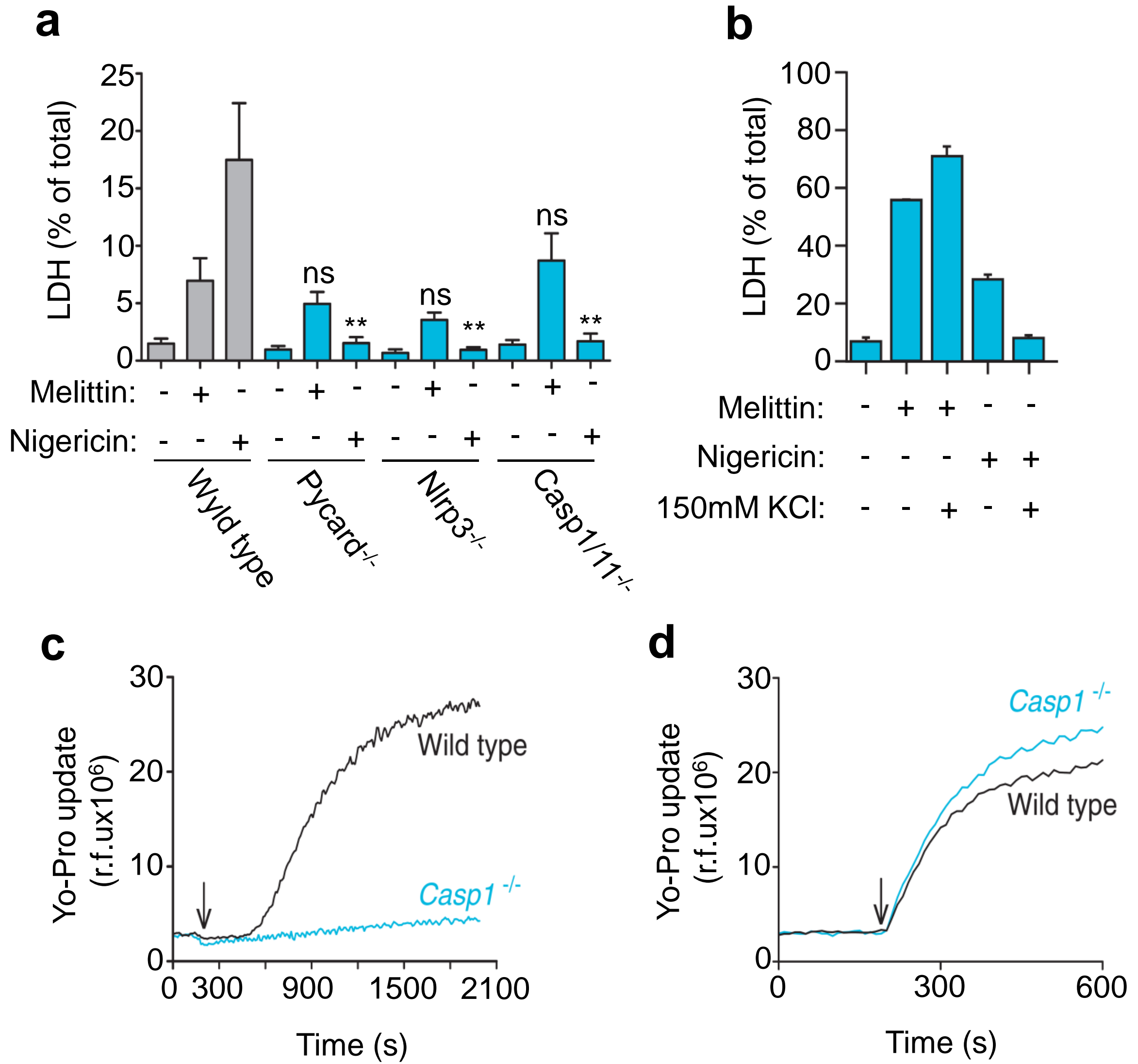

a
LDH (% of total)
25
20
15
10
5
0
ns
ns
ns
**
**
**
Melittin: - + - - + - - + - - + -
Nigericin: - - + - - + - - + - - +
Wyld type
Pycard-/-
Nlrp3-/-
Casp1/11-/-
b
LDH (% of total)
100
80
60
40
20
0
Melittin: - + + - -
Nigericin: - - - + +
150mM KCl: - - + - +
c
Yo-Pro update (r.f.ux10⁶)
30
20
10
0
0 300 900 1500 2100
Time (s)
Wild type
Casp1 -/-
d
Yo-Pro update (r.f.ux10⁶)
30
20
10
0
0 300 600
Time (s)
Casp1 -/-
Wild type

Figure 28. Melittin-induced cell death is independent of the inflammasome and caspase-1. (a) Cell death measured as increase of extracellular LDH in wild type, *Pycard*$^{-/-}$, *Nlrp3*$^{-/-}$ or *Casp1/11*$^{-/-}$ BMDMs primed with LPS (1 µg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 µM) or melittin (0.5 µM). **(b)** Cell death measured as increase of extracellular LDH in THP-1 primed with LPS (1 µg/ml, 4 h), and then stimulated or not for 30 min with nigericin (25 µM) or melittin (5 µM) in normal K^+ buffer (2 mM KCl) or high K^+ buffer (150 mM KCl). **(c,d)** Kinetics of Yo-Pro uptake in wild type or *Casp1/11*$^{-/-}$ BMDMs primed as in (a), and stimulated with nigericin (5 µM) **(c)** or melittin (20 µM) **(d)**; the arrow denotes the time of nigericin or melittin administration; $^{**}p < 0.01$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was done for a.

1.2. The inflammatory response in sepsis

1.2. The inflammatory response in severe sepsis

To gain insight into the inflammatory response during infection, we analysed a cohort of intra-abdominal origin septic patients (n= 35, Table 4). Peritoneal isolation of microorganisms in this cohort of patients showed that the majority of detected infections were polymicrobial developed by different gram negative bacteria (Table 5). Especially *E. coli* isolation was present in almost 50% of patients, followed by *Prevotella sp., Klebsiella sp.,* and *Bacteroides sp.* Less frequent isolated gram negative microorganisms were *P. aeruginosa, Enterobacter sp., Citrobacter sp.,* and *Proteus mirabilis*. Between gram positive isolated bacteria, *Streptococcus sp.,* and *Enterococcus sp.* were frequently found. In addition, *Staphylococcus sp.* and *Clostridium perfringens* isolations were only found in a single patient. Furthermore, *Candida sp.* fungal isolation was also frequent; and in 3 patients microorganism isolation was negative, but exploratory laparoscopy showed symptoms of intra-abdominal infection.

Septic patients presented elevated levels of CRP and PCT in the plasma 24 h after sepsis initiation when compared to a control group of abdominal surgery patients that did not developed sepsis (Figure 29a). Septic patients presented an averaged APACHE II score of 18.6 ± 7.7 (range 5 to 52) and SOFA score of 6.4 ± 2.8 (range 2 to 12), with dysfunction of one or more vital organs, together with an increase in the blood of neutrophils, lactate, NTproBNP, creatinine, bilirubin and fibrinogen (Figure 31 and Table 4 and 6). On the contrary, the amount of hemoglobin and bicarbonate in blood was reduced when compared to the standards of healthy individuals (Figure 31). As expected, IL-6 and IL-8 cytokines were also elevated in the plasma of septic patients when compared to control surgery and healthy groups (Figure 29b), suggesting that our cohort of septic patients presented elevated systemic inflammation and supporting the diagnostic use of these cytokines to identify septic patients **(Harbarth et al. 2001)**. At this time point, the inflammasome-related cytokines IL-1β, IL-18 and the alarmin HMGB1 were elevated in septic patients when compared to control groups (Figure 29c). However, the concentration of IL-18 in plasma was not associated to the death of septic patients (1.20±0.24 vs 1.02±0.17 ng/ml survival n= 24 vs non-survival n= 12 respectively, $p=0.5798$, with an area under the curve in the receiver operating characteristic (ROC) analysis of 0.559±0.096). In addition, circulating ASC specks in plasma were elevated in septic patients when compared to control

surgery and healthy groups (Figure 29d). These results confirm the previous evidences where ASC specks are elevated in the plasma during systemic inflammation (Baroja-Mazo et al. 2014). The percentage of monocytes with active caspase-1 was found elevated in septic patients when compared to healthy controls and other patients that develop different autoinflammatory diseases (Figure 29e). However, these data show that during the first 24h after intra-abdominal sepsis development there is an activation of the inflammasome that contribute to the cytokine storm and inflammatory status found during initial phase of sepsis.

Surprisingly, we did not find significant increase of monocytes with active caspase-1 in samples from patients suffering from different monogenic autoinflammatory disorders such as CAPS, APLAID, PAAND and FMF when compared with healthy controls (Figure 29e). However, PBMCs primed with LPS displayed an increase of caspase-1 activation in monocytes from the auto-inflammatory patients when compared to monocytes isolated from healthy donors (Figure 30a and b). Caspase-1 activation was increased after ATP-induced activation (Figure 30a and b) as well as after CdtB treatment in monocytes from PAAND and FMF patients (Figure 30b). These results support previous evidences showing that the inflammasome pathway is constitutively upregulated in monocytes from autoinflammatory patients after LPS priming **(Chae et al. 2015; Masters et al. 2009; Dinarello 2009)**.

Despite elevated concentration of IL-1β in the plasma of septic patients, PBMCs from septic patients present a decreased release of IL-1β upon NLRP3 inflammasome activation induced by extracellular ATP stimulation (Figure 32a). We found that approximately half of the septic patients analysed for cytokine production (10 out of 23) released very low IL-1β after ATP stimulation (from 0 to 81 pg/ml) compared to the median release of IL-1β from the septic patients (264.3 pg/ml). This allowed us to initially stratify septic patients into two groups, a group of patients that released a small concentration of IL-1β upon NLRP3 activation (<100 pg/ml) and another group that released higher concentration of IL-1β with no significant differences when compared to control groups (Figure 32b). ROC analysis segregating septic patients into these two groups identified cut-off values for these parameters with a high sensitivity and specificity to identify a group of septic patients with immunocompromised innate immunity (Table 7), presenting also a

defect in the production of other cytokines. ROC analysis showed that IL-1β release after NLRP3 inflammasome activation was the most significant parameter to differentiate septic patients from control groups (Table 7). The release of IL-1β was significant in the ROC analysis to differentiate the group of immunocompromised patients inside the septic group, with an area under the curve of 0.98 (Table 7). ROC analysis allowed us to stratified 11 immunocompromised septic patients that presented at least 4 of the 5 parameters under the cut off value and all identified within the first 24 h of sepsis onset (Table 7). We next found that this group of immunocompromised patients accumulated over 80% of the deaths registered on the group of septic patients during their stay at the Surgical Critical Unit (Figure 32c, Table 6). The deaths on immunocompromised septic patients started from day 9 onwards, while the only death registered on non-immunocompromised patients was at day one (Figure 32d). Different biochemical and clinical scores used to assess the severity of septic patients at day 1 were not able to discriminate the group of immunocompromised septic patients (Figure 32e, f and 33a, b), such as CRP (Figure 11e), SOFA and APACHE II scores (Figure 11f), percentage of monocytes, number of neutrophils (Figure 33a), PCT, lactate, NT-proBNP, creatinine bilirubin, hemoglobin, carbonate (HCO_3) and fibrinogen (Figure 33b). Infection type was also not different among both groups (Tables 2 and 3). Furthermore, plasma cytokines, monocytes with active caspase-1 or circulating ASC-specks were also similar between immunocompromised and non-immunocompromised patients (Figure 33c,d). However, immunocompromised septic patients presented more renal dysfunction (54.5 vs 25% of non-immunocompromised septic patients), more respiratory dysfunction (81.8 vs 50%), and more cardiovascular dysfunction (90.9 vs 41.7%), as well as immunocompromised patients suffer more nosocomial infections (63.6 vs 41.7%). However, any of these differences were not statistically significant (Table 6) as well as differences in distribution of isolated bacteria between immunocompromised and non-immunocompromised patients (Table 5). Therefore, with the markers and clinical scores currently employed to determine sepsis severity during the first 24 h of disease development, the clinician cannot accurately predict the patients presenting an immunocompromised status with a high probability of death. Mortality was not the only late-outcome associated to early immunocompromised septic patients, and the evolution of clinical parameters as ΔSOFA (calculated as the change in SOFA from day 1 to day 5) was stable or decreased in immunocompromised septic patients that died (Figure 32g). This confirms that ΔSOFA is a good late-prognostic marker for sepsis related deaths **(Alan E.**

Jones 2009); meanwhile innate immunoparalysis emerge as an early prognostic marker. Also, immunocompromised septic patients presented a longer stay at the surgical critical unit and required more days of mechanic ventilation (Figure 32h), suggesting a worst outcome for the patients that presented early impairment of NLRP3 activation.

We have shown that inflammasome markers and acute phase proteins increased during the first 24 hours of infection. However, plasma concentration of CRP, PCT and IL-6 decreased after 3 and 5 days of sepsis (Figure 34a), denoting a gradual decrease of sepsis-related systemic inflammation. The decrease of CRP, PCT and IL-6 in plasma with the time was similar in both immunocompromised and non-immunocompromised groups (Figure 34c). Immunocompromised septic patients that survive (n=4), once recovered from the septic episode (120 days after), do not had detectable IL-6 in the plasma (Figure 34b) and were able to normally activate the NLRP3 inflammasome (Figure 34d). This result suggests that NLRP3 inflammasome immunoparalysis during sepsis is transitory.

FIGURES:

The inflammatory response in sepsis

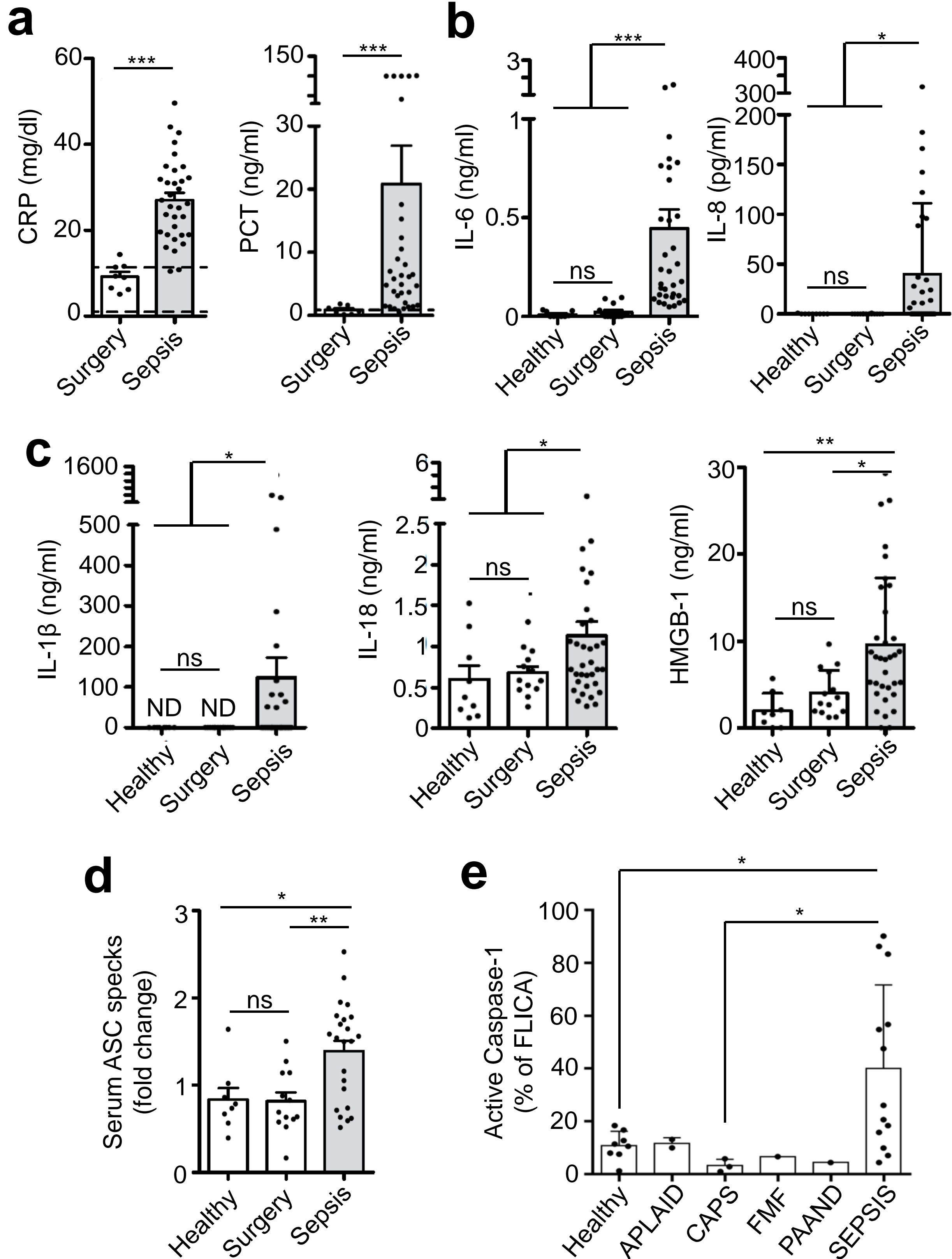
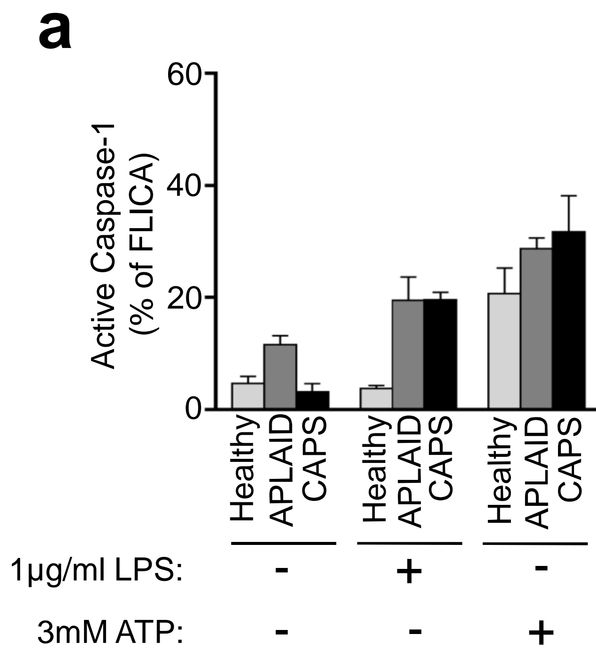

a
CRP (mg/dl)
PCT (ng/ml)
Surgery
Sepsis
b
IL-6 (ng/ml)
IL-8 (pg/ml)
Healthy
Surgery
Sepsis
ns
c
IL-1β (ng/ml)
IL-18 (ng/ml)
HMGB-1 (ng/ml)
ND
d
Serum ASC specks
(fold change)
e
Active Caspase-1
(% of FLICA)
Healthy
APLAID
CAPS
FMF
PAAND
SEPSIS

Figure 29. Septic patients present elevated inflammasome markers, pro-inflammaotry cytokines and acute phase proteins in the plasma. (**a-c**) Plasma concentration of CRP, PCT **(a)**, IL-6, IL-8 **(b)**, IL-1β, IL-18 and HMGB1 **(c)** in healthy controls, abdominal surgery patients within the first 24 h after surgery and intra-abdominal origin septic patients within the first 24 h of admission at the surgical critical unit; dotted lines in (a) represent standard threshold for CRP and PCT in healthy population. **(d)** Fold increase of circulating ASC specks in the plasma of surgery controls and septic patients compared with healthy controls. **(e)** Active caspase-1 measured by FLICA staining in monocytes from healthy controls (n=8), APLAID patients (n=2), CAPS patients (n=3), FMF patient (n=1), PAAND patient (n=1) and septic patients (n=13). Each dot represents an individual patient; *p< 0.05; **p< 0.01; ***p< 0.001; *ns*, no significant difference (p> 0.05); Mann-Whitney test for a, Kruskal-Wallis test for b, c, d, e.


a
Active Caspase-1
(% of FLICA)
0
20
40
60
Healthy
APLAID
CAPS
1µg/ml LPS:
3mM ATP:
-
+

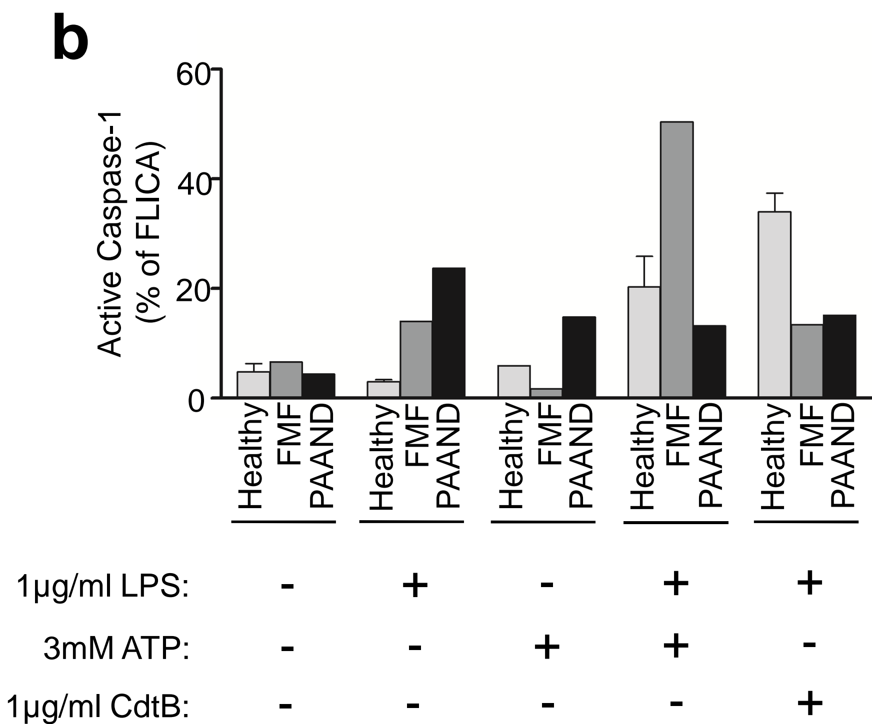

b
Active Caspase-1
(% of FLICA)
0
20
40
60
Healthy
FMF
PAAND
1µg/ml LPS:
3mM ATP:
1µg/ml CdtB:
-
+

Figure 30. Monocytes from patients with auto-inflammatory diseases have a distinct caspase-1 activation profile compared with healthy controls. (a,b) Active caspase-1 measured by FLICA staining in monocytes from CAPS and APLAID patients **(a)** or from FMF and PAAND patients **(b)** in comparison with healthy controls (n=6). Cells were primed *in vitro* with 1 µg/ml LPS during 2 h, and them stimulated or not for 30 minutes with 3 mM of ATP **(a,b)** or for 1 hour with 1µg/ml CdtB **(b)**.

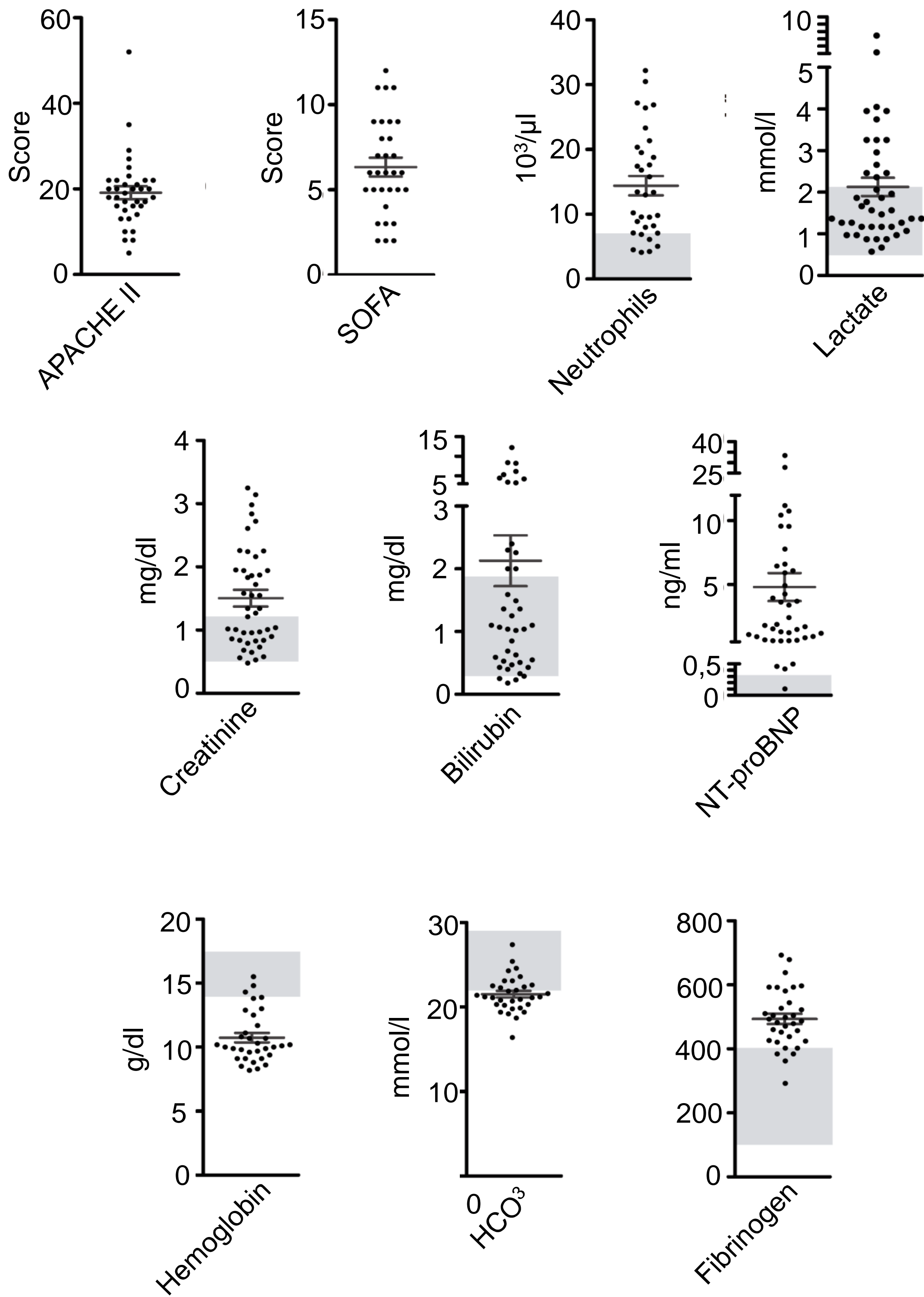

Score
APACHE II
Score
SOFA
10³/µl
Neutrophils
mmol/l
Lactate
mg/dl
Creatinine
mg/dl
Bilirubin
ng/ml
NT-proBNP
g/dl
Hemoglobin
mmol/l
HCO³
Fibrinogen

Figure 31. Clinical parameters of septic patients within the first 24 h of admission at the surgical critical unit (day 1). SOFA and APACHEII clinical score from septic patients enrolled in our study. Number of circulating neutrophils, concentration of lactate, NT-proBNP, creatinine, bilirubin, haemoglobin, $HCO3^-$ and fibrinogen in peripheral blood samples. Grey shadow on graphs indicates the normal range in healthy population for each parameter analysed. Each dot represents an individual patient.

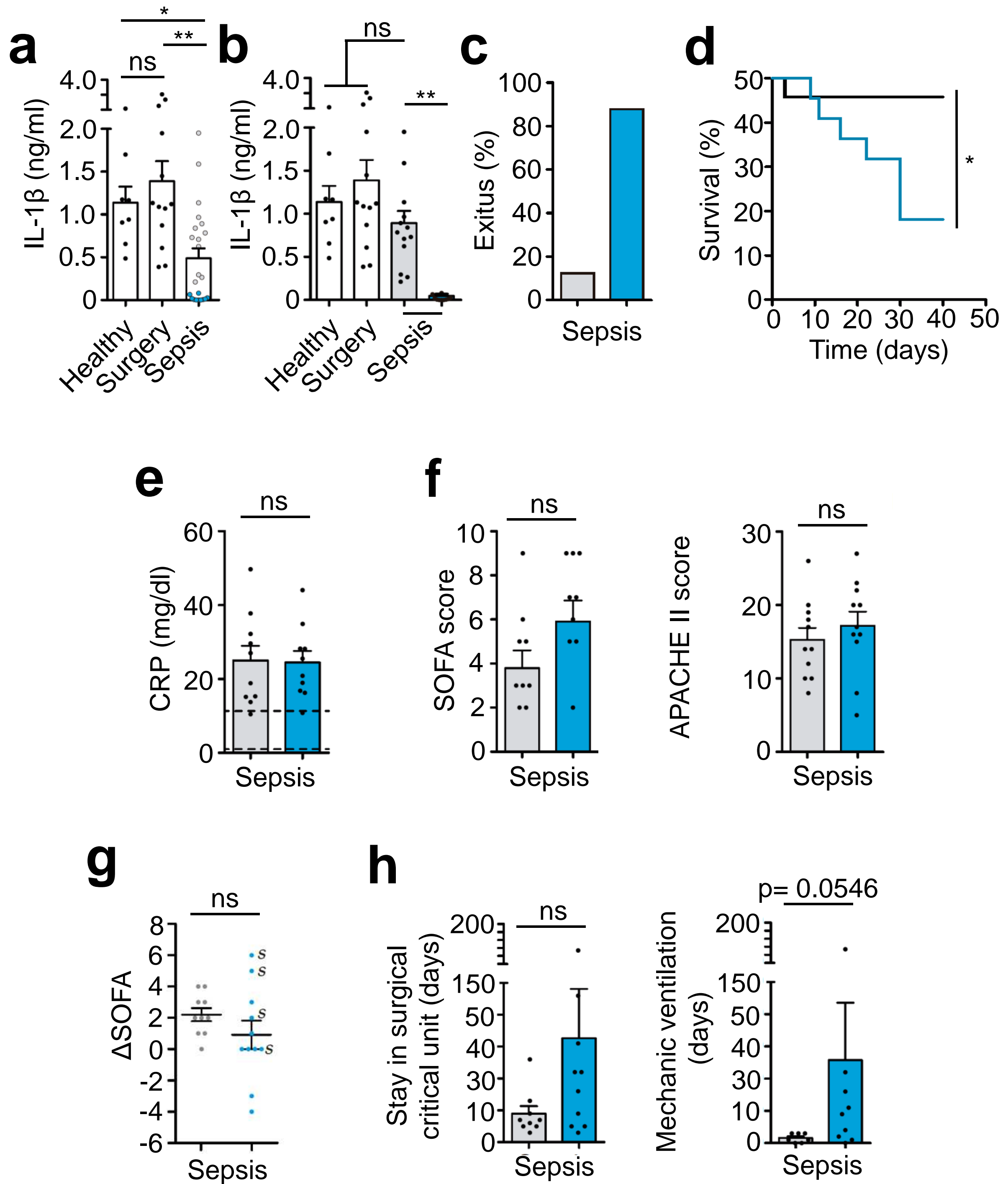

a
b
c
d
IL-1β (ng/ml)
Healthy
Surgery
Sepsis
ns
*
**
Exitus (%)
Survival (%)
Time (days)
e
CRP (mg/dl)
f
SOFA score
APACHE II score
g
ΔSOFA
h
Stay in surgical critical unit (days)
Mechanic ventilation (days)
p= 0.0546

Figure 32. Inflammasome activation is compromised in septic patients with high mortality. (a) ELISA for IL-1β in supernatants from PBMCs isolated from septic patients within the first 24 h of admission at the surgical critical unit and control groups after NLRP3 inflammasome activation by LPS (1 µg/ml, 2 h) and ATP (3 mM, 30 min) treatment. There is a group of septic patients that release little or no IL-1β (blue dots) and other group of septic patients that release IL-1β (grey dots). **(b)** IL-1β from same samples as in (a) but where the septic patients were separated into two groups: non-immunocompromised (the grey dots in (a), represented by a grey bar) and immunocompromised (the blue dots in (a), represented by a blue bar). **(c)** Percentage of mortality in non-immunocompromised (grey bar) and immunocompromised (blue bar) septic patients respect the total mortality of septic patients. **(d)** Kaplan-Meier representation of non-immunocompromised (grey line) and immunocompromised (blue line) septic patients' survival. **(e)** Concentration of C-reactive protein (CRP) in plasma of non-immunocompromised (grey bar) and immunocompromised (blue bar) septic patients at day 1; dotted lines represent threshold concentration of CRP for healthy population. **(f)** SOFA and APACHEII scores in non-immunocompromised (grey bar) and immunocompromised (blue bar) septic patients at day 1. **(g)** ΔSOFA calculated as the change in SOFA from day 1 to day 5; *s*: survival patient. **(h)** Days of stay on the surgical critical unit and days of mechanical ventilation for non-immunocompromised (grey bar) and immunocompromised (blue bar) septic patients. Each dot represents an individual patient; *$p < 0.05$; **$p < 0.01$; *ns*, no significant difference ($p > 0.05$); Long-rank test for b; Kruskal-Wallis test for a-b; and Mann-Whitney test for e-h.

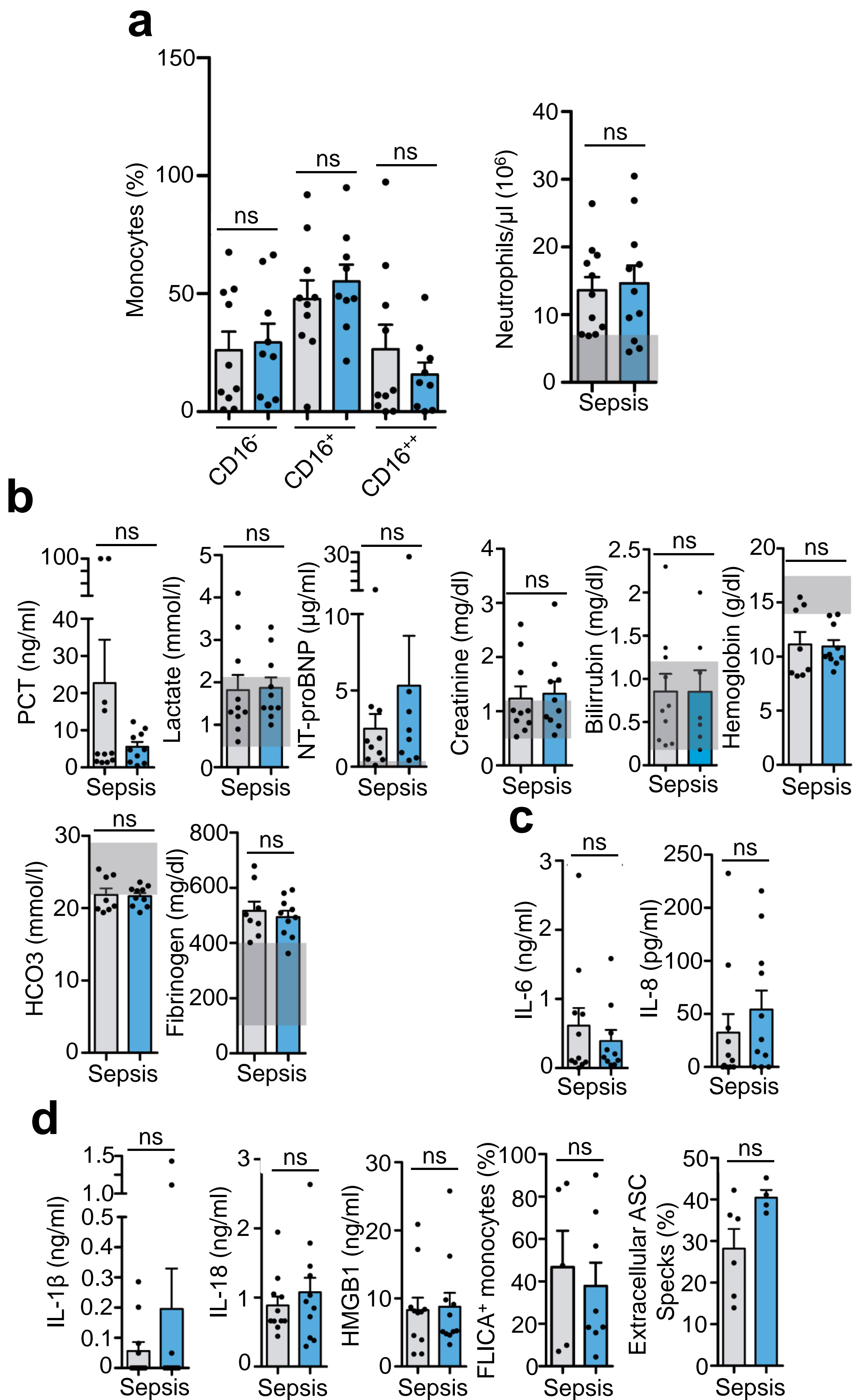
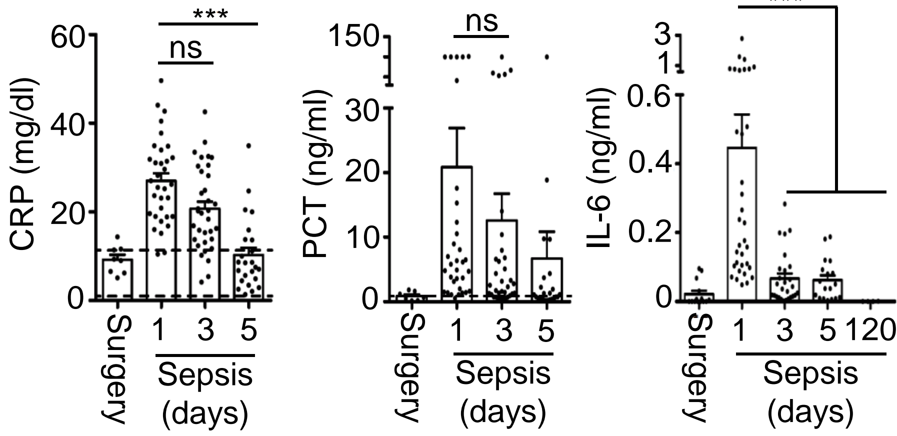
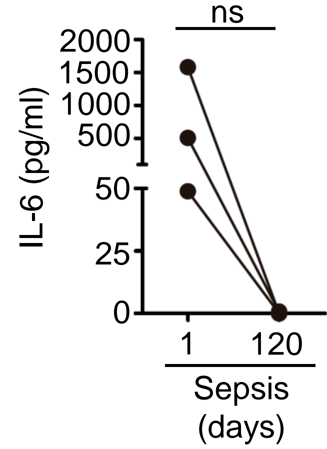
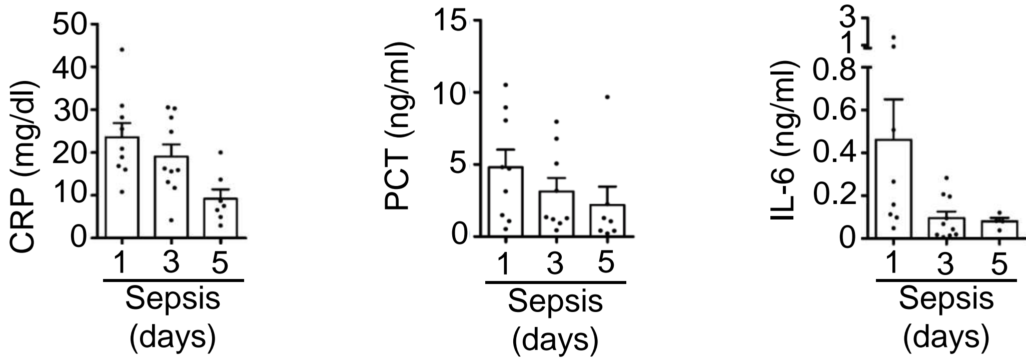
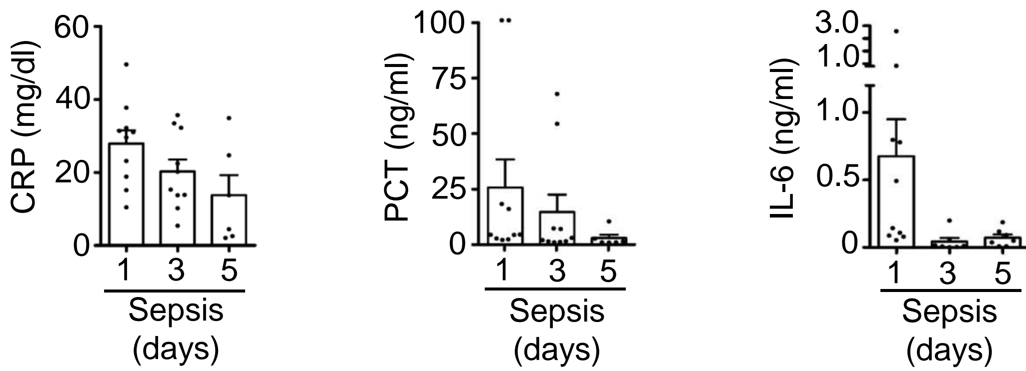

a
Monocytes (%)
150
100
50
0
ns
CD16-
CD16+
CD16++
Neutrophils/µl (10^6)
40
30
20
10
0
Sepsis
b
PCT (ng/ml)
Lactate (mmol/l)
NT-proBNP (µg/ml)
Creatinine (mg/dl)
Bilirubin (mg/dl)
Hemoglobin (g/dl)
HCO3 (mmol/l)
Fibrinogen (mg/dl)
c
IL-6 (ng/ml)
IL-8 (pg/ml)
d
IL-1β (ng/ml)
IL-18 (ng/ml)
HMGB1 (ng/ml)
FLICA+ monocytes (%)
Extracellular ASC
Specks (%)

Figure 33. Inflammatory response in immunocompromised septic patients. **(a)** Percentage of CD16 expressing populations on CD14^{+} monocytes (left) and number of circulating neutrophils (right) from non-immunocompromised (grey bars) and immunocompromised (blue bars) septic patients at day 1. **(b)** Different clinical markers in the blood from non-immunocompromised (grey bars) and immunocompromised (blue bars) septic patients at day 1; grey shadow on graphs indicate the normal range for each parameter analysed. **(c)** inflammasome activation non-dependent cytokines measured in plasma (IL-6 and IL-8). **(d)** inflammasome dependent pro-inflammatory cytokines in plasma (IL-1β, IL-18 and HMGB1), percentage of monocytes with active caspase-1 (FLICA^{+} monocytes) and presence of circulating ASC specks on plasma from non-immunocompromised (grey bars) and immunocompromised (blue bars) septic patients at day 1. Each dot represents an individual patient; *ns*, no significant difference ($p > 0.05$); Mann-Whitney test for a-d.


a
CRP (mg/dl)
PCT (ng/ml)
IL-6 (ng/ml)
Surgery
Sepsis
(days)
ns
***
**


b
IL-6 (pg/ml)
ns
Sepsis
(days)


c
Immunocompromised septic patients
CRP (mg/dl)
PCT (ng/ml)
IL-6 (ng/ml)
Sepsis
(days)


Non-immunocompromised septic patients
CRP (mg/dl)
PCT (ng/ml)
IL-6 (ng/ml)
Sepsis
(days)

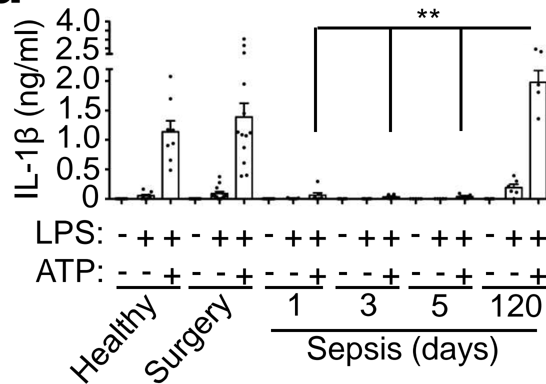

d
IL-1β (ng/ml)
LPS:
ATP:
Healthy
Surgery
Sepsis (days)
**

Figure 34. Immunoparalysis during sepsis is transitory. (a) Concentrations of CRP, PCT and IL-6 in plasma of septic patients at day 1, 3, 5 during sepsis and at day 120 after sepsis recovery; dotted lines represent normal concentration of CRP and PCT. Levels of these markers in septic patients were compared with abdominal surgery controls at 24 hours after surgery. **(b)** IL-6 concentration in plasma of immunocompromised septic patients at day 1 during sepsis and at day 120 after sepsis recovery. **(c)** Concentration of CRP, PCT and IL-6 in plasma of septic patients at day 1, 3, 5 during sepsis in immunocompromised and non-immunocompromised patients. **(d)** ELISA for IL-1β in PBMCs supernatants after NLRP3 inflammasome activation by LPS (1 µg/ml, 2h) and ATP (3 mM, 30 min) treatment from control groups and immunocompromised septic patients at day 1, 3, 5 during sepsis and at day 120 after sepsis recovery. Each dot represents an individual patient; **$p < 0.01$; ***$p < 0.001$; *ns*, no significant difference ($p > 0.05$); Kruskal-Wallis test for a and d.

TABLES:

The inflammatory response in sepsis

	Healthy controls	Abdominal surgery controls	Abdominal origin septic patients
N	16	11	35
Age, mean (range) ± SD	38.88 (21-65) ±13.98	60.7 (38-82) ± 14.10	69.33 (43-91) ±13.34
p value *vs* septic group	$p< 0.005$ ***	$p> 0.05^{ns}$	
Gender, N (%)			
Male	8 (50%)	6 (55%)	21 (60%)
Female	8 (50%)	5 (45%)	14 (40%)
p value *vs* septic group	$p> 0.05^{ns}$	$p> 0.05^{ns}$	
Clinical data (*only for septic patients*)			
Location, N (%)			
Stomach			3 (8.1%)
Duodenum			1 (2.7%)
Gallbladder			5 (13.5%)
Liver			1 (2.7%)
Small intestine			6 (16.2%)
Cecum			1 (2.7%)
Colon			18 (48.7%)
Sigmoid colon			1 (2.7 %)
Abdominal, unidentified			1 (2.7%)
Type of admission			
Medical, N (%)			13 (37.1%)
Surgical, N (%)			22 (62.7%)
APACHEII score at admission mean (range) ± SD			18.65 (5-52) ± 7.749
SOFA score at admission mean (range) ± SD			6.4 (2-12) ± 2.830
Days in Surgical Critical Unit mean (range) ± SD			18.33 (2-167) ± 28.47
Days of mechanic ventilation mean (range) ± SD			12.75 (0-167) ± 31.31
Exitus N (%)			12 (34.3%)

Table 4. Demographics and clinical characteristics of enrolled patients with severe sepsis of abdominal origin and control groups.

SD, standard deviation; *ns*, not significant difference ($p > 0.05$); Chi-square (χ^2) test was used, except for age that a one-way ANOVA test was used.

	Septic patients	Non-IC septic patients	IC septic patients
Peritoneal isolation, N (%)			
Gram-negative bacteria			
Escherichia coli	17 (48.6)	4 (36.4)	5 (41. 7)
Prevotella sp.	5 (14.3)	0	1 (8. 3)
Klebsiella sp.	5 (14.3)	1 (9.1)	3 (25)
Bacteroides sp.	5 (14.4)	2 (18.2)	1 (8.3)
Pseudomonas aeruginosa	2 (5.7)	0	1 (8.3)
Enterobacter sp.	2 (5.7)	0	0
Citrobacter sp.	2 (5.7)	0	0
Proteus mirabilis	1 (2.8)	1 (9.1)	0
Gram-positive bacteria			
Clostridium perfringens	1 (2.8)	0	0
Streptococcus sp.	8 (22.8)	1 (9.1)	3 (25)
Enterococcus sp.	8 (22.8)	2 (18.2)	1 (8.3)
Staphylococcus sp.	1 (2.8)	0	1 (8.3)
Fungi			
Candida sp.	4 (11.4)	2 (18.2)	1 (8.3)
Negative	3 (8.6)	2 (18.2)	0
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$

Table 5. Peritoneal microbial isolation at the moment of the initial surgery in non-immunocompromised and immunocompromised septic patients

IC, immunocompromised; *ns*, not significant difference ($p > 0.05$) with Chi-square (χ^2) test.

	Septic patients	Non-IC septic patients	IC septic patients
N	35	12	11
Age			
Mean (range) ± SD	69.33 (43-91) ± 13.34	68.73 (48-83) ± 14.79	74.64 (53-90) ± 11.64
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$
Gender, N (%)			
Male	21 (60)	6 (50)	6 (54.5)
Female	14 (40)	6 (50)	5 (45.5)
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$
Oncologic patients			
N (%)	11 (31.4)	5 (41.7)	6 (54.5)
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$
Organ dysfunnction, N (%)			
Renal	17 (48.6)	3 (25)	6 (54.5)
Respiratory	24 (68.6)	6 (50)	9 (81.8)
Cardiovascular	26 (74.3)	5 (41.7)	10 (90.9)
Hepatic	16 (45.7)	4 (33.3)	4 (36.4)
Hematological	5 (14.3)	1 (8.3)	0 (0)
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$
Infection acquired, N (%)			
Nosocomial	19 (54.3)	5 (41.7)	7 (63.6)
Community	16 (45.7)	7 (58.3)	4 (36.4)
p value against septic group		$p > 0.05^{ns}$	$p > 0.05^{ns}$
p value non-IC *vs* IC			$p > 0.05^{ns}$
Mortality			
N (%)	12 (34.3)	1 (8.3)	7 (63.6)
Initial multi-organic failure*	6 (50)	1 (100)	3 (42.8)
Secondary infections**	6 (50)	0	4 (57.2)

Table 6. Demographic and clinical data of non-immunocompromised and immunocompromised septic patients

IC, immunocompromised; SD, standard deviation; *ns*, not significant difference ($p > 0.05$) with Chi-square (χ^2) test.

*Dysfunction of one or more organs without clear recuperation after initial admission.

** Recuperation of initial organ dysfunction after admission, mortality due to acquisition of new infectious processes that could result in another multiple organ dysfunction.

	Median control	Median sepsis	ROC	AUC±SE	*p*	Cut-off	Sensitivity (%)	Specificity (%)
IL-1β	1121	264.3***	Control *vs* sepsis	0.83±0.06	0.00018	<856	78.2	76.2
			non-IC *vs* IC	0.98±0.02	<0.0001	<146	90.9	100
ASC-specks	31.55	8.6***	Control *vs* sepsis	0.95±0.03	<0.0001	<15.6	82.6	100
			non-IC *vs* IC	0.90±0.06	0.00111	<7.3	81.8	91.7
HMGB1	10.36	3.53^{ns}	Control *vs* sepsis	0.62±0.11	0.2482	<9.8	64.29	55
			non-IC *vs* IC	1	<0.0001	<3.5	100	100
IL-6	124	58.9*	Control *vs* sepsis	0.70±0.08	0.023	<70.9	60.9	73.7
			non-IC *vs* IC	1	<0.0001	<51.5	100	100
TNF-α	409.3	287.7^{ns}	Control *vs* sepsis	0.66±0.08	0.083	<253.5	65.2	73.7
			non-IC *vs* IC	1	<0.0001	<183.5	100	100
IL-8	872.2	1488^{ns}	Control *vs* sepsis	0.53±0.10	0.7918	>998.2	61.1	70.6
			non-IC *vs* IC	0.89±0.07	0.00576	<1243	85.7	81.8
ΔSOFA	ND	2	non-IC *vs* IC	0.92±0.07	0.00459	<1.35	87.5	87.5

Table 7. Receiver operating characteristic (ROC) analysis for IL-1β, ASC-speck formation, HMBG1, IL-6, TNF-α and IL-8.

AUC, area under the curve; SE, standard error; non-IC, non-immunocompromised septic patient; IC, immunocompromised septic patient; ND, not determined; Mann Whitney test to compare control *vs* sepsis: *p<0.05; ***p<0.0001; *ns*, not significant difference (p> 0.05). Control group has healthy donors and abdominal surgery controls (*n*= 27), and sepsis group included non-IC and IC septic patients (*n*= 23).

The median and cut-off values are expressed in pg/ml normalized to $5x10^4$ monocytes, except for HMBG1 that is ng/ml normalized to $5x10^4$ monocytes, for ASC-specks is the percentage of monocytes with intracellular ASC speck formation and for ΔSOFA is the variation of SOFA score between day 1 and 5 (SOFA day 1 – SOFA day 5).

2. EXPRESSION OF THE PURINERGIC P2X7 RECEPTOR IN THE INFLAMMATORY RESPONSE

2.1. P2X7 receptor surface is increased in leukocytes from septic patients

2.1. P2X7 receptor surface expression is increased in leukocytes from septic patients

Flow cytometry analysis of PBMCs was initiated by gating cells from a classical SSC-FSC dot-plot (Figure 35a). We first analysed monocytes that were then gated as $CD3^{-}CD14^{+}$ cells with the highest size measured by SSC^{high} (Maecker, McCoy, and Nussenblatt 2012) (Figure 35b and c).

In order to investigate the possible causes of immune immunoparalysis during sepsis, we aimed to study the expression of P2X7R on monocytes from septic patients, as this is the receptor for extracellular ATP, the ligand we used to activate the NLRP3 inflammasome in monocytes from septic patients. We first found that the surface expression of P2X7R was increased in the monocytes of septic patients when compared to control groups (Figure 36a), despite the percentage of $P2X7^{+}$ monocytes was similar among septic patients and control groups (Figure 36b). This increase was also observed in the levels of soluble P2X7R detected in plasma (Figure 36c) and the expression of P2X7R on the surface of monocytes was reduced upon sepsis recovery (Figure 36d). As expected, $CD14^{+}CD16^{++}$ inflammatory monocytes population increased during sepsis (Figure 37a), independently of the immunoparalysis found in some septic patients (Figure 37b). However, in all septic patients, the surface expression of P2X7R appeared elevated in the three populations of monocytes ($CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{++}$) (Figure 38a). The increase in surface expression of P2X7R in monocytes during sepsis was similar among immunocompromised and non-immunocompromised septic patients (Figure 38b). In spite of this evidence, the increase in surface expression of P2X7R in immunocompromised monocytes from septic patients could not explain the lack of IL-1β release after NLRP3 inflammasome stimulation by extracellular ATP. It could be expected that the higher the P2X7R expression is, the higher release of IL-1β after ATP stimulation we should observe. This positive correlation was found in surgery control patients and non-immunocompromised septic patients and was not present in immunocompromised septic patients (Figure 39a). LPS stimulation of monocytes from healthy individuals, but not IL-6, TNFα or IFNγ stimulation, increased surface expression of P2X7R (Figure 39b). This effect was increased starting at 48h after LPS priming (Figure 39c). These results suggest that

bacterial infection and not cytokines could be responsible to increase the surface expression of P2X7R during sepsis.

As previous reports have shown differential changes in PBMCs populations beyond monocytes during sepsis **(Hotchkiss et al. 2001)**, we aimed to analyse the percentage of different PBMCs populations in our cohort of septic patients and compared with healthy and surgery controls (Figure 40a). Monocytes ($CD3^{-}$ $CD14^{+}$) slightly increased during 24 hours of sepsis development when compared to healthy individuals, but not when compared with patients after an abdominal surgery (Figure 40b). $CD3^{+}$ $CD4^{+}$ T cells significantly increased early in sepsis, and their levels were restored 120 days after infection (Figure 40a and c). However, the increase of $CD3^{+}$ $CD4^{+}$ T cells in sepsis was observed in some septic patients where they could represent up to 50% of total PBMCs, and in other patients they present either normal percentage of $CD3^{+}$ $CD4^{+}$ T cells or very low percentage (Figure 40c). On the contrary, the percentage of $CD3^{+}CD8^{+}$ T cells decreased significantly from 24 hours and up to 5 days of sepsis development (Figure 40d), but this decrease was also found in patients after an abdominal surgery (Figure 40a and d). $CD3^{-}CD19^{-}CD14^{-}$ $CD16^{++}$ cells (mainly NK cells) slightly decreased during sepsis (Figure 40a and e), and $CD3^{-}$ $CD19^{+}$ B cells maintained their levels during sepsis (Figure 40a and f).

As we previously shown that almost all monocytes were positive for P2X7R expression in both healthy donors and septic patients; we aimed to study if the percentage of P2X7R positive cells in other populations of PBMCs could change during sepsis respect healthy and surgery controls (Figure 41a). We found that the percentage of P2X7R positive cells inside the different populations of PBMCs increased in sepsis when compared to controls (Figure 31a). Septic $CD3^{-}$ $CD19^{+}$ (B cells) and $CD3^{-}$ $CD19^{-}CD14^{-}CD16^{++}$ (mainly NK cells) experimented the higher increase in P2X7R positive cells, meanwhile P2X7R expression was weakly increased in $CD3^{+}$ T cells (Figure 41a). As we observed this increase of P2X7R expression in different PBMCs populations during sepsis, we aimed to *in vitro* evaluate the expression of P2X7R in PBMCs from healthy volunteers before and after activation. PBMCs were stimulated *in vitro* with several compounds that would activate the different types of lymphocytes, then the percentage of P2X7R positive cells was analysed inside PBMCs populations' together with CD25 and CD69 activation markers for B and T cells respectively **(Afeltra et al. 1993; Radulovic et al. 2013; Borrego et al. 1999; Brisslert et al.**

2006) (Figure 41b and c). We found that stimulation with anti-CD3/CD28 was able to significantly induce the percentage of P2X7R positive $CD3^+$ (T cells) and $CD3^-$ $CD19^+$ (B cells) lymphocytes, but not $CD3^-CD19^-CD14^-CD16^{++}$ cells (mainly NK cells) (Figure 41c). However, anti-CD3/CD28 was not able to induce activation of these cell populations (Figure 41b). In addition, PWM weakly triggered $CD3^-CD19^+$ cell activation, meanwhile IL-2 addition increased $CD3^-CD19^-CD14^-CD16^{++}$ cell activation (Figure 41b). In contraposition, PMA alone or in combination with the Ca^{2+} ionophore ionomycin, was able to increase the percentage of active CD3+, $CD3^-CD19^+$ and $CD3^-CD19^-CD14^-CD16^{++}$ cells (Figure 41b). However, treatment with IL-2, LPS, PWM, or PMA was not able to significantly increase the percentage of P2X7R positive cells inside these cell populations (Figure 41c).

$CD3^+$ T cells were plotted in a CD4-CD8 dot-plot to obtain $CD3^+CD4^+$ and $CD3^+CD8^+$ cells (Figure 42a, b and c). These populations have been considered classically as T helper (Th) and T cytotoxic (Tc) cells respectively (**Maecker, McCoy, and Nussenblatt 2012**). We found that the percentage of T cells with P2X7R expression increased in both $CD3^+CD4^+$ and $CD3^+CD8^+$ populations in septic patients when compared to healthy controls (Figure 42 d, e and f). In this context, a significant increase of P2X7R percentage was found in $CD3^+CD4^+$ cells from septic patients at day 1 in comparison with controls without differences between immunocompromised and non-immunocompromised patients (Figure 42e). However, percentage of P2X7R returned to normal values after 120 days of infection (Figure 42e). The same results were obtained when $CD3^+CD8^+$ were analysed 120 days after infection (Figure 42f). These results suggest that percentage of $CD3^+P2X7R^+$ cells increased in a transitory manner. Interestingly, the amount of CD8 marker calculated as the mean of fluorescent intensity in $CD3^+CD8^+$ cells decreased after 120 days of sepsis development in comparison with septic patients at day 1, meanwhile CD8 expression was higher in healthy controls (Figure 42g). Similarly, the expression of CD4 decreased during sepsis and at 120 days after sepsis development, although here the differences were not significant (Figure 42g). This reveal that after sepsis recovery, $CD3^+$ T cells present a decrease of CD4 and CD8, that could be involved in an impairment of $CD3^+$ T cell activation upon sepsis.

T regulatory (Treg) cells could be detected using $CD4^{++}CD25^+CD127^-$ surface markers (**Liu et al. 2006; Corthay 2009**). This cell population was gated from PBMCs in a CD4-CD25 dot-plot (Figure 43a and b). $CD4^{++}CD25^+$ cells were gated in a CD127-P2X7R dot-plot, and $CD127^-$ cells were selected to analyse P2X7R expression (Figure 43c). CD127

expression is opposite to FOXP3 expression, being FOXP3 a key transcriptional regulator for the development and function of Treg cells **(Liu et al. 2006).** We observed that during sepsis there were a decrease of $CD4^{++}CD25^{+}CD127^{-}$ cells (Figure 43d). However, during sepsis the percentage of $CD4^{++}CD25^{+}CD127^{-}$ cells that expressed P2X7R was increased when compared to healthy controls (Figure 43e). This increase of P2X7R positive Treg was independent of the innate-immunocompromised state of the patients (Figure 43e).

CD19 is a characteristic marker for B cells (Maecker, McCoy, and Nussenblatt 2012) and in this Thesis $CD19^{+}$ cells were gated from the $CD3^{-}$ population (Figure 44a). The percentage of $P2X7R^{+}$ cells in $CD3^{-}CD19^{+}$ B cells was higher in septic patients when compared to healthy and surgery controls, independently if patients suffered innate immunoparalysis or not (Figure 44b and c). The increase of $CD3^{-}CD19^{+}$ B cells positive for P2X7R expression was reverted 120 days after sepsis development, where the percentage of $P2X7R^{+}$ B cells returned to normal levels (Figure 44c).

The majority of NK cells in humans are $CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ cells **(Maecker, McCoy, and Nussenblatt 2012)** and in this work, we first gated $CD19^{-}$ cells from $CD3^{-}$ population (Figure 44a and 45a). $CD3^{-}CD19^{-}$ cells were then plotted in a CD14-CD16 dot-plot where $CD14^{-}CD16^{++}$ cells were selected as NK cells (Figure 45b). We could observe a trend to increase the percentage of $P2X7R^{+}$ cells in the population of $CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ cells from septic patients (Figure 45c and d). This effect was independent on the innate immunocompromised status of the patients (Figure 45e). The percentage of $P2X7R^{+}$ NK cells returned to normal levels at 120 days after sepsis, indicating that this phenotypic change is transitory (Figure 45d). Interestingly, similarly to $CD3^{+}CD8^{+}$ and $CD3^{+}CD4^{+}$ cells, CD16 marker in $CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ cells decreased in patients after 120 days of sepsis recovery, being smaller than in healthy controls (Figure 45e).

Because the main PBMCs populations increase the percentage of $P2X7R^{+}$ cells during sepsis, we then found that the percentages of $P2X7R^{+}$ cells strongly correlated between PBMCs populations in septic patients, meanwhile in healthy donors the expression of P2X7R in the different PBMCs populations presented a weak correlation (Figure 46a and b). The increase of P2X7R expression in $CD3^{-}CD14^{+}$ monocytes and the augment of $P2X7R^{+}$ $CD3^{+}CD8^{+}$ cells correlated with the increase of $P2X7R^{+}$ cells in the rest of PBMCs

populations (Figure 46a). The increase of $P2X7R^+CD3^+CD4^+$ cells correlated with the rise of $P2X7R^+$ cells in $CD3^-CD14^+$ and $CD3^+CD8^+$ cells but not when compared with the other PBMCs populations (Figure 46a). The increase of $P2X7R^+$ cells in $CD3^-CD19^+$ and $CD3^-CD19^-CD14^-CD16^{++}$ cells positively correlated between them and with $CD3^-CD14^+$ and $CD3^+CD8^+$ cells (Figure 46a). This observation could suggest the importance of the communication between monocytes and lymphocytes in the coordinated inflammatory response against infections by increasing P2X7R in these different populations **(Martinez, Helming, and Gordon 2009)**. On the contrary, in healthy controls, the percentage of $P2X7R^+$ cells only correlated between $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (Figure 46b).

As we found a correlation between the expression of P2X7R in $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in healthy controls, we aimed to analyse if $CD4^{++}CD25^+CD127^-$ Treg cells presented a similar correlation (Figure 47a and b). As we expected, $CD4^{++}CD25^+CD127^-$ cells presented a positive correlation in the percentage of $P2X7R^+$ cells when compared with $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in septic patients, but not in healthy controls (Figure 47a and b).

FIGURES:

P2X7 receptor surface is increased

in leukocytes from septic patients

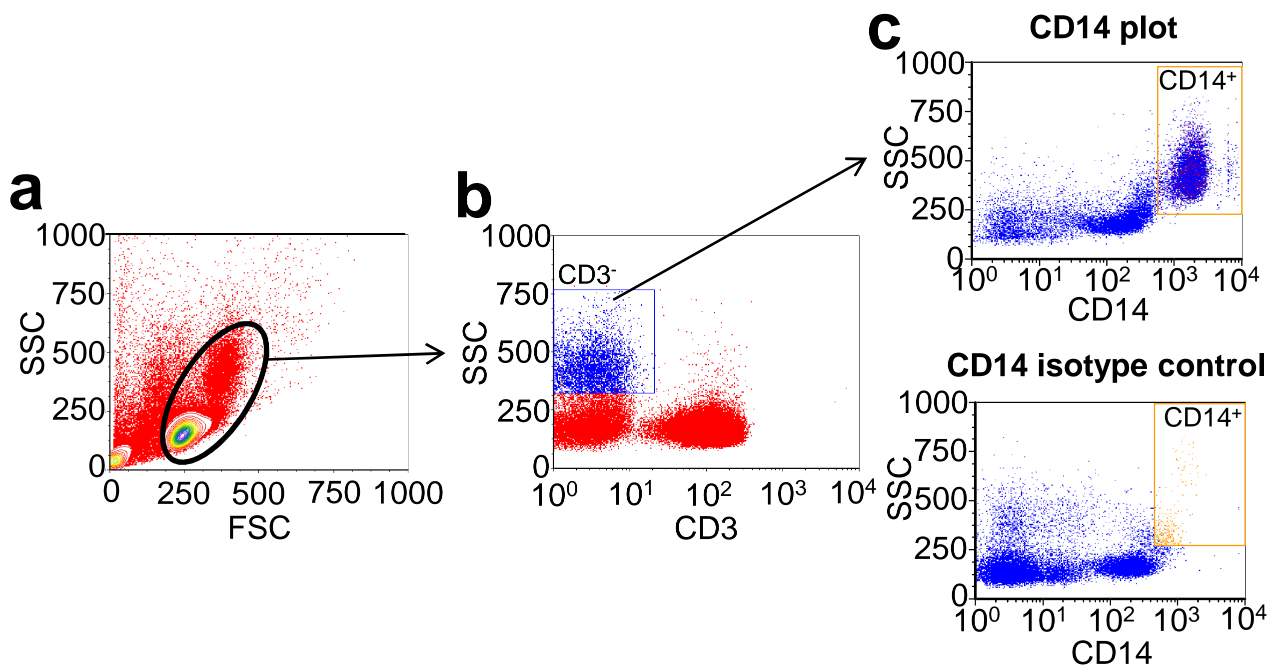

a
1000
750
500
250
0
SSC
0
250
500
750
1000
FSC
b
1000
750
500
250
0
SSC
CD3-
10^0
10^1
10^2
10^3
10^4
CD3
c
CD14 plot
CD14+
1000
750
500
250
0
SSC
10^0
10^1
10^2
10^3
10^4
CD14
CD14 isotype control
CD14+
SSC
CD14

Figure 35. Monocytes $CD3^{-}CD14^{+}$ gating strategy. (a) Representative dot-plot for PBMCs gating (the oval represents the viable initial PBMCs population gated). **(b)** Representative SSC-CD3 dot-plot for PBMCs gated in (a); $CD3^{-}$ cells with SSC^{high} were gated (blue dotes). **(c)** Representative SSC-CD14 dot-plot for cells gated in (b); $CD14^{+}$ cells were gated (orange quadrant); top dot-plot is a stained sample, whereas bottom dot-plot represent a sample stained with anti-CD14 control isotype.

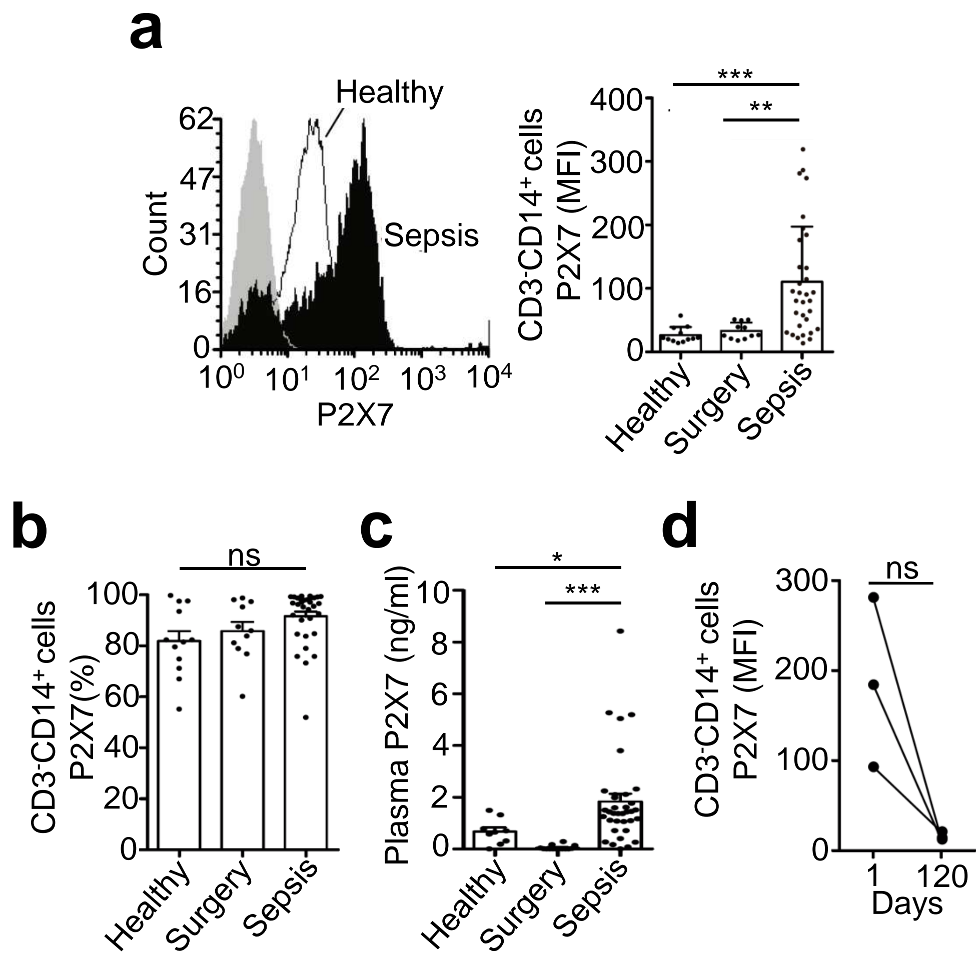

a
Healthy
Sepsis
Count
62
47
31
16
0
10^0 10^1 10^2 10^3 10^4
P2X7
CD3⁻CD14⁺ cells P2X7 (MFI)
***
**
400
300
200
100
0
Healthy
Surgery
Sepsis
b
ns
CD3⁻CD14⁺ cells P2X7(%)
100
80
60
40
20
0
Healthy
Surgery
Sepsis
c
*
***
Plasma P2X7 (ng/ml)
10
8
6
4
2
0
Healthy
Surgery
Sepsis
d
ns
CD3⁻CD14⁺ cells P2X7 (MFI)
300
200
100
0
1
120
Days

Figure 36. P2X7R upregulates in monocytes during sepsis. (a) Representative histogram plot (left) for surface P2X7R staining in $CD3^{-}CD14^{+}$ cells from healthy (white), septic patient (black) and non-P2X7R stained $CD3^{-}CD14^{+}$ cells (grey). Quantification of P2X7R mean intensity fluorescence (MFI) on $CD3^{-}CD14^{+}$ cells in control and septic patients (right plot). **(b)** Percentage of positive $CD3^{-}CD14^{+}$ cells for P2X7R in control and septic patients. **(c)** Concentration of soluble P2X7R in plasma of control and septic patients. **(d)** Quantification of P2X7R MFI in $CD3^{-}CD14^{+}$ cells at 1 day during sepsis and 120 days after recovery. Each dot represents an individual septic patient or healthy donor; $^{*}p< 0.05$; $^{**}p< 0.01$; $^{***}p< 0.001$; *ns*, no significant difference ($p> 0.05$); Kruskal-Wallis test was used in a-c; Mann-Whitney test for d.

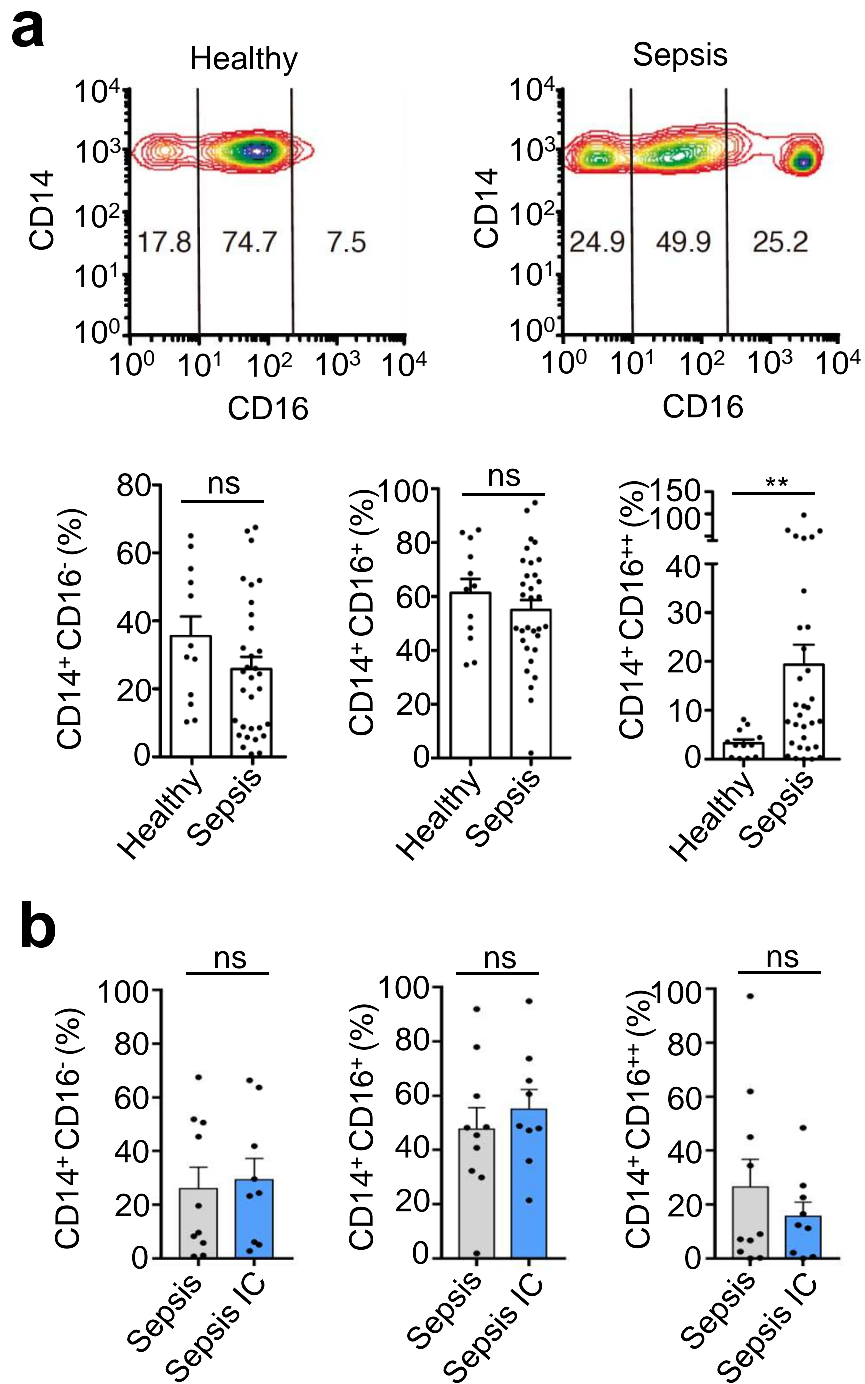

a
Healthy
Sepsis
CD14
CD16
17.8
74.7
7.5
24.9
49.9
25.2
ns
ns
**
CD14+CD16- (%)
CD14+CD16+ (%)
CD14+CD16++ (%)
Healthy
Sepsis
b
Sepsis IC

Figure 37. Percentage of monocytes phenotypes during sepsis. **(a)** Representative plots for $CD14^{+}CD16^{-/+/++}$ monocytes in a control healthy donor and in a septic patient at day 1 (top); and percentage of $CD14^{+}CD16^{-/+/++}$ monocyte populations from healthy individual and septic patients at day 1 (bottom); Numbers in top plots represent the percentage for the different CD16 populations. **(b)** Percentage of $CD14^{+}CD16^{-/+/++}$ monocyte populations from non-immunocompromised (grey bars) and immunocompromised (IC, blue bars) septic patients at day 1. Each dot represents an individual patient; $^{**}p < 0.01$; *ns*, no significant difference ($p > 0.05$); Mann-Whitney test was used for a-b.

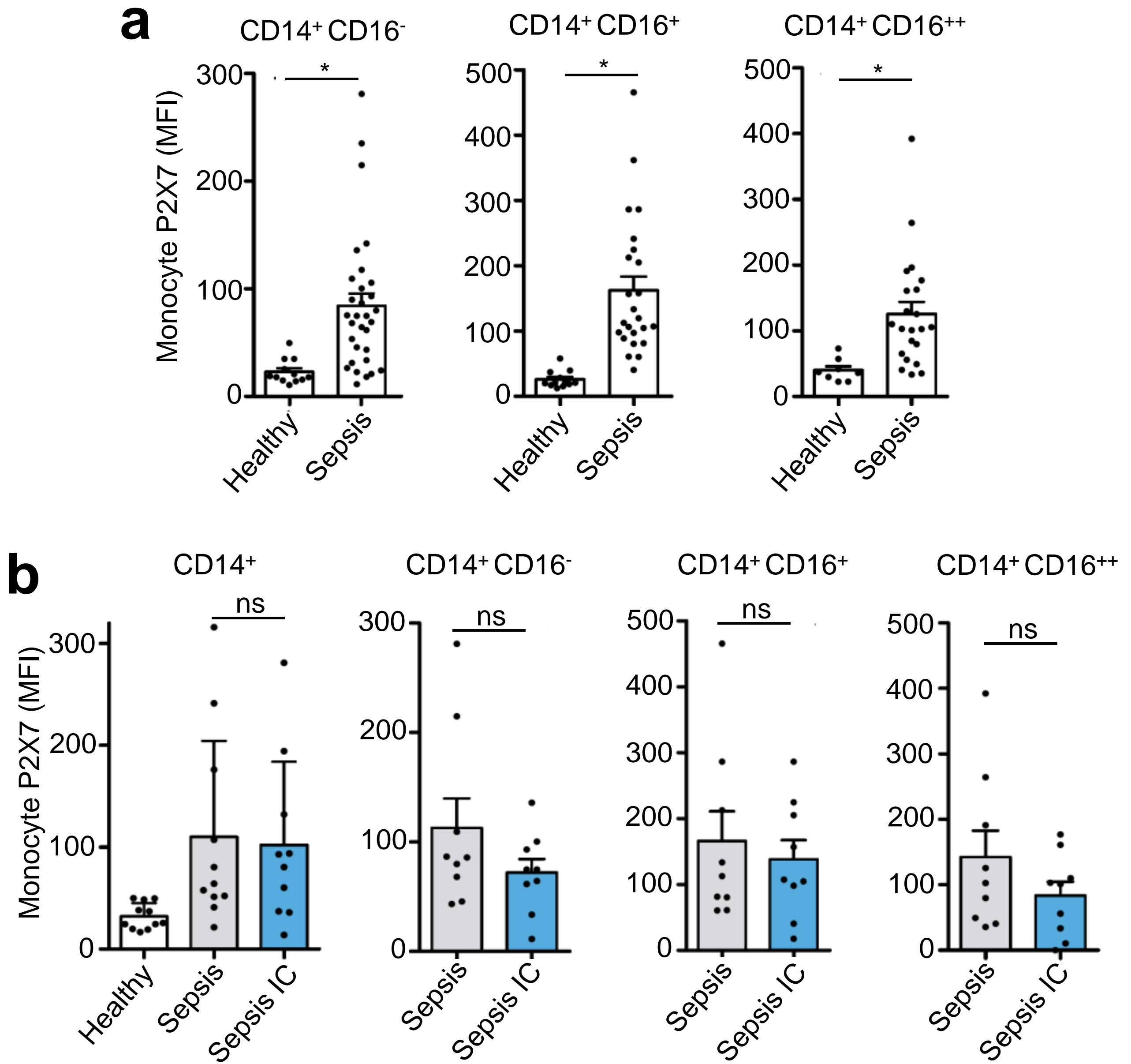
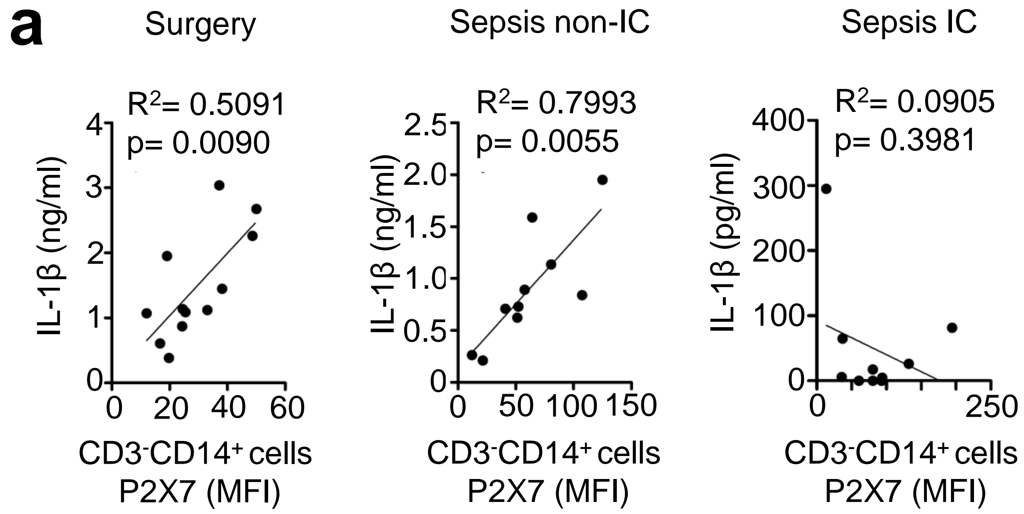
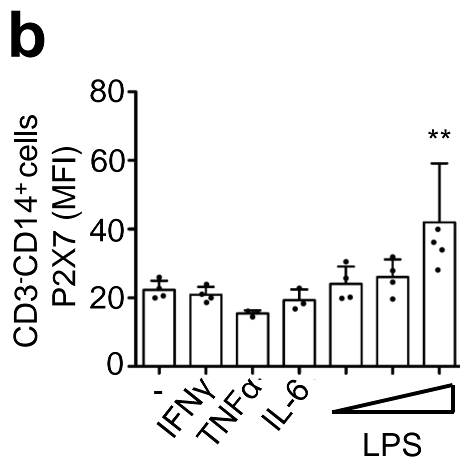

a
CD14+ CD16-
CD14+ CD16+
CD14+ CD16++
Monocyte P2X7 (MFI)
*
Healthy
Sepsis
b
CD14+
ns
Sepsis IC

Figure 38. P2X7R expression in monocytes during sepsis. (a) Quantification of P2X7R MFI on the different CD16 monocyte populations from healthy individual and septic patients at day 1. **(b)** Quantification of P2X7R MFI on non-immunocompromised (grey bars) and immunocompromised (IC, blue bars) septic patients at day 1 in $CD3^{-}CD14^{+}$ monocytes (left) or on the different $CD16^{+}$ monocyte populations as indicated. Each dot represents an individual patient; *$p < 0.05$; *ns*, no significant difference ($p > 0.05$); *$p < 0.05$; *ns*, not significant difference ($p > 0.05$); Mann-Whitney test was used for a-b.


a
Surgery
Sepsis non-IC
Sepsis IC
R2= 0.5091
p= 0.0090
R2= 0.7993
p= 0.0055
R2= 0.0905
p= 0.3981
IL-1β (ng/ml)
IL-1β (pg/ml)
CD3-CD14+ cells
P2X7 (MFI)


b
CD3-CD14+ cells
P2X7 (MFI)
**
-
IFNγ
TNFα
IL-6
LPS

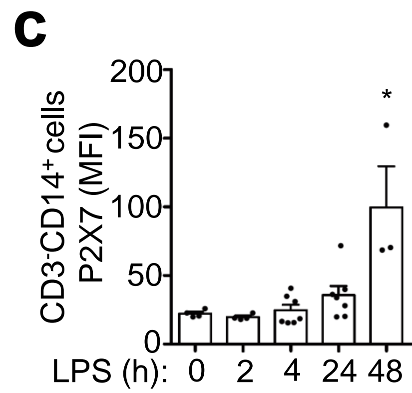
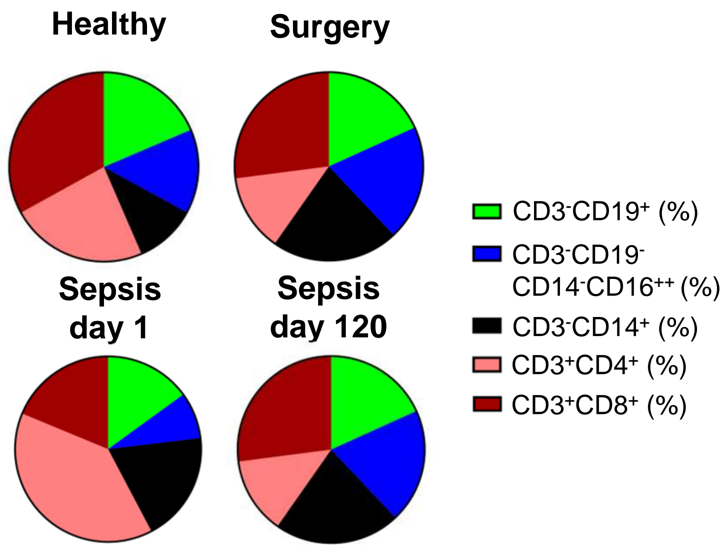
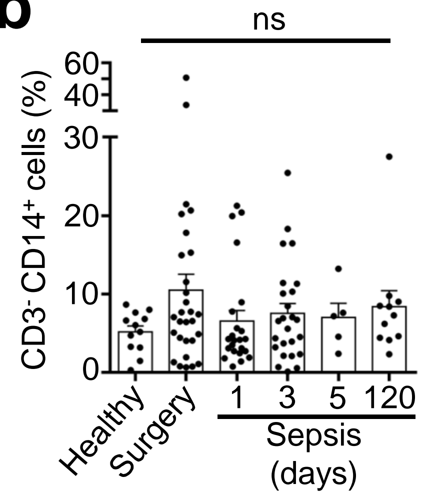
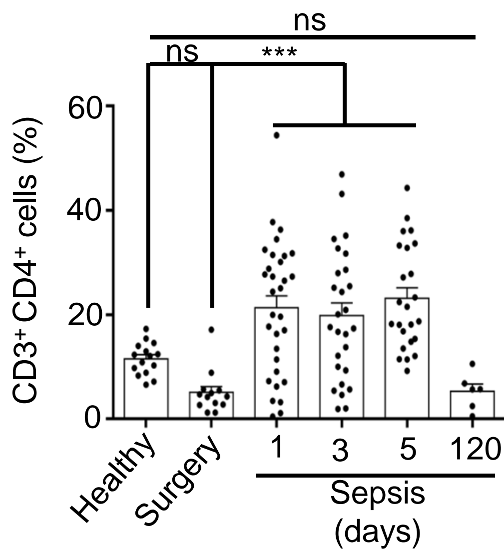
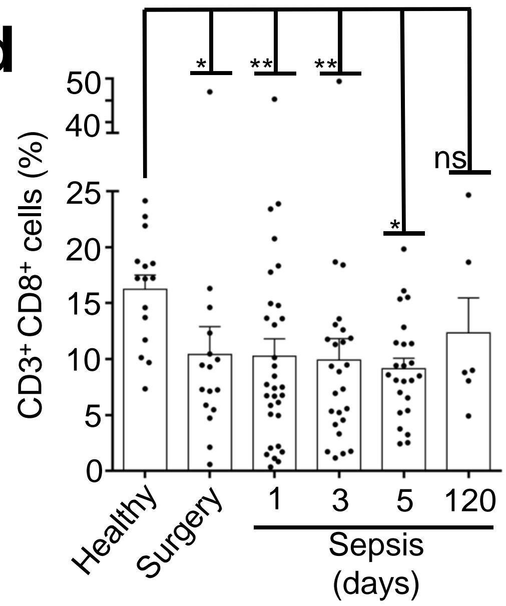

c
CD3-CD14+ cells
P2X7 (MFI)
*
LPS (h): 0 2 4 24 48

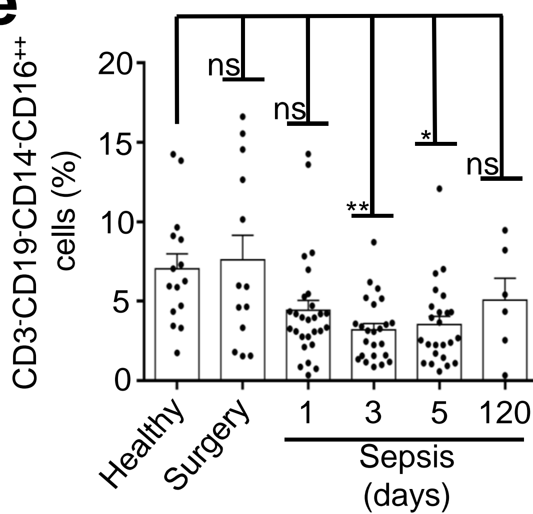
Figure 39. Increase of P2X7R expression is induced by bacterial infection. (a) Correlation between the concentration of IL-1β released from PBMCs treated with LPS (1 µg/ml, 2h) and ATP (3 mM, 30 min) and the quantification of P2X7R MFI in $CD3^{-}CD14^{+}$ cells from the indicated control and septic patient groups; **(b)** Quantification of P2X7R MFI in $CD3^{-}CD14^{+}$ cells from healthy donors treated with IFNγ, TNF-α, IL-6 (all at 20 ng/ml), or with increasing concentrations of LPS (10, 100, 1000 ng/ml) for 24 h. **(c)** Quantification of P2X7R MFI in $CD3^{-}CD14^{+}$ cells from healthy donors treated with LPS (1 µg/ml) for the indicated times. IC: immunocompromised. Each dot represents an individual septic patient or healthy donor; *$p < 0.05$; **$p < 0.01$; Pearson correlation was used in a; Kruskal-Wallis test was used in b-c.


a
Healthy
Surgery
Sepsis day 1
Sepsis day 120
CD3-CD19+ (%)
CD3-CD19- CD14-CD16++ (%)
CD3-CD14+ (%)
CD3+CD4+ (%)
CD3+CD8+ (%)


b
ns
CD3- CD14+ cells (%)
Healthy
Surgery
1
3
5
120
Sepsis (days)


c
ns
ns
***
CD3+ CD4+ cells (%)
Healthy
Surgery
1
3
5
120
Sepsis (days)


d
*
**
**
*
ns
CD3+ CD8+ cells (%)
Healthy
Surgery
1
3
5
120
Sepsis (days)


e
ns
ns
**
*
ns
CD3-CD19-CD14-CD16++ cells (%)
Healthy
Surgery
1
3
5
120
Sepsis (days)

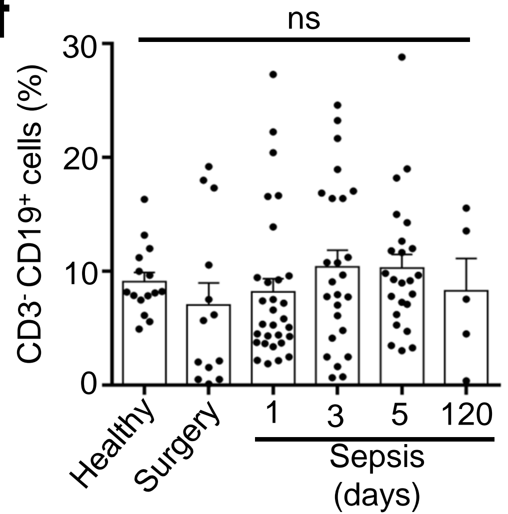

f
ns
CD3- CD19+ cells (%)
Healthy
Surgery
1
3
5
120
Sepsis (days)

Figure 40. Percentage of PBMCs subpopulations in septic patients. (a) Sector graphs for the percentage of different PBMCs populations from healthy and surgery controls or septic patients at day 1 or after 120 days of infection (as is indicated in the graph). Data is average of n=15 healthy donors, n= 13 surgery controls, n= 30 septic patients at day 1, and n= 6 septic patients at 120 days. Green, blue, black, magenta and brown colours were used for $CD3^-$ $CD19^+$, $CD3^-$ $CD19^-CD14^-CD16^{++}$, $CD3^-$ $CD14^+$, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells respectively as indicated in the legend. **(b-f)** Percentage of $CD3^-$ $CD14^+$ **(b)**, $CD3^+CD4^+$ **(c)**, $CD3^+CD8^+$ **(d)**, $CD3^-CD19^-CD14^-$ $CD16^{++}$ **(e)**, and $CD3^-CD19^+$ **(f)** populations from total of PBMCs in healthy and surgery controls as well as septic patients at day 1, 3, 5 and 120. Each dot represents a single patient; *p< 0.05; **p< 0.01; ***p< 0.001; *ns*, not significant difference (p> 0.05); Kruskal-Wallis test was used for b-f.

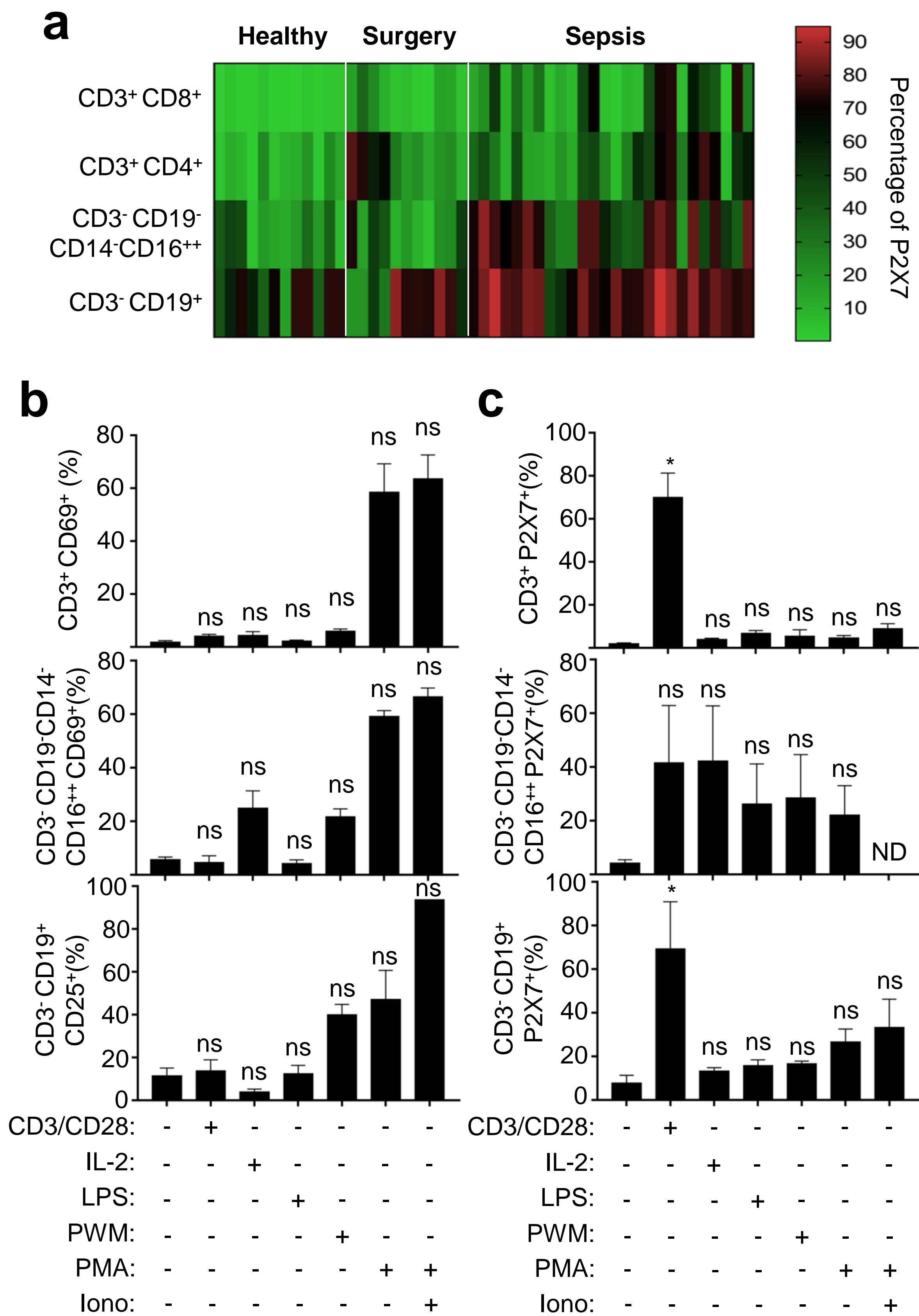

a
Healthy
Surgery
Sepsis
CD3+ CD8+
CD3+ CD4+
CD3- CD19- CD14-CD16++
CD3- CD19+
Percentage of P2X7
b
c
CD3+ CD69+ (%)
CD3- CD19-CD14- CD16++ CD69+(%)
CD3- CD19+ CD25+(%)
CD3+ P2X7+(%)
CD3- CD19-CD14- CD16++ P2X7+(%)
CD3- CD19+ P2X7+(%)
ns
*
ND
CD3/CD28:
IL-2:
LPS:
PWM:
PMA:
Iono:

Figure 41. P2X7R expression increased in different PBMCs subpopulations during sepsis. (a) Heat-map representing the percentage of P2X7R$^+$ cells in different PBMCs subpopulations from septic patients at day 1 and in healthy and surgery controls. Red colour represent a high percentage of P2X7R$^+$ cells, meanwhile green colour represent a low percentage of P2X7R$^+$ cells as denoted in the scale. **(b)** Percentage of cells with CD69 or CD25 activation markers in the indicated subpopulations upon PBMC stimulated for 48 h with antibodies for CD3/CD28 (20μl/ml) , recombinant IL-2 (20ng/ml), LPS (1μg/ml), PWM (1μg/ml), or PMA (10ng/ml) with or without ionomycin (500ng/ml). **(c)** Percentage of P2X7R$^+$ cells in the indicated subpopulations upon PBMC stimulated as in (b). $^*p< 0.05$; *ns*, not significant difference ($p> 0.05$); ND: Not detected; Kruskal-Wallis test was used for b,c.

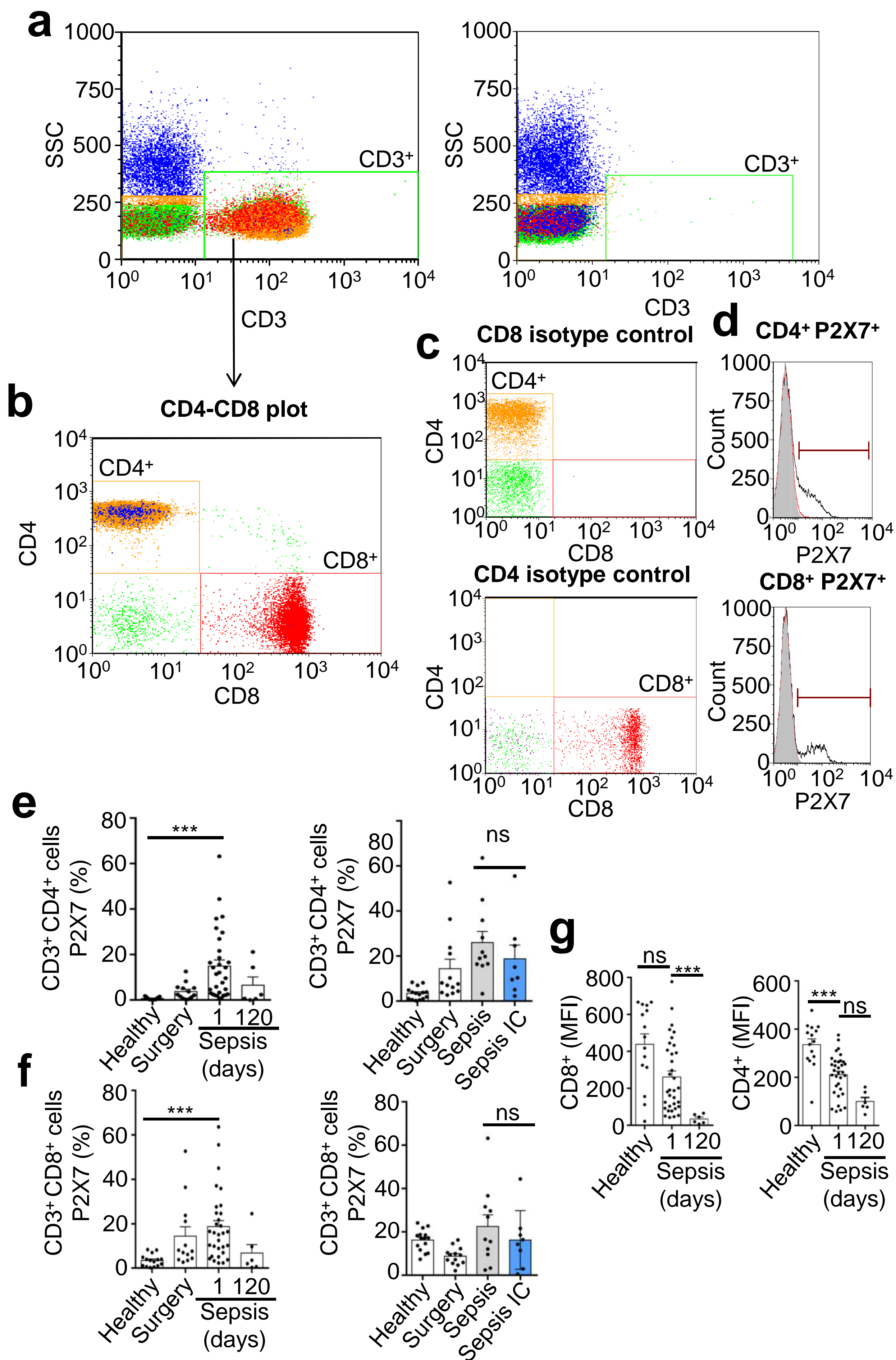

a
SSC
CD3
CD3+
b
CD4-CD8 plot
CD4
CD8
CD4+
CD8+
c
CD8 isotype control
CD4 isotype control
d
CD4+ P2X7+
CD8+ P2X7+
Count
P2X7
e
CD3+CD4+ cells P2X7 (%)
Healthy
Surgery
Sepsis (days)
Sepsis
Sepsis IC
ns
***
f
CD3+CD8+ cells P2X7 (%)
g
CD8+ (MFI)
CD4+ (MFI)

Figure 42. Percentage of T lymphocytes expressing P2X7R increase during sepsis. (a,b,c) Representative dot-plots for $CD3^+$ T cells gating strategy. **(a)** Representative SSC-CD3 dot-plot for PBMCs; $CD3^+$ cells with SSC^{low} were gated (green gate, left panel); dot-plot representing a sample stained with anti-CD3 control isotype (right panel). (b) Representative CD4-CD8 dot-plot for $CD3^+$ cells with SSC^{low}. $CD3^+CD4^+$ (orange gate) or $CD3^+CD8^+$ (red gate) were gated. (c) dot-plot representing a sample stained with anti-CD8 control isotype (top panel) or anti-CD4 control isotype (bottom panel). **(d)** Representative histogram plot for surface P2X7R staining in $CD3^+CD4^+$ (top) or $CD3^+CD8^+$ (bottom) cells from healthy (red), septic patient (black) and non-P2X7R stained cells (grey). **(e)** Percentage of $P2X7R^+CD3^+CD4^+$ cells from control individuals and septic patients at day 1 and 120 after sepsis development (left panel) or in septic immunocompromised (blue bar) and non-immunocompromised (grey bar) (right panel). **(f)** Percentage of $P2X7R^+CD3^+CD8^+$ cells in groups as in (e). **(g)** Quantification of CD8 (left) or CD4 (right) mean intensity fluorescence (MFI) in $CD3^+CD8^+$ (left) or $CD3^+CD4^+$ (right) cells from healthy controls and septic patients at day 1 or 120 after infection; IC: Immunocompromised; each dot represents a single patient; ***$p < 0.001$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was used for e, f, g.

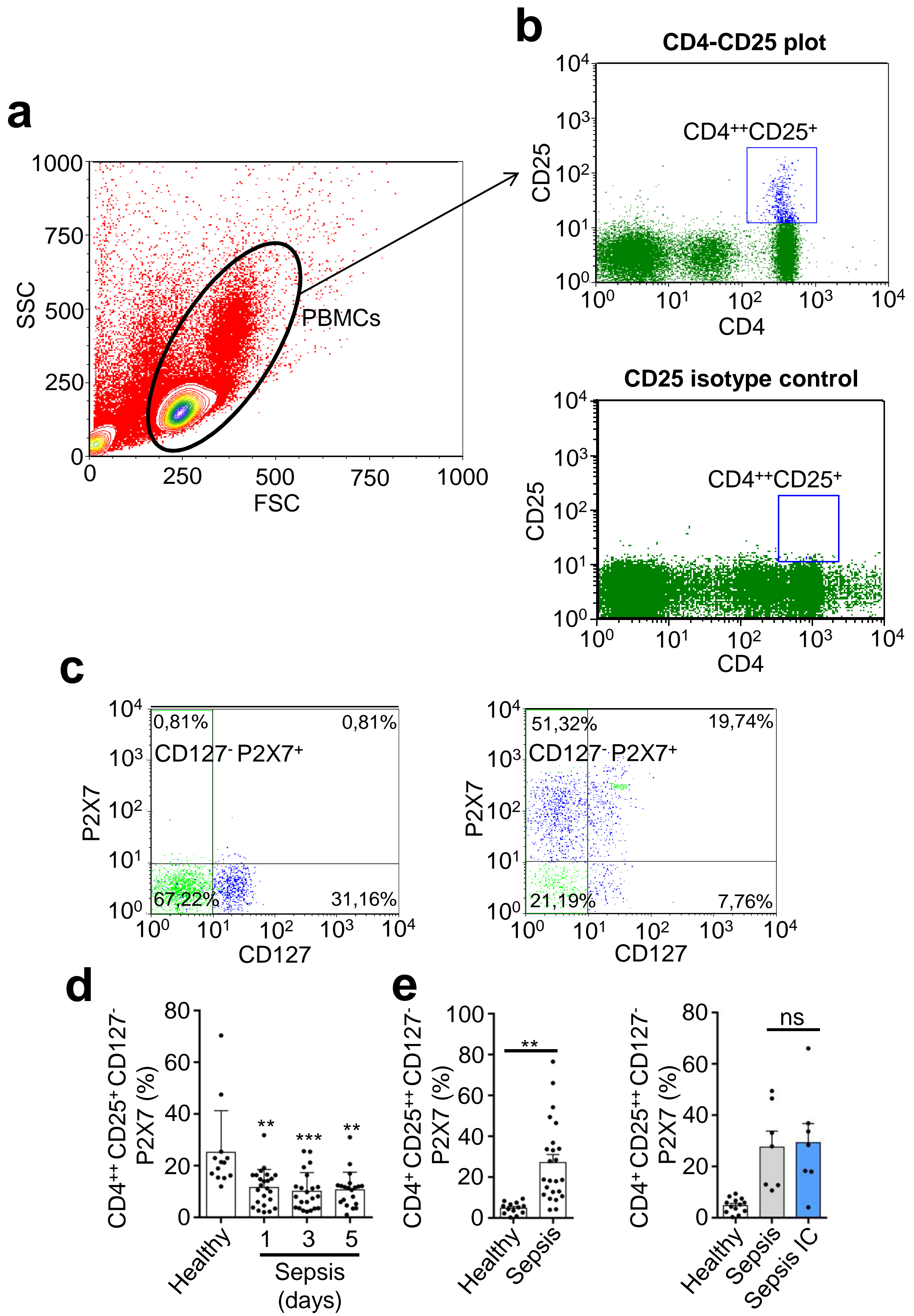

a
b
CD4-CD25 plot
CD4++CD25+
CD25
CD4
PBMCs
SSC
FSC
CD25 isotype control
c
0,81%
0,81%
CD127- P2X7+
67,22%
31,16%
51,32%
19,74%
21,19%
7,76%
P2X7
CD127
d
e
CD4++CD25+CD127- P2X7 (%)
Healthy
Sepsis
(days)
CD4+CD25++CD127- P2X7 (%)
Sepsis IC
ns

Figure 43. Percentage of T regulatory $CD4^{++}CD25^{+}CD127^{-}$ cells expressing P2X7R increase during sepsis. (a) Representative dot-plot for PBMCs gating (the oval represents the viable initial PBMCs population gated). **(b)** Representative CD25-CD4 dot-plot for PBMCs gated in (a); **$CD4^{++}CD25^{+}$** cells were gated (blue gate, top); anti-CD25 control isotype (bottom panel). **(c)** Representative P2X7R-CD127 dot-plot for **$CD4^{++}CD25^{+}$** cells gated in (b) from a healthy control (left panel) and a septic patient (right panel). **(d)** Percentage of $CD4^{++}CD25^{+}CD127^{-}$ cells in healthy individuals and septic patients at days 1, 3, and 5 after infection. **(e)** Percentage of $P2X7R^{+}$ in $CD4^{++}CD25^{+}CD127^{-}$ cells in healthy or septic patients (left panel) or septic patients separated as immunocompromised (blue bar) or non-immunocompromised (grey bar) septic patients (right panel). IC: Immunocompromised; each dot represents a single patient; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was used for e; Mann-Whitney test was used for d

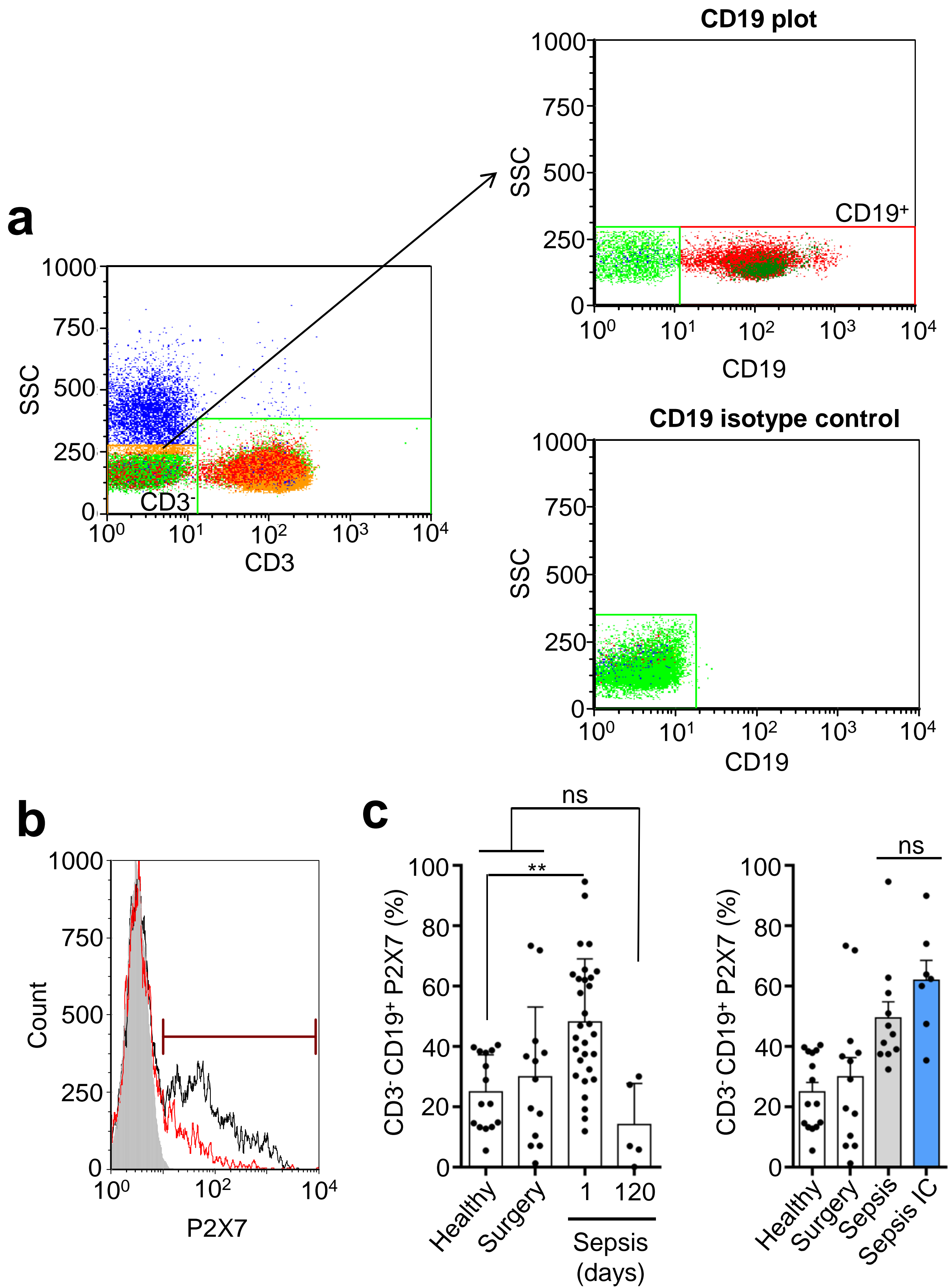

a
CD19 plot
1000
750
500
250
0
SSC
CD19+
CD19
CD19 isotype control
CD3-
CD3
b
Count
P2X7
c
ns
**
CD3- CD19+ P2X7 (%)
Healthy
Surgery
1
120
Sepsis
(days)
Sepsis
Sepsis IC

Figure 44. Percentage of $CD3^{-}$ $CD19^{+}$ B cells expressing P2X7R increase during sepsis. (a) Representative SSC-CD3 dot-plot (left) where $CD3^{-}$ cells with SSC^{low} cells were gated (COLOR gate); representative SSC-CD19 dot-plot (right) for $CD3^{-}SSC^{low}$ gated cells; $CD19^{+}$ cells were gated (red quadrant); top right dot-plot is a CD19 stained sample, whereas bottom right dot-plot represent a sample stained with anti-CD19 control isotype. **(b)** Representative histogram plot for surface P2X7R staining in $CD3^{-}CD19^{+}$ cells from healthy (red), septic patient (black) and non-P2X7R stained cells (grey). **(c)** Percentage of $P2X7R^{+}$ in $CD3^{-}CD19^{+}$ cells from control individuals and septic patients at day 1 and 120 after sepsis development (left panel) or in septic immunocompromised (blue bar) and non-immunocompromised (grey bar) (right panel). IC: Immunocompromised; Kruskal-Wallis test was used for c; each dot represents a single patient; $^{**}p < 0.01$; *ns*, not significant difference ($p > 0.05$).

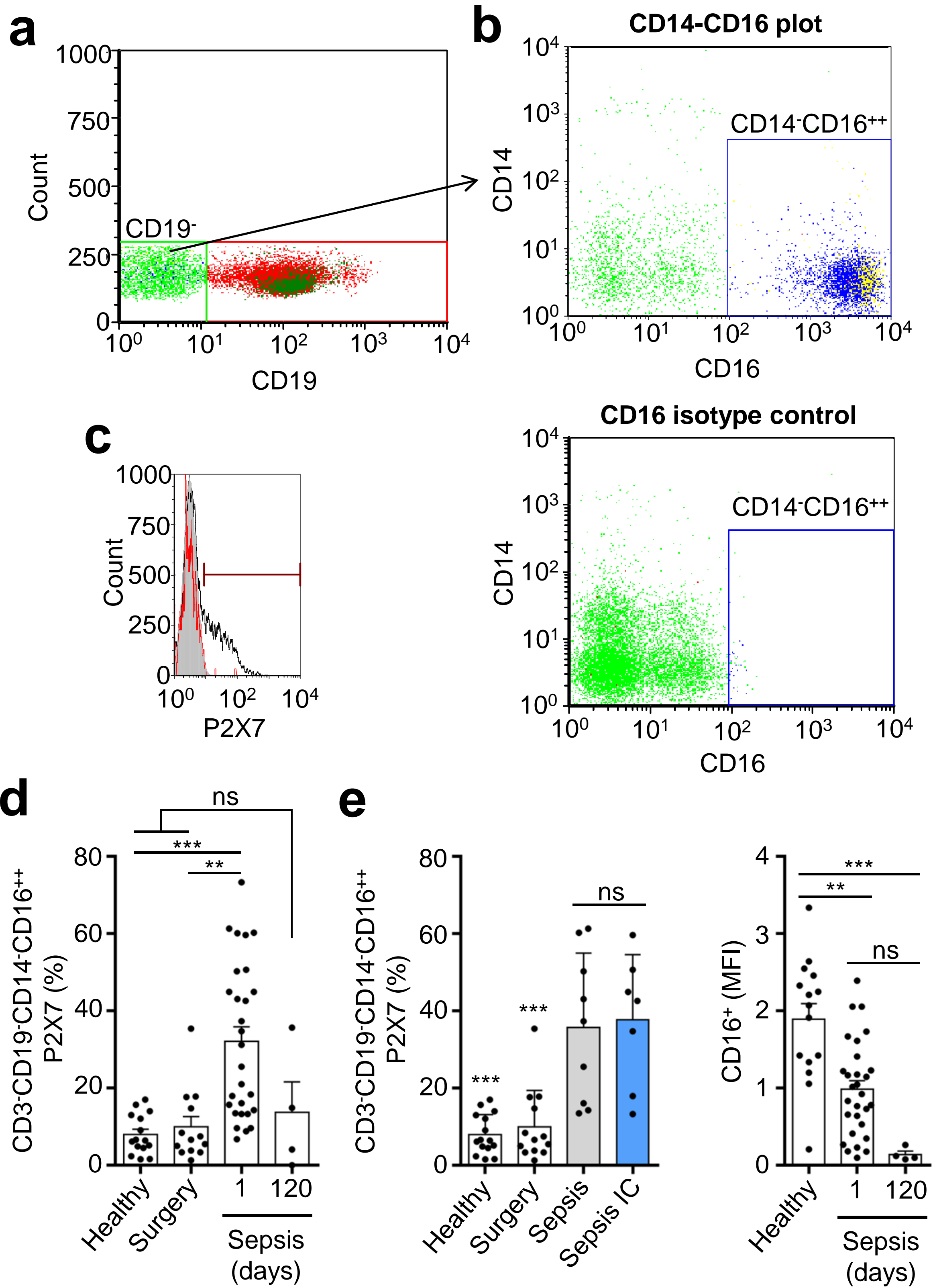
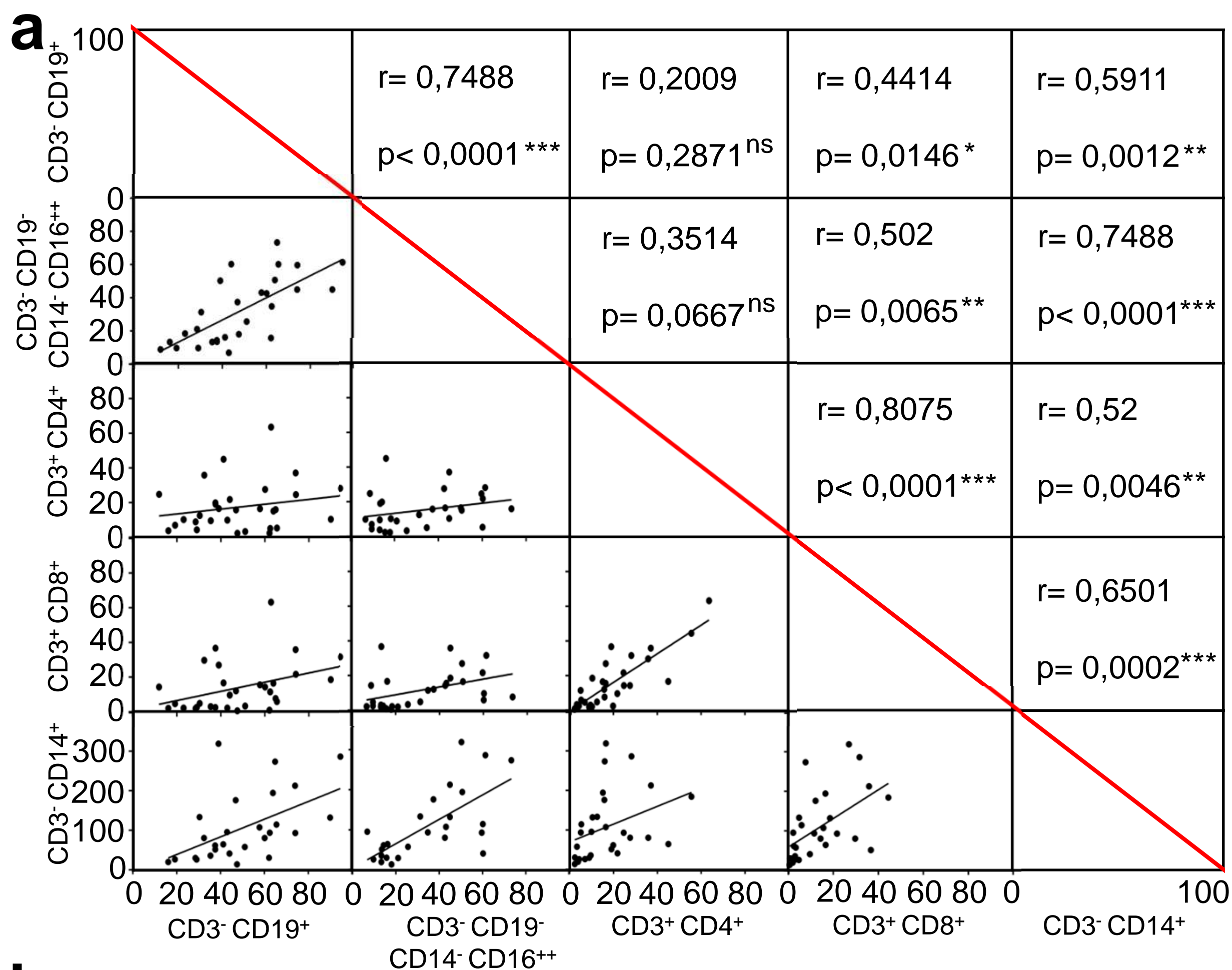

a
Count
CD19-
CD19
b
CD14-CD16 plot
CD14
CD14-CD16++
CD16
CD16 isotype control
c
Count
P2X7
d
ns
CD3-CD19-CD14-CD16++ P2X7 (%)
Healthy
Surgery
Sepsis (days)
e
Sepsis
Sepsis IC
CD16+ (MFI)

Figure 45. Percentage of $CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ NK cells expressing P2X7R increase during sepsis. (a) Representative count-CD19 dot-plot for $CD3^{-}$ cells gated as in Figure 22a; **$CD3^{-}$** $CD19^{-}$ cells were gated (green quadrant). (b) Representing CD14-CD16 dot-plot of **$CD3^{-}$**$CD19^{-}$ cells gated in (a) stained with anti-CD14 and anti-CD16 (top) or control CD16 isotype control (bottom); **$CD3^{-}CD19^{-}CD14^{-}CD16^{++}$** are gated in the blue quadrant. **(c)** Representative histogram plot for surface P2X7R staining in **$CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ NK cells** from healthy (red), septic patient (black) and non-P2X7R stained cells (grey). **(d,e)** Percentage of $P2X7R^{+}$ in **$CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ NK cells** from control individuals and septic patients at day 1 and 120 after sepsis development (d) or in septic immunocompromised (blue bar) and non-immunocompromised (grey bar) (e, left panel); **q**uantification of CD16 (e, right panel) mean intensity fluorescence (MFI) in **$CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ NK cells** from healthy controls and septic patients at day 1 or 120 after infection; IC: Immunocompromised; Kruskal-Wallis test was used for d, e; each dot represents a single patient; **$p < 0.01$; ***$p < 0.001$; *ns*, not significant difference ($p > 0.05$).


a
CD3- CD19+
CD3- CD19- CD14- CD16++
CD3+ CD4+
CD3+ CD8+
CD3- CD14+
r= 0,7488
p< 0,0001***
r= 0,2009
p= 0,2871ns
r= 0,4414
p= 0,0146*
r= 0,5911
p= 0,0012**
r= 0,3514
p= 0,0667ns
r= 0,502
p= 0,0065**
r= 0,7488
p< 0,0001***
r= 0,8075
p< 0,0001***
r= 0,52
p= 0,0046**
r= 0,6501
p= 0,0002***

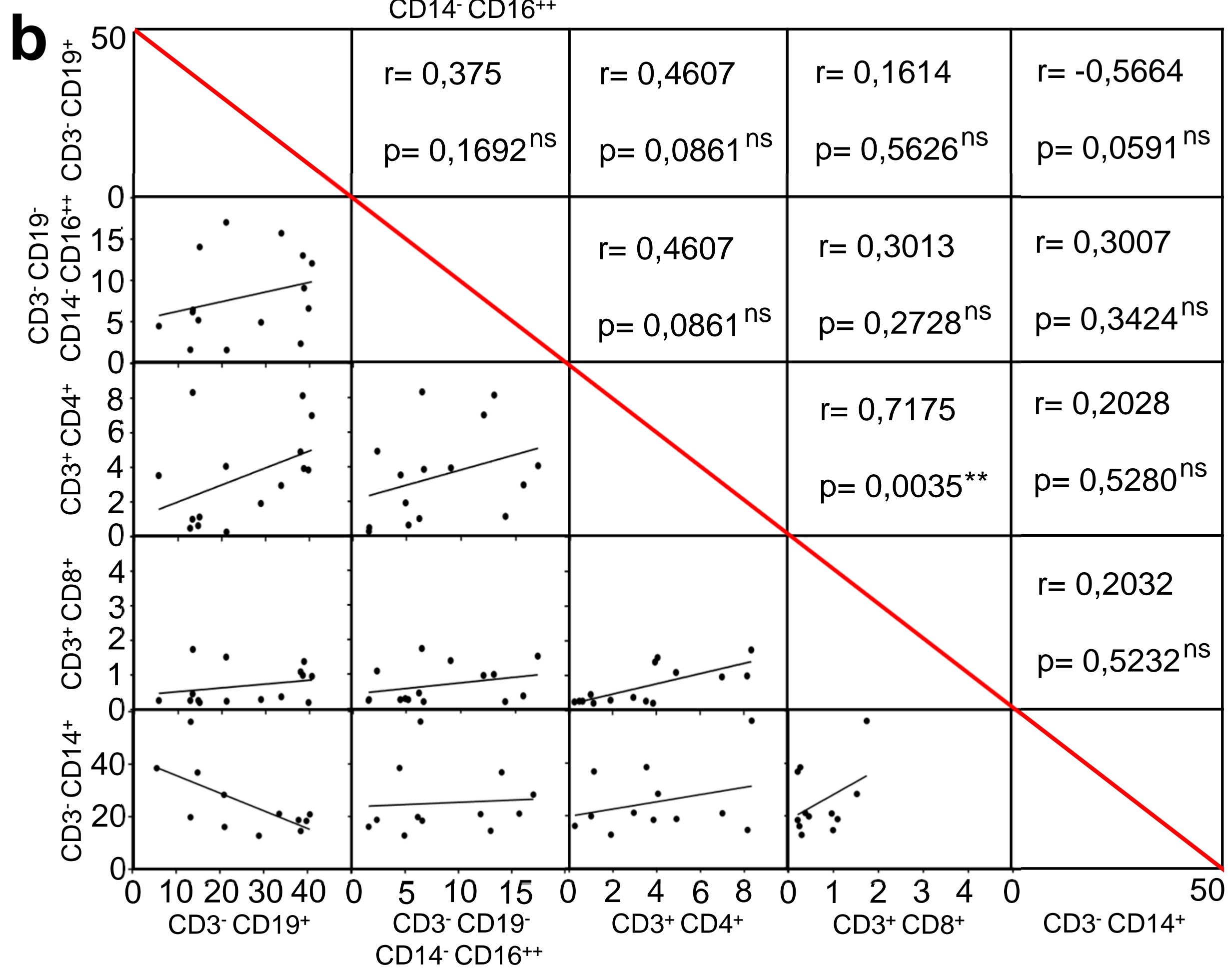
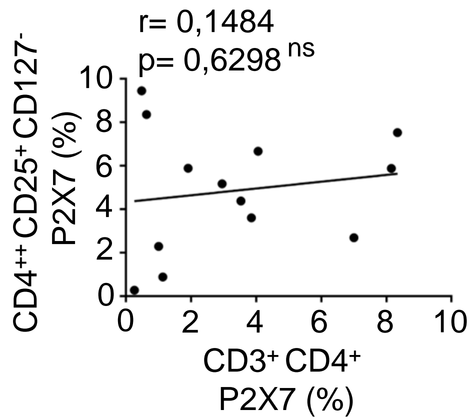
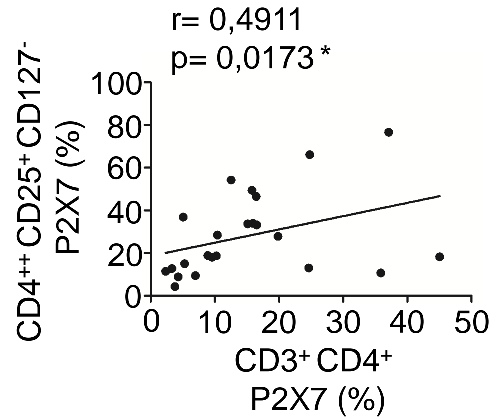

b
CD3- CD19+
CD3- CD19- CD14- CD16++
CD3+ CD4+
CD3+ CD8+
CD3- CD14+
r= 0,375
p= 0,1692ns
r= 0,4607
p= 0,0861ns
r= 0,1614
p= 0,5626ns
r= -0,5664
p= 0,0591ns
r= 0,4607
p= 0,0861ns
r= 0,3013
p= 0,2728ns
r= 0,3007
p= 0,3424ns
r= 0,7175
p= 0,0035**
r= 0,2028
p= 0,5280ns
r= 0,2032
p= 0,5232ns

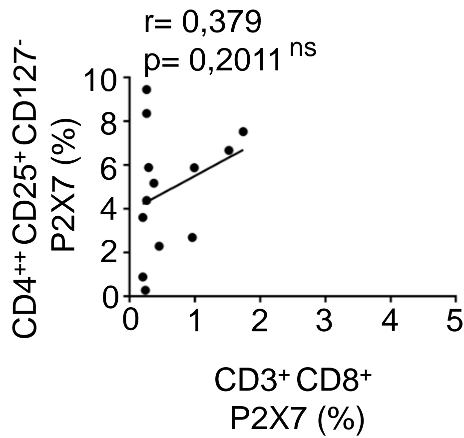
Figure 46. Correlation of P2X7R expression in different PBMCs subpopulations from septic patients and healthy individual. (a,b) Correlation between P2X7R mean intensity fluorescence (MFI) in $CD3^{-}CD14^{+}$ cells and the percentage of $P2X7R^{+}$ in $CD3^{+}CD8^{+}$, $CD3^{+}CD4^{+}$, $CD3^{-}CD19^{+}$, and $CD3^{-}CD19^{-}CD14^{-}CD16^{++}$ cells in septic patients (a) or healthy individuals (b): r spearman test was used for correlations; each dot represents a single patient; *p< 0.05; **p< 0.01; ***p< 0.001; *ns*, not significant difference (p> 0.05).

a





b



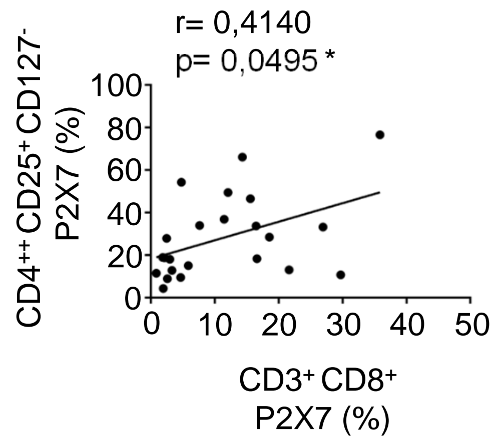


Figure 47. Correlations of P2X7R expression in Tregs and CD4 and CD8 T cell subpopulations from septic patients and healthy individual. (a,b) Correlation between the percentage of $P2X7R^{+}$ in $CD3^{+}CD4^{+}$ (a) or in $CD3^{+}CD8^{+}$ (b); healthy individuals are on the left panels and septic patients on the right panels; r spearman test was used for correlations; each dot represents a single patient; *$p < 0.05$; *ns*, not significant difference ($p > 0.05$).

2.2. P2X7 receptor function associates with immunoparalysis during sepsis

2.2. P2X7 receptor function associates with innate immunoparalysis during sepsis

As we have described that during sepsis there is a functional inflammasome immunoparalysis in some patients, and taking into account the well-established role of P2X7R activating the NLRP3 inflammasome (Roger, Pelegrin, and Surprenant 2008; Pelegrín 2011) and that immunocompromised septic patients P2X7R expression in monocytes do not correlate with ATP-induced IL-1β release (Figure 39a). We then aimed to study the role of P2X7R in immunocompromised septic patients. We found a positive correlation between P2X7R expression in monocytes from immunocompromised septic patients with mitochondrial membrane depolarization on the monocytes (Figure 48a and b). This correlation was absent in non-immunocompromised septic patients (Figure 48a), were there was a correlation with ATP-induced IL-1β release (Figure 39a). We then found that P2X7R stimulation on monocytes and macrophages resulted in a fast-mitochondrial membrane depolarization that was reverted using P2X7R antagonists and the anti-P2X7R blocking nanobody 13A7 (Figure 48c and d). Similarly, using a P2X7R potentiator nanobody 14D5 (Danquah et al. 2016), we were able to increase mitochondrial depolarization in response to suboptimal ATP concentrations for P2X7R (Figure 48d). Mitochondrial membrane depolarization induced by ATP was independent of LPS-priming and the NLRP3 inflammasome (Figure 48e). These data suggest that P2X7R activation in resting monocytes and macrophages results in mitochondrial dysfunction.

According with previous results in septic patients, where we found that immunoparalysis induced by sepsis is transitory (Figure 34), macrophage mitochondrial membrane potential was restored after washing extracellular ATP for 4-12 h (Figure 49a). Interestingly, when we stimulated BMDMs with LPS just after the initial ATP treatment (ATP was not present during the LPS stimulation time), the release of IL-6 was decreased when compared to macrophages not treated with ATP (Figure 49b). This suggest that ATP stimulation before LPS priming is able to affect macrophage priming in a similar way to what happened in immunosuppressed septic patients. If initial ATP was washed, and cells were incubated for 2h before LPS stimulation, then the macrophages recover the ability to normally produce IL-6 after LPS stimulation (Figure 49b). So, the effect of ATP affecting mitochondrial

membrane potential and the ability to produce IL-6 were both transitorily recovered (Figure 49a and b). Furthermore, the antioxidant PDTC was able to protect ATP induced mitochondrial membrane depolarization (Figure 49c) and restored the production of IL-6 after P2X7R stimulation with ATP before LPS priming (Figure 49d). Suggesting that damaging the mitochondria in myeloid cells upon P2X7R activation is a signal to immunocompromise bacterial activation of these cells.

FIGURES:

P2X7 receptor function associates with immunoparalysis during sepsis

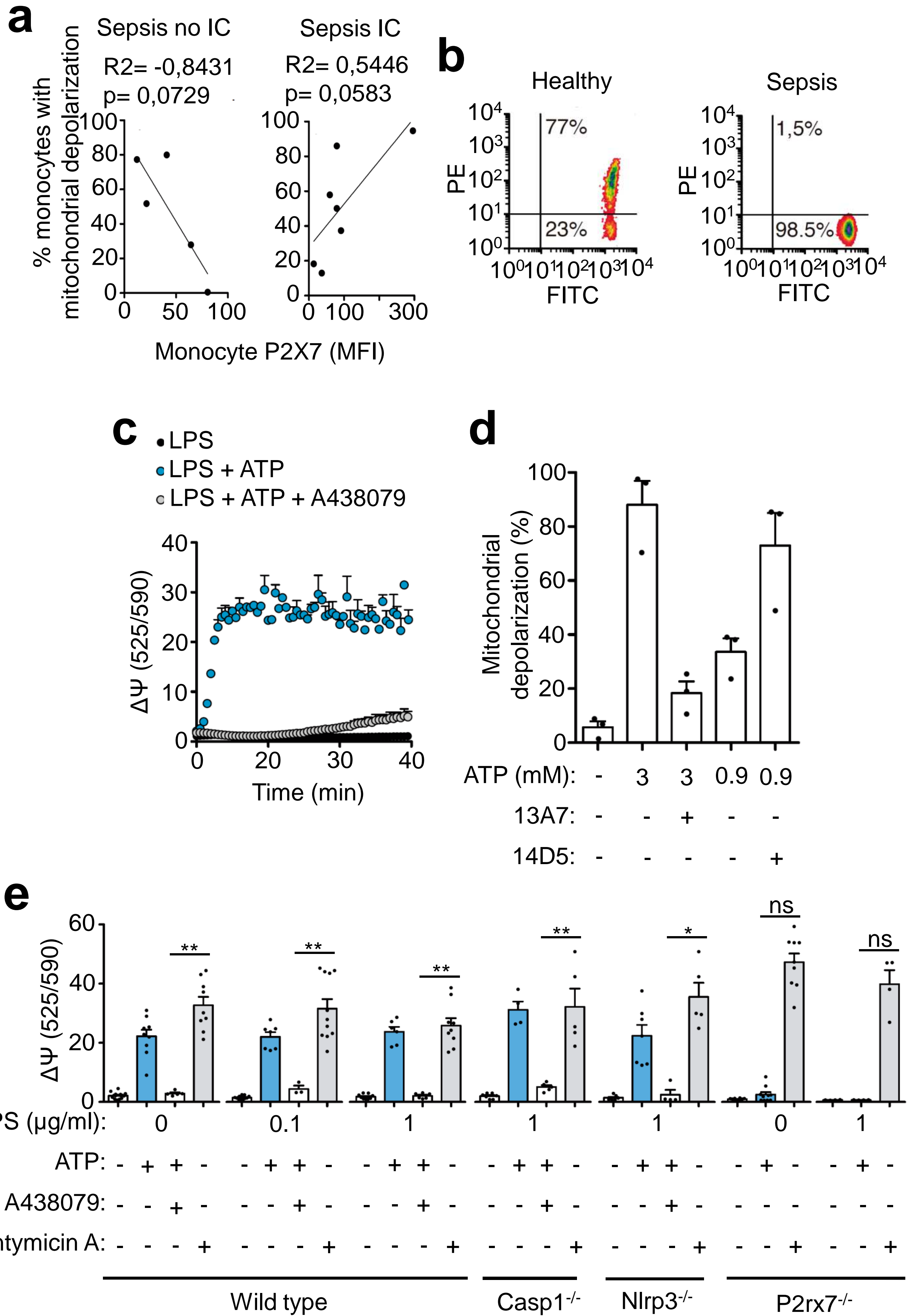
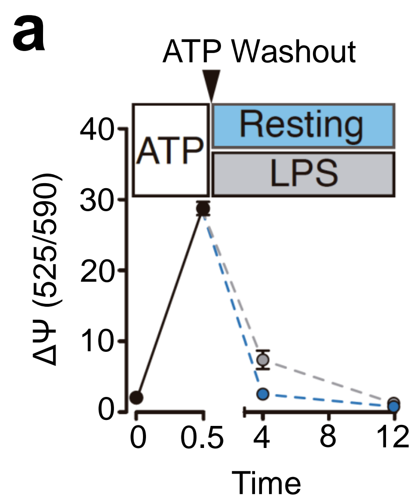
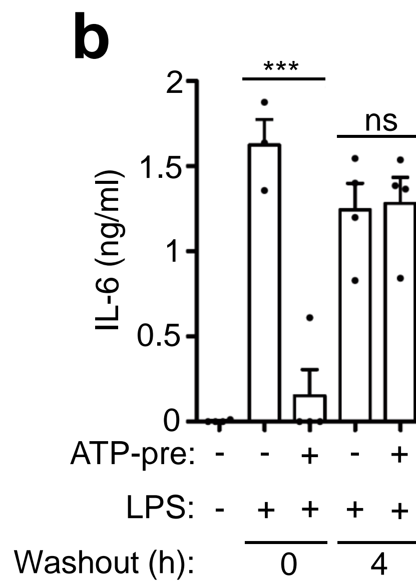
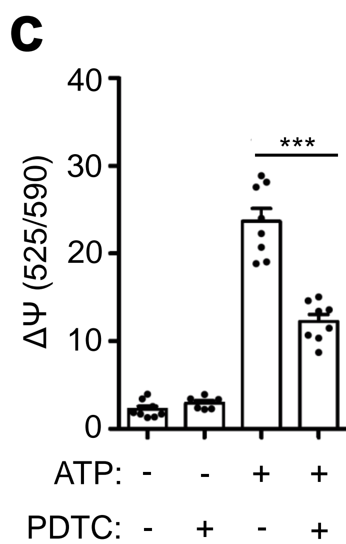

a
Sepsis no IC
R2= -0,8431
p= 0,0729
Sepsis IC
R2= 0,5446
p= 0,0583
% monocytes with mitochondrial depolarization
Monocyte P2X7 (MFI)
b
Healthy
Sepsis
PE
FITC
77%
23%
1,5%
98.5%
c
LPS
LPS + ATP
LPS + ATP + A438079
ΔΨ (525/590)
Time (min)
d
Mitochondrial depolarization (%)
ATP (mM):
13A7:
14D5:
e
ΔΨ (525/590)
LPS (µg/ml):
ATP:
A438079:
Antymicin A:
Wild type
Casp1-/-
Nlrp3-/-
P2rx7-/-

Figure 48. P2X7R activation induces mitochondrial membrane depolarization. (**a**) Correlation between the percentage of monocytes with mitochondrial membrane depolarization and the quantification of P2X7 receptor mean intensity fluorescence (MFI) in monocytes from the indicated septic patient groups; IC: immunocompromised. **(b)** Mitochondrial membrane potential from blood monocytes stained with JC-10 in a control healthy donor (left) and in a septic patient at day 1 (right); PE- cells represent the monocytes with mitochondrial depolarization. **(c)** Kinetics of mitochondrial depolarization after ATP (3 mM) stimulation of LPS-primed BMDMs incubated or not with the P2X7 receptor antagonist A438079 (10 µM). **(d)** Percentage of mouse BMDMs with mitochondrial membrane depolarization after ATP stimulation at the indicated concentrations for 30 min and incubated or not with anti-P2X7 nanobodies (13A7, blocking nanobody; 14D5, potentiating nanobody; each at 200 nM). **(f)** Mitochondrial membrane depolarization from wild-type or knock-out BMDMs as indicated primed or not with LPS (4 h) and treated for 30 min with ATP (3 mM, blue bars), ATP and A438079 (10 µM, white bars), or antimycin A (5 µM, grey bars). Each dot represents an individual patient in a, a single independent experiment in d,f, or average of two independent experiments in b; *p< 0.05; **p< 0.01; *ns*, no significant difference (p> 0.05); Pearson correlation was used in a; and Kruskal-Wallis test was used in e.


a
ATP Washout
ATP
Resting
LPS
ΔΨ (525/590)
Time
0
0.5
4
8
12


b
***
ns
IL-6 (ng/ml)
ATP-pre: - - + - +
LPS: - + + + +
Washout (h): 0 4


c
***
ΔΨ (525/590)
ATP: - - + +
PDTC: - + - +

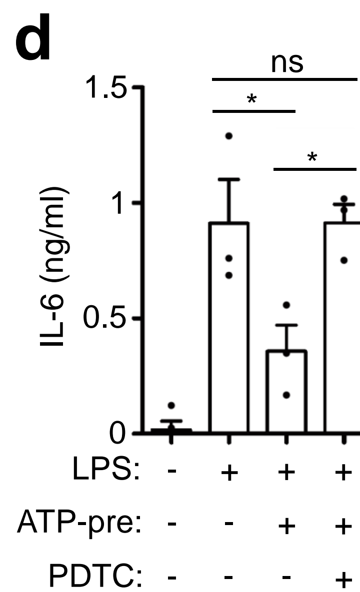

d
ns
*
*
IL-6 (ng/ml)
LPS: - + + +
ATP-pre: - - + +
PDTC: - - - +

Figure 49. Mitochondrial dysfunction mediated by P2X7R induces immunoparalysis of macrophages. **(a)** Mitochondrial membrane depolarization in BMDMs treated with ATP (3 mM, 30 min), then washed out and incubated for the indicated times in the absence or presence of LPS (1 µg/ml) as indicated. **(b)** IL-6 release from BMDMs treated as in (a). **(c)** Mitochondrial membrane depolarization in BMDMs treated with ATP (3 mM, 30 min) in the presence or absence pyrrolidine dithiocarbamate (PDTC, 40 µM). **(d)** IL-6 release from BMDMs treated as in (c) and then primed with or without LPS (1 µg/ml, 4h). Each dot represents a single independent experiment. *p< 0.01; ***p< 0.001; *ns*, no significant difference (p> 0.05); Mann-Whitney test was used for b; Kruskal-Wallis test was used for c and d.

2.3. P2X7 receptor activation affects cell adhesion

2.3. P2X7 receptor activation affects cell adhesion

One important function of myeloid cells in the inflammatory response is their ability to adhere to different extracellular substrates, this allow for example the extravasation of monocytes into areas of infection and injury after binding to activated endothelial cells **(Dowling and Kiely 2015).** In this chapter we aimed to study the role of P2X7R activation in cell-substrate adhesion. We initially evaluated the adhesion of BMDMs and HEK293 cells at different confluences by RTCA (Figure 50a and b). We observed a positive relation between the cellular confluences and the increase of cell adhesion in BMDMs measured by RTCA (Figure 50a). Macrophage adhesion was also revealed by detecting the number of cells by the MTT assay that remains adhered to the plate after a shaking adhesion protocol (Figure 50a). The detection of BMDMs by RTCA and the MTT assay was similar (Figure 50a). Similarly, HEK293 cells also presented the same relation between cell adhesion and confluence measured by RTCA and MTT assays (Figure 50b). This observation reveals that cell adhesion increased with cell confluence independently of the detection method used to quantify.

Additionally, we evaluated if ATP addition was able to induce changes on cell adhesion in BMDMs and HEK293 cells stably expressing or not the P2X7R. We observed a decrease of BMDMs adhesion when ATP concentration increased over 1mM (Figure 51a). The effective dose (EC_{50}) for ATP inducing BMDM detachment was 3mM (Figure 51a). ATP stimulation also result in a decrease of HEK293 cell adhesion only if they expressed P2X7R (Figure 51b). ATP was dose-dependently decreasing HEK293-P2X7R cell adhesion, and the highest the ATP concentrations was used the higher cell detachment was found (Figure 51c). Because in previous chapters of the Thesis we have shown that P2X7R is able to induce mitochondrial depolarization and MTT assay was used to quantify the number of cells in these adhesion experiments (Figure 51a-c), we wanted to make sure that P2X7R activation damaging the mitochondria will not affect the MTT reduction to formazan that requires functional succinate dehydrogenase enzyme of the mitochondria **(Price and Mcmillan 1990)**. Therefore, it could be that the reduction in cell adhesion measured by a decrease on the MTT assay could be misinterpreted due to a reduction of mitochondria function of the remaining attached cells due to P2X7R activation. Therefore, we next quantified the number

of adherent cells after ATP stimulation by staining the cells with the nuclear dye Yo-Pro-1 after plasma membrane permeabilization with triton X-100 to favour Yo-Pro-1 staining. Measurement of Yo-Pro-1 fluorescence from adherent cells after activation with different concentrations of ATP revealed a similar dose dependence reduction than the one found by MTT on cells that remain attached to the tissue culture plate (Figure 51c and d).

EC_{50} for ATP treatment in HEK293cells for cell detachment was of 1.42mM (Figure 51d), a slightly smaller than mouse BMDMs, this could be due because HEK293 cells are stable for rat P2X7, and rat P2X7R present a smaller ATP EC_{50} than mouse P2X7R, albeit HEK293 stable cell line constitutively express high levels of P2X7R on the plasma membrane **(Young, Pelegrin, and Surprenant 2007).**

To corroborate that P2X7R was affecting cell adhesion, the specific P2X7R antagonists A438079 and A740003 were incubated before and during the treatment with ATP, and these antagonists were able to maintain the number of adherent BMDMs and HEK293-P2X7R in the presence of ATP similar to control cells (Figure 52a). Additionally, the same effect was observed when cell adhesion was detected by the Yo-Pro-1 assay, where both A438079 and A740003 inhibitors were able to block ATP-induced cell detachment (Figure 52b).

We next aimed to determine the required time of P2X7R stimulation to induce cell detachment and a time course for cell adherence after ATP treatment was done in BMDMs (Figure 53a and b). In Figure 52a the early time point assayed was 5 minutes after ATP treatment, were we already found a loose of 40% of cells when compared to untreated cells (Figure 53a). This result indicates that ATP affects cell adhesion at a short time (less than 5 minutes) and this reduction was maintained stable over the time up to 2 hours. We also observed that after 30 minutes of culture in assay buffer (ET) without ATP it caused a P2X7R-independent cell detachment (Figure 53a), suggesting that the ET buffer is not ideal to keep the cells over 30 min. We then wanted to analyse cell detachment during the initial 5 minutes of ATP stimulation (Figure 53b). Results indicated that cell detachment started at least after 2 minutes of ATP stimulation (Figure 53b). These finding revealed that ATP affect cell adhesion in a short time after P2X7R stimulation.

Because P2X7R is an unspecific cation channel permeable for Na^+, Ca^{2+} and K^+ **(Browne et al. 2013)**, and its activation induces an efflux of K^+, together an influx of Na^+ and Ca^{2+}, we wanted to study if any of these ionic fluxes could be involved in the rapid cell detachment found upon P2X7R activation. For that, we stimulated HEK293 or HEK293-P2X7R with ATP with different ionic buffer composition, where Na^+ was replaced by $NMDG^+$ (to avoid Na^+ influx), Ca^{2+}-free buffer to avoid Ca^{2+} influx or a buffer with 147 mM of K^+ to avoid K^+ efflux. We observed that in HEK293 cells, there were no significant changes after ATP treatment independently of the buffer used (Figure 54). However, in HEK293-P2X7R cells, ATP was able to detach the cells independently of the buffer used (Figure 54b), suggesting that ionic fluxes trough P2X7R are not affecting cell adhesion.

These results indicated that P2X7R activation affected cell adhesion to the plastic of tissue culture plates. However, in physiological conditions cells interact and adhere to ECMs **(Taherian et al. 2011).** We next assessed BMDMs or HEK293-P2X7R adhesion into different ECM components as type I collagen, laminin or fibronectin (Figure 55a). We observed that HEK293-P2X7R adhesion increased after ECM coating, being significant higher when plates were coated with fibronectin (Figure 55a). On the contrary, BMDMs did not present any difference in cell adhesion after ECM coating respect to the plastic on tissue plates (Figure 55a). These results indicated that macrophages can adhere easily to any substrate because they are highly adherent cells. When BMDMs were activated with ATP, we found cell detachment only when collagen was used to coat the plate (Figure 55b). Therefore, this result suggests that P2X7R activation in BMDMs was not able to induce cell detachment from laminin or fibronectin.

As LPS increase macrophage cell adhesion **(Kounalakis and Corbett 2006)**, we wanted to evaluate the effect of LPS priming in the P2X7R induced macrophage detachment. RTCA revealed that LPS priming was inducing in fact an increase of BMDMs adhesion (Figure 35a). This increase in BMDMs upon LPS stimulation was not evidenced by our adhesion protocol, suggesting that RTCA could be also measuring cell spreading after LPS priming. However, LPS significantly increased BMDM adhesion to fibronectin (Figure 56b), indicating than LPS could be upregulating adhesion molecules in BMDM that specifically binds fibronectin. Cell detachment induced by P2X7R significantly decreased when ATP was applied after 4 hours of LPS priming, but not after IL-4 priming where cell detachment was similar to resting BMDMs (Figure 56c). As expected, pyroptotic cell death

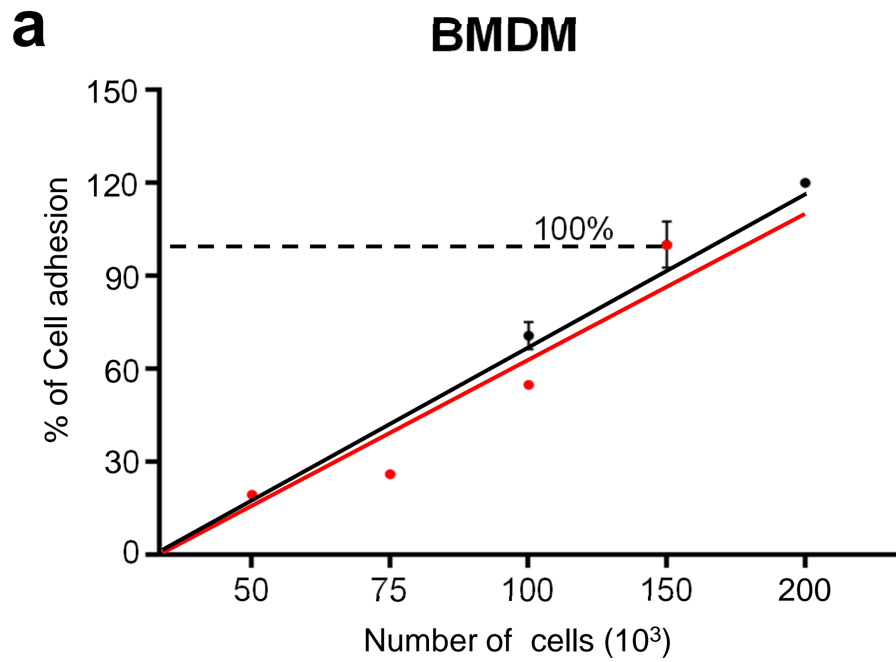
measured by the presence of extracellular LDH and IL-1β that were significantly increased on LPS + ATP treated BMDMs (Figure 56d). Therefore, the high decrease in LPS-primed macrophages adhesion could be due to a combination of P2X7R inducing loose of cellular adherence and pyroptotic cell death. If this is true, pyroptosis should induce an unspecific type of cell detachment. In fact, LPS-primed BMDMs were detached upon P2X7R activation independently on the ECM substrate used, including laminin and fibronectin (Figure 56e), where we found that P2X7R did not affect macrophage adherence (Figure 55b).

Binding to type I collagen, laminin or fibronectin requires the recognition through αxβ1 integrins that are present in cell membrane of macrophages (Albelda and Buck 1990; Hynes 2002). Moreover, It has been shown that P2X7R activation by extracellular ATP induces shedding of different adhesion molecules, facilitating cell migration (Sengstake, Boneberg, and Illges 2006; Mishra et al. 2016). Western-blot analysis for β1 integrin in resting or LPS-primed BMDMs supernatants revealed the presence of extracellular β1 integrin (120-130 kDa) only after ATP treatment (Figure 56f). This result also demonstrates that the shedding of β1 integrin was independent on LPS priming (Figure 56f). The release of soluble β1 integrin could be involved in the loose of cell adherence found upon P2X7R activation, as a previous work describe (Furlan-freguia et al. 2011). Our data support a model where in resting macrophages P2X7R could mediate the shedding of β1 integrin losing adhesion to collagen that is highly dependent on this integrin. Meanwhile macrophages still using other β integrins to bind to laminin or fibronectin. Upon LPS priming, pyroptotic cell death induced by P2X7R will be also added to unspecific loose adherence to different substrates (Figure 57).

FIGURES:

2X7 receptor activation

Affects cell adhesion


a
BMDM
150
120
90
60
30
0
% of Cell adhesion
100%
50
75
100
150
200
Number of cells (10^3)

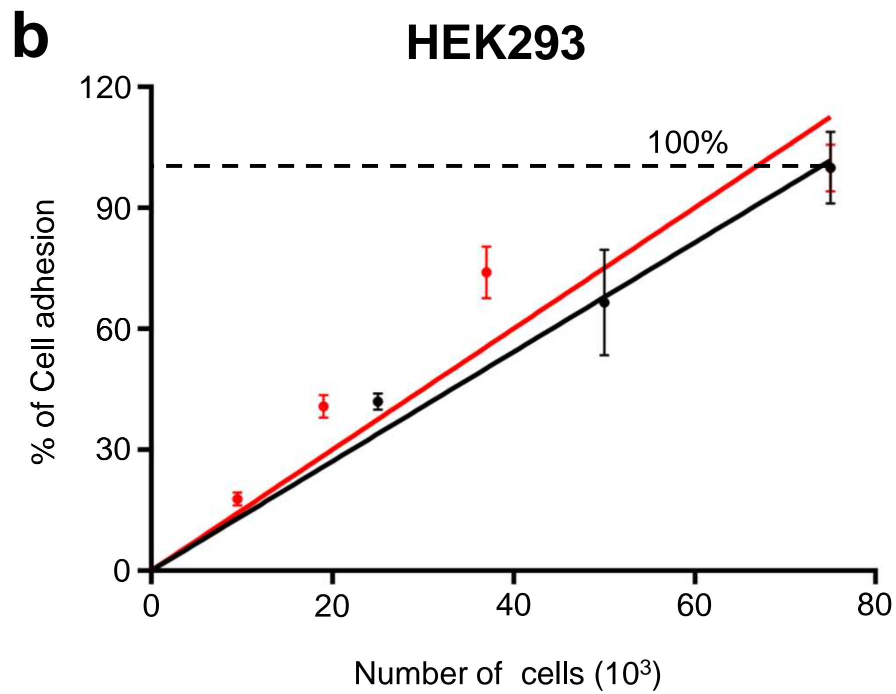
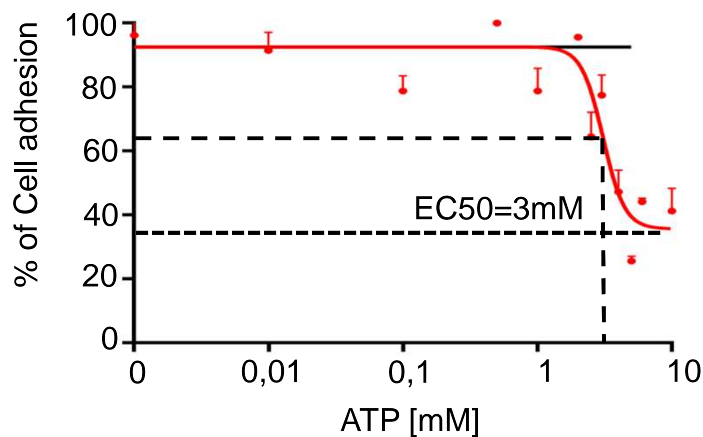
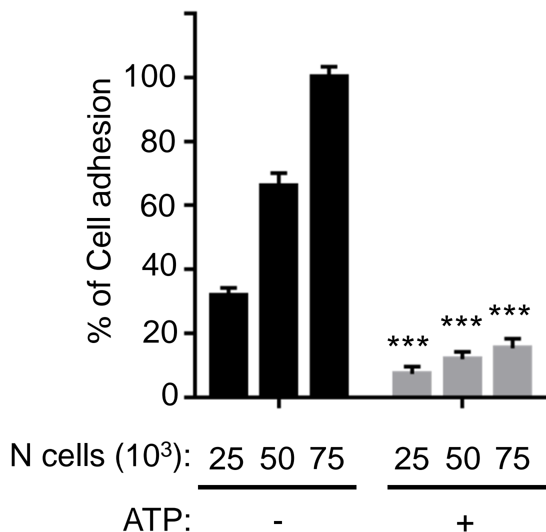
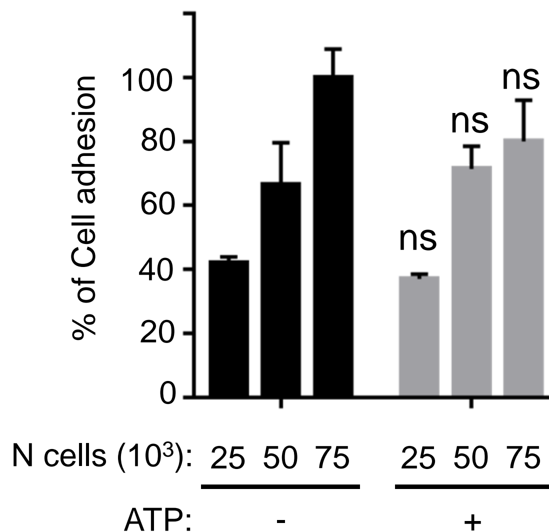
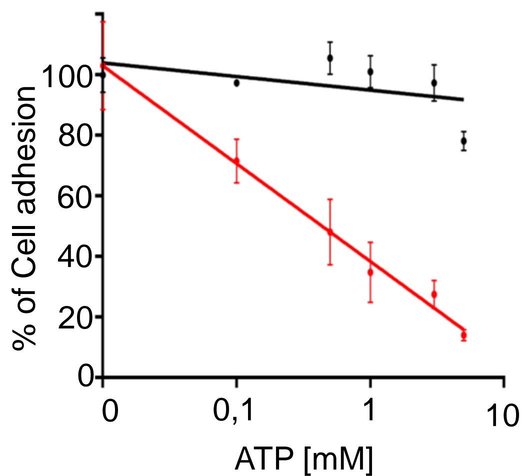

b
HEK293
120
90
60
30
0
% of Cell adhesion
100%
0
20
40
60
80
Number of cells (10^3)

Figure 50. Macrophage and HEK293 cell adhesion. (a,b) Percentage of cell adhesion measured by the MTT assay (black line) or RTCA (red line) in BMDM (a) or HEK293 (b) plated at different densities.


a
BMDM
% of Cell adhesion
100
80
60
40
20
0
EC50=3mM
0
0,01
0,1
1
10
ATP [mM]


b
HEK293-P2X7
% of Cell adhesion
100
80
60
40
20
0
***
***
***
N cells (10³): 25 50 75 25 50 75
ATP: - +


HEK293
% of Cell adhesion
100
80
60
40
20
0
ns
ns
ns
N cells (10³): 25 50 75 25 50 75
ATP: - +


c
HEK293-P2X7 MTT
% of Cell adhesion
100
80
60
40
20
0
0
0,1
1
10
ATP [mM]

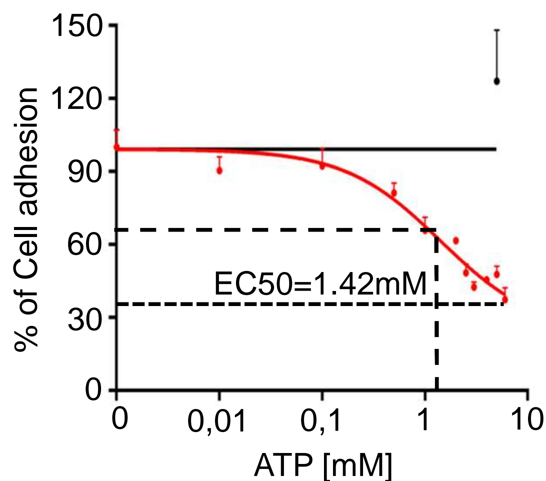
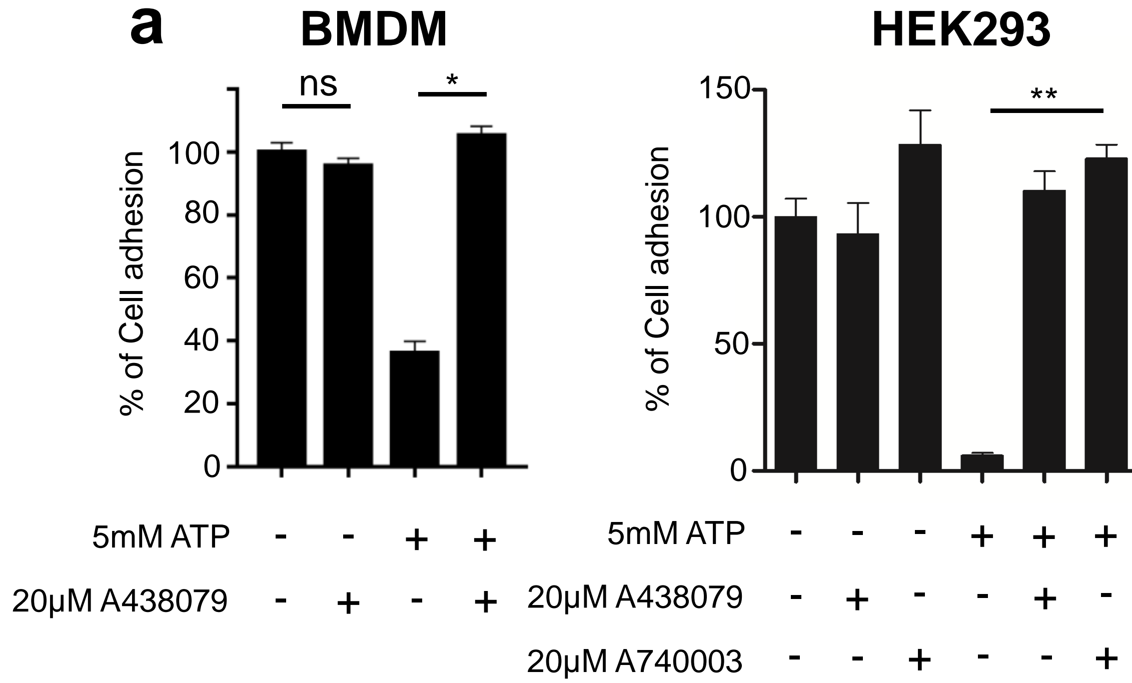

d
HEK293-P2X7 Yo-Pro
% of Cell adhesion
150
120
90
60
30
0
EC50=1.42mM
0
0,01
0,1
1
10
ATP [mM]

Figure 51. Cell adhesion is reduced in an ATP dose dependent manner. (a) Percentage of cell adhesion measured by the MTT assay in BMDM treated with different ATP concentrations. **(b)** Percentage of cell adhesion measured by the MTT assay in HEK-P2X7R (left) or HEK293 (right) pleated at different cell densities and stimulated as indicated for XX min with ATP (3 mM). **(c,d)** Percentage of cell adhesion measured by the MTT assay (c) or Yo-Pro-1 staining (d) in HEK293-P2X7R cells untreated (black trace) treated with different ATP concentrations (red trace); each experiment represent the average of n=3 independent observations; ***$p < 0.001$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was used in b.


a
BMDM
ns
*
% of Cell adhesion
100
80
60
40
20
0
5mM ATP - - + +
20µM A438079 - + - +
HEK293
**
150
100
50
0
% of Cell adhesion
5mM ATP - - - + + +
20µM A438079 - + - - + -
20µM A740003 - - + - - +

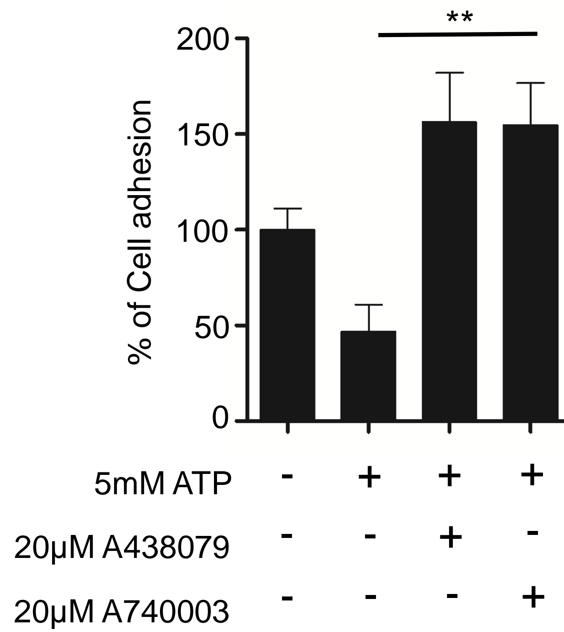
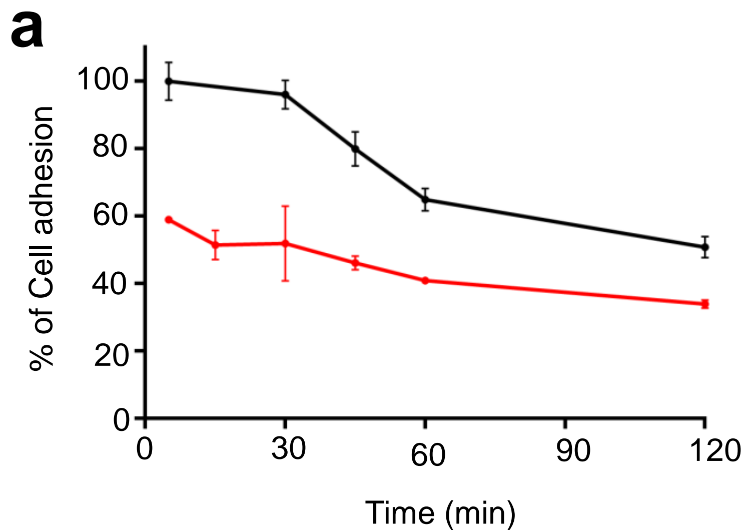

b
**
200
150
100
50
0
% of Cell adhesion
5mM ATP - + + +
20µM A438079 - - + -
20µM A740003 - - - +

Figure 52. Cell detachment is dependent on the P2X7R activation. (a) Percentage of cell adhesion measured by MTT assay (a) or Yo-Pro-1 staining (b) in BMDM (a, left panel) or in HEK293-P2X7R cells (a, right panel, b) treated for 10 min with the of P2X7R-specific inhibitors A438079 or A790003 as indicated (both at 20 μM) and then activated for 30 min with ATP (5 mM). Kruskal-Wallis test was used in a,b; each value represent average of n=3 independent experiments; *p< 0.05; **p< 0.01; *ns*, not significant difference (p> 0.05).


a
100
80
60
40
20
0
0
30
60
90
120
% of Cell adhesion
Time (min)

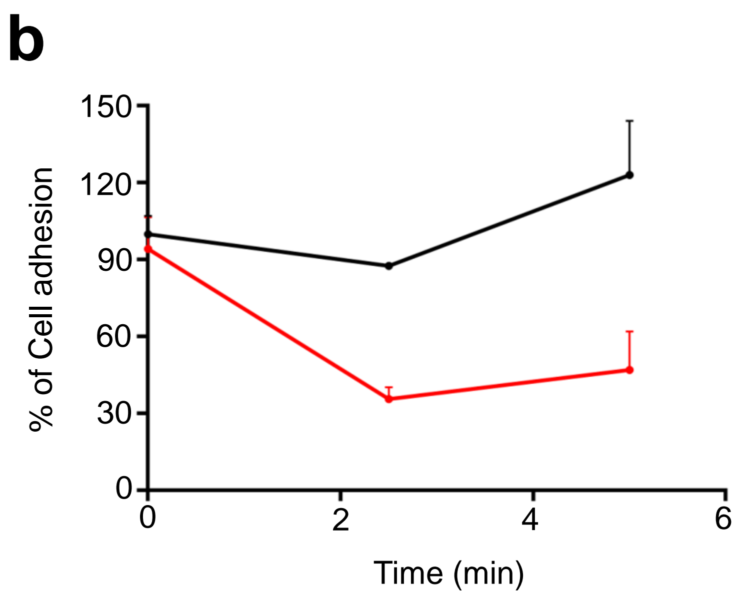
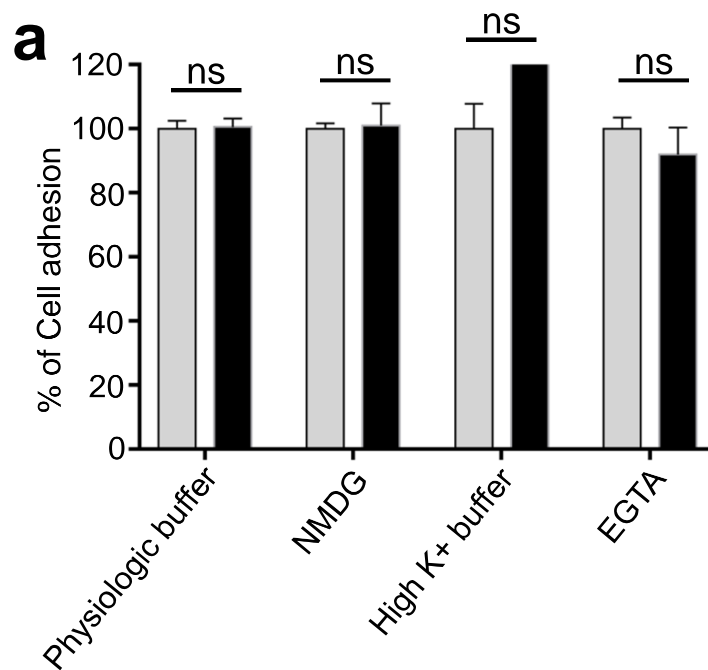

b
150
120
90
60
30
0
0
2
4
6
% of Cell adhesion
Time (min)

Figure 53. Kinetic for ATP induced cell detachment. (a) Cell adhesion measured by MTT in BMDM cells untreated (black trace) or treated from 5 to 120 minutes with ATP (5 mM, red trace). **(b)** Cell adhesion measured by Yo-Pro-1 staining assay in HEK293-P2X7R cells untreated (black trace) or treated from up to 5 minutes with ATP (5 mM, red trace). Each time evaluated represents average of n=3 independent experiments.


a
ns
ns
ns
ns
120
100
80
60
40
20
0
% of Cell adhesion
Physiologic buffer
NMDG
High K+ buffer
EGTA

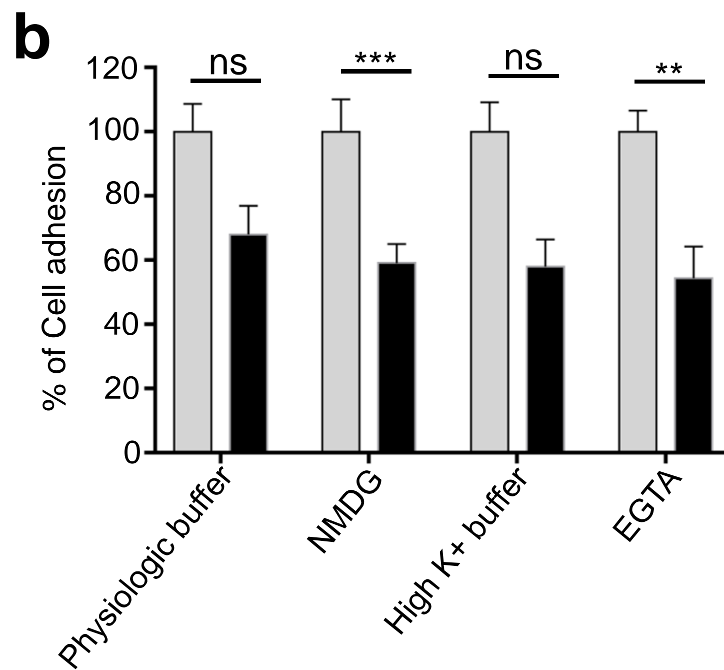
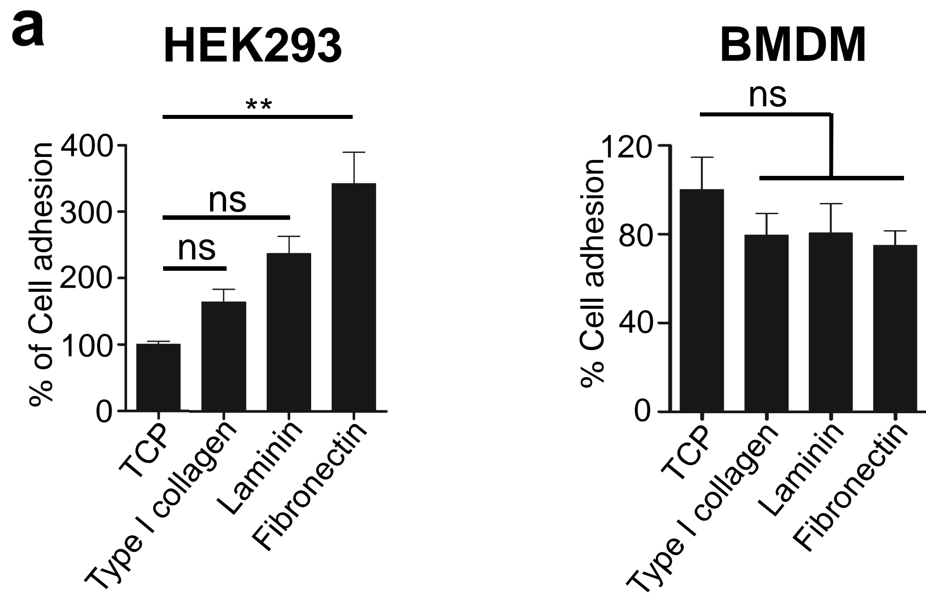

b
ns
***
ns
**
120
100
80
60
40
20
0
% of Cell adhesion
Physiologic buffer
NMDG
High K+ buffer
EGTA

Figure 54. Ionic flux across P2X7R does not contribute to cell adhesion. (a,b) Cell adhesion measured by MTT in HEK293 (a) or HEK293-P2X7R (b) cells treated or not for 30 min with ATP (5 mM) in ET physiological buffer, a buffer with $NMDG^+$ instead Na^+, a 147 mM K^+ buffer, and a buffer without Ca^{2+} and EGTA. Data is average of n=3 independent experiments; **$p < 0.01$; ***$p < 0.001$; *ns*, not significant difference ($p > 0.05$); Mann-Whitney test was used.


a
HEK293
**
ns
ns
% of Cell adhesion
400
300
200
100
0
TCP
Type I collagen
Laminin
Fibronectin
BMDM
ns
% Cell adhesion
120
80
40
0
TCP
Type I collagen
Laminin
Fibronectin

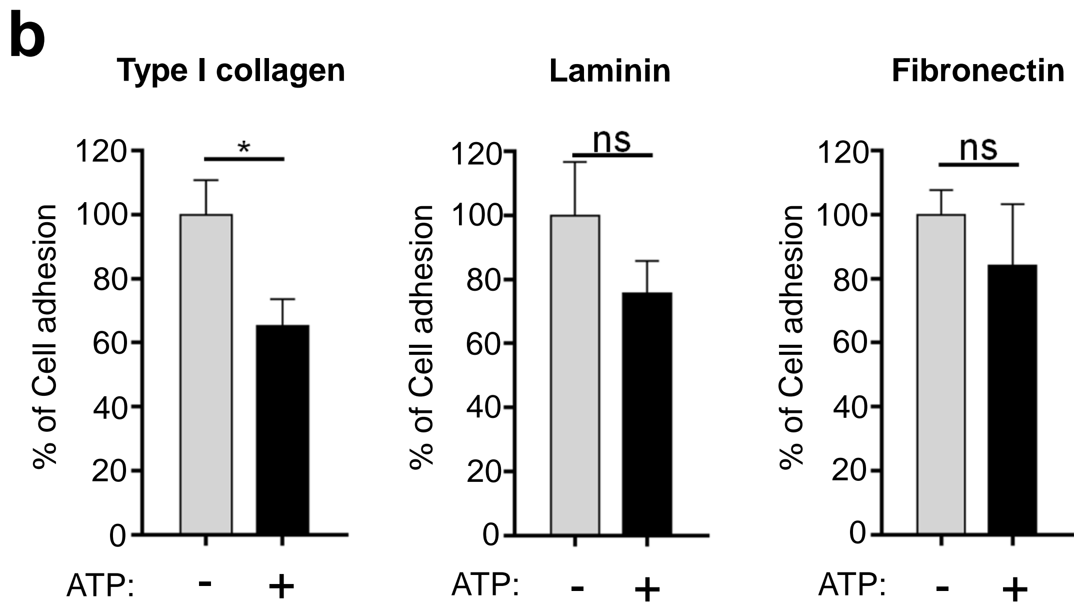

b
Type I collagen
Laminin
Fibronectin
*
ns
ns
% of Cell adhesion
120
100
80
60
40
20
0
ATP:
-
+

Figure 55. P2X7R activation does not affect macrophage cell adhesion to collagen and laminin (a) Percentage of cell adhesion measured in HEK293 cells (left panel) or BMDMs (right panel) to uncoated plates (tissue culture plate, TCP) or coated with type I collagen, laminin or fibronectin. **(b)** BMDMs adhesion plated in different ECM as in a, but treated for 30 min with or without ATP (5 mM). Each value represent the average of n=3 independent experiments; *$p < 0.05$; **$p < 0.01$; *ns*, not significant difference ($p > 0.05$); Kruskal-Wallis test was used for a; Mann-Whitney test was used for b.

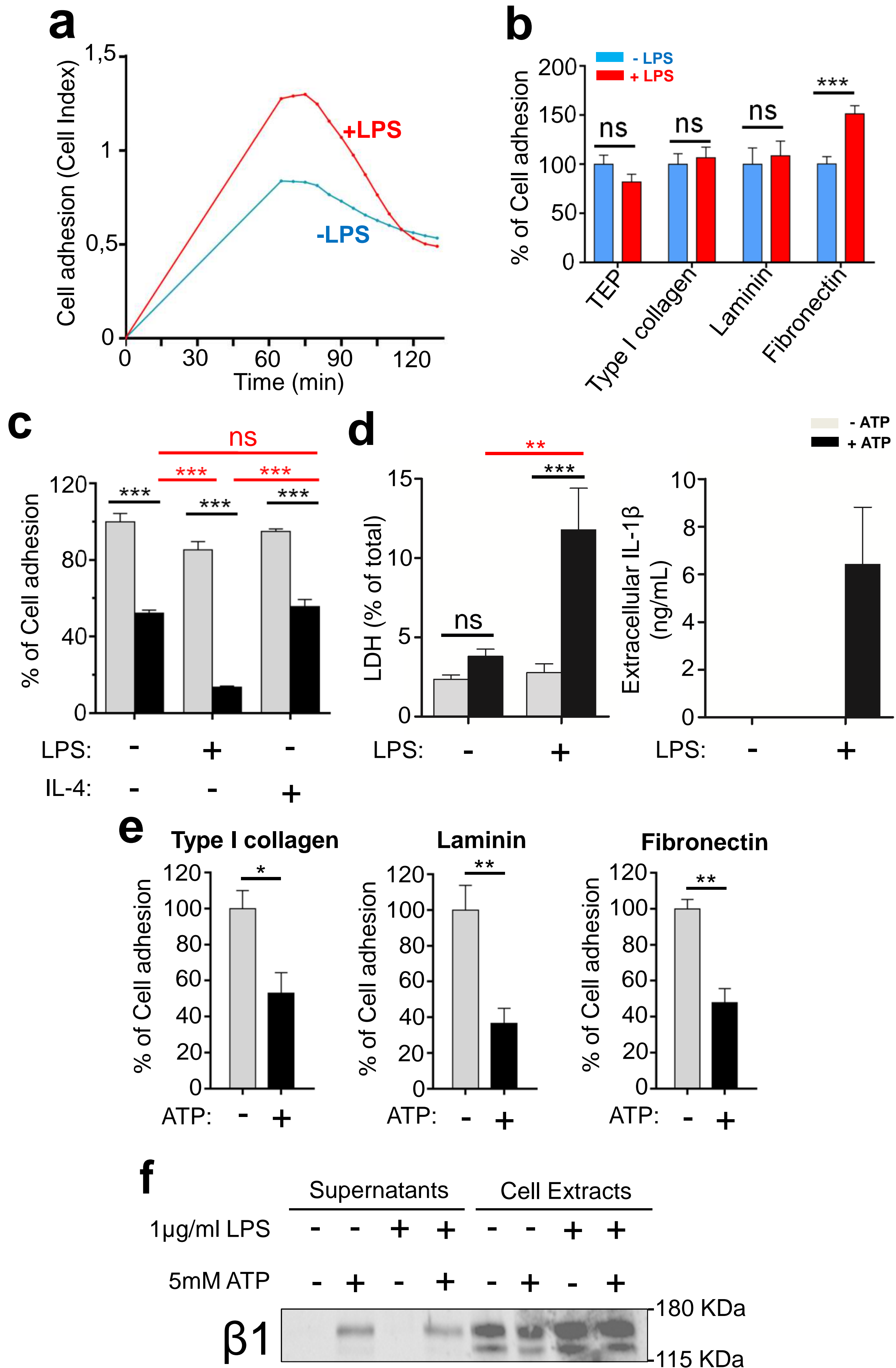
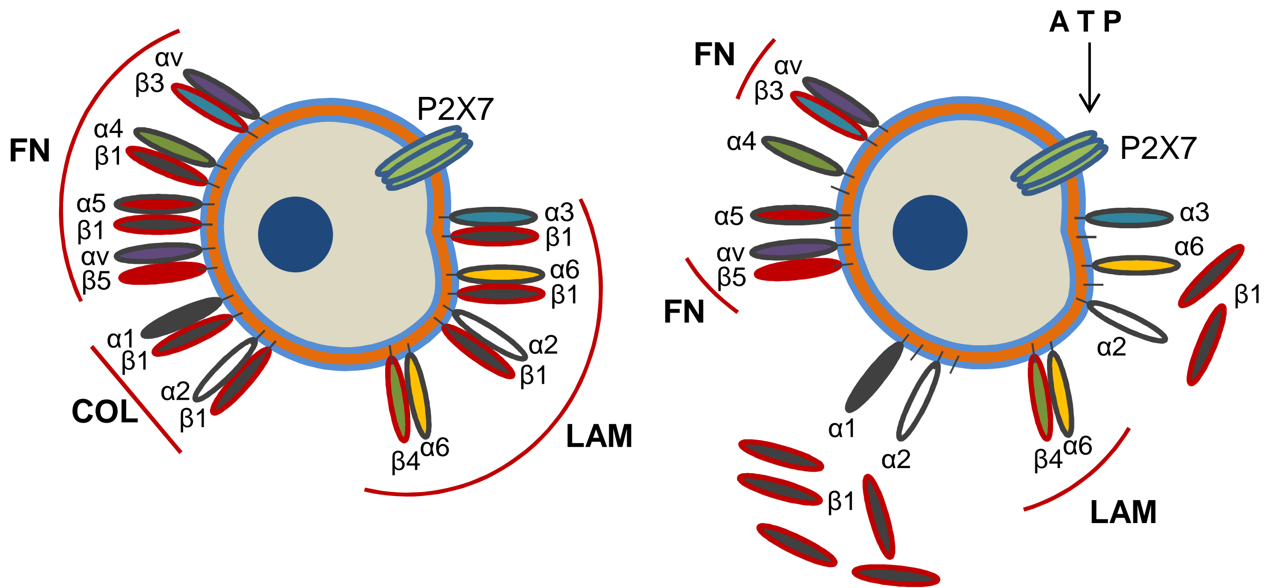

a
Cell adhesion (Cell Index)
1,5
1
0,5
0
+LPS
-LPS
0
30
60
90
120
Time (min)
b
- LPS
+ LPS
% of Cell adhesion
200
150
100
50
0
ns
ns
ns
***
TEP
Type I collagen
Laminin
Fibronectin
c
ns
***
***
***
***
***
% of Cell adhesion
120
80
40
0
LPS: - + -
IL-4: - - +
d
**
***
ns
- ATP
+ ATP
LDH (% of total)
15
10
5
0
Extracellular IL-1β (ng/mL)
10
8
6
4
2
0
LPS: - +
LPS: - +
e
Type I collagen
Laminin
Fibronectin
% of Cell adhesion
120
100
80
60
40
20
0
*
**
**
ATP: - +
f
Supernatants
Cell Extracts
1µg/ml LPS - - + + - - + +
5mM ATP - + - + - + - +
β1
180 KDa
115 KDa

Figure 56. LPS-activated macrophages interspecifically loose adhesion after ATP stimulation. (a) Cell adhesion measured by RTCA in BMDMs treated or not for 120 min with LPS (1µg/ml). **(b)** Percentage of cell adhesion measured by MTT assay in BMDMs treated or not for 4 h with LPS (1µg/ml) plated in uncoated plates (tissue culture plate, TCP) or plates coated with type I collagen, laminin or fibronectin. **(c)** Percentage of cell adhesion measured by MTT assay in BMDMs plated in uncoated plates and primed or not for 4 h with LPS (1µg/ml) or IL-4 (20 ng/ml) and then stimulated for 30 min with ATP (5 mM). **(d)** Extracellular LDH (left panel) or IL-1β (right panel) in BMDMs treated as in (c) as indicated. **(e)** Percentage of cell adhesion measured by MTT assay in LPS-primed BMDMs plated into type I collagen, laminin, or fibronectin plates and then stimulated for 30 min with ATP (5 mM). **(f)** Western blot for β1 integrin in BMDMs cell lysates or supernatants treated as in (d). Values represent average of n=3 independent experiments for b-e; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *ns*, not significant difference ($p > 0.05$); black statistics indicate intragroup differences; red statistics indicate differences between two groups; Kruskal-Wallis test was used for b; Two-way ANOVA was used for c,d; Mann-Whitney test was used for e.


Resting Mφ
FN
αv
β3
α4
β1
α5
β1
αv
β5
α1
β1
COL
α2
β1
P2X7
α3
β1
α6
β1
α2
β1
β4
α6
LAM
ATP
FN
αv
β3
α4
α5
αv
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FN
P2X7
α3
α6
β1
α2
α1
α2
β1
β4
α6
LAM

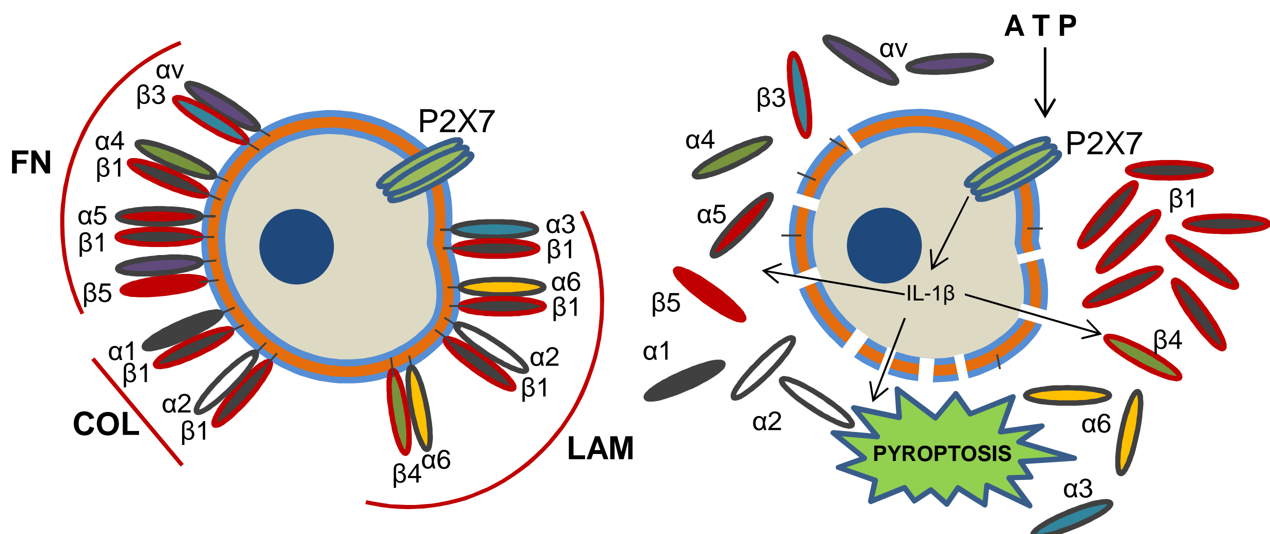
LPS-primed Mφ
FN
αv
β3
α4
β1
α5
β1
β5
α1
β1
COL
α2
β1
P2X7
α3
β1
α6
β1
α2
β1
β4
α6
LAM
ATP
αv
β3
α4
α5
β5
α1
α2
P2X7
β1
IL-1β
β4
PYROPTOSIS
α6
α3

Figure 57. Model of P2X7R role in the adhesion of macrophages. β1 integrin shedding after ATP stimulation of P2X7R in a resting macrophage (upper panel) or in a LPS-primed macrophage where P2X7R associates to pyroptosis in addition to β1 integrin shedding. Mφ: macrophage; COL: collagen; FN: fibronectin; LAM: laminin.

Discussion

This Thesis is organized in different sections, because it covers different aspects of the P2X7R signalling and the NLRP3 inflammasome in immunity, focusing at the beginning on the effect of AMP on NLRP3 activation, where we found that melittin was an activator of NLRP3 inflammasome, resulting in an attenuated inflammasome response that does not result in caspase-1 dependent cell death, then by characterizing inflammasome involvement and P2X7 contribution to immunosuppression of the inflammatory response in sepsis from abdominal origin, and finishing on the expression and function of P2X7R in leukocytes from septic patients as well as its role on cellular adhesion.

Lytic cell death induced by melittin bypasses pyroptosis and induces NLRP3 inflammasome activation and IL-1β release.

We have described that the cytotoxic AMP known as melittin produces macrophage plasma membrane disruption with a concomitant decrease in intracellular K^+ concentration, driving the canonical NLRP3 inflammasome activation. However, melittin-induced plasma membrane permeabilization also leads to rapid cytotoxicity that impairs the execution of pyroptosis, denoting that necrotic cell death induced by this AMP toxin, similar to other plasma membrane disrupting agents, could preclude pyroptosis **(Martín-sánchez et al. 2016)**. AMP are produced by different cells from distinct organisms to aid in the clearance of pathogen infections **(Zasloff 2002)**. In this Thesis, we examined the function of four cationic natural amphipathic antimicrobial peptides in the activation of the NLRP3 inflammasome: (a) LL-37, the only human cathelicidin peptide produced by a variety of cells, including monocytes; (b) temporin A, an antimicrobial peptide produced by the skin of the European frog *Rana temporaria*; (c) Indolicidin, a peptide present in the cytoplasmic granules of bovine neutrophils; and (d) melittin, a peptide toxin produced by bees and with high microbicide activity **(Zasloff 2002; Rinaldi et al. 2002; Raghuraman and Chattopadhyay 2007)**. The lytic mechanism of these peptides is driven by their small, cationic and amphipathic structures that insert and destabilize membrane structures by different mechanisms **(Zasloff 2002).** Although this mechanism of action is shared by these four antimicrobial peptides, we identified melittin as the most active, inducing IL-1β release from LPS-primed macrophages, confirming previous work **(Palm and Medzhitov 2013)**. LL-37 is also known to induce IL-1β

release via modulation of the P2X7R in human monocytes (Elssner et al. 2004); however, our results indicated that LL-37 was not able to induce IL-1β in mouse bone marrow derived macrophages. This may be due to different activation pathways to induce the NLRP3 inflammasome in different types of myeloid cells; in fact, strong differences were reported in NLRP3 activation after cell swelling when THP-1 and BMDMs were compared (Compan et al. 2012). Furthermore, THP-1 is a derived monocytic cell line, and although treatment with phorbol esters differentiates THP-1 into a macrophage-like type, it is known that monocytes regulate the NLRP3 inflammasome in a different manner (Netea et al. 2009). While in macrophages, NLRP3 requires two-step signalling for its activation, monocytes activate NLRP3 through an alternative pathway that involves the TRIF–RIPK1–FADD–CASP8 pathway upon TLR4 engagement (Gaidt et al. 2016). Therefore, the differences in activating NLRP3 among monocytes and macrophages could help explain the LL-37 disability to induce IL-1β release in BMDMs. Furthermore, THP-1 is a tumour cell line (TIB-202; American Type Culture Collection) and, as such, more susceptible to the action of membrane-active antimicrobial peptides than primary cells such as BMDMs. Melittin is the main component of bee venom and is responsible for inducing an allergic reaction characterized by local inflammation (Müller 2010). NLRP3 inflammasome is known to play a role in this inflammatory response *in vivo* (Palm and Medzhitov 2013) and in our study we found that melittin activates the canonical NLRP3 inflammasome pathway via intracellular K^+ depletion, a common key step for activation of this inflammasome (Muñoz-Planillo et al. 2013; Pétrilli et al. 2007). NLRP3 activates caspase-1 by recruitment of the adaptor protein ASC via homotypic domain interactions (Próchnicki, Mangan, and Latz 2016; Carlos de Torre-Minguela, del Castillo, and Pelegrín 2017), and although melittin- induced caspase-1 activation and IL-1β secretion required the adaptor ASC protein (Palm and Medzhitov 2013) and our study, we also found that melittin induced cell death was independent of ASC and caspase-1. ASC aggregation into large speck- like structures is the result of a prion-like oligomerization process that amplifies inflammasome signalling and executes a specific type of cell death called pyroptosis, driven by gasdermin D cleavage by caspase-1 (Dick et al. 2016; Cai et al. 2014; Lu et al. 2014). The lack of caspase-1-induced pyroptosis after NLRP3 activation by melittin could likely be associated to its rapid effect on plasma membrane destabilization that occurs in parallel to NLRP3 activation. Melittin is a peptide able to induce cell death by the formation of pores in the plasma membrane eukaryotic cells, leading to osmotic cell lysis (van den Bogaart et al. 2008; Subbalakshmi, Krishnakumari, and Nagaraj 1996; Raghuraman

and Chattopadhyay 2007). We found plasma membrane destabilization by melittin to occur more rapidly than caspase-1 plasma membrane disruption by the production of the lytic amino-terminal fragment of gasdermin D. However, melittin- induced plasma membrane pore formation is important for inducing caspase-1 activation and IL-1β secretion by K^+ efflux. Similarly, mixed-lineage kinase domain-like protein induces necroptosis and activates the NLRP3 inflammasome, and in this process, IL-1β release is independent of gasdermin D plasma membrane permeabilization (Gutierrez et al. 2017). Melittin-induced direct cell lysis is independent of pyroptosis and excludes pyroptosis because when the plasma membrane is already permeabilized by melittin, caspase-1 activates and processes gasdermin D and, therefore, pyroptosis execution by caspase-1 is bypassed. However, melittin is still the initial trigger for NLRP3 activation and IL-1β release. It also has been reported that LL-37 inhibits pyroptosis after NLRP3 activation through caspase-1 blocking (Hu et al. 2014), denoting different mechanisms of action for antimicrobial peptides, preventing pyroptosis. Hydroxyapatite crystals also induce the activation of both NLRP3 inflammasome and IL-1β release independently of cell lysis, and caspase-1 is not required for hydroxyapatite induced cytotoxicity (Jin et al. 2011). Following caspase-1 activation, together with IL-1β there is also a processing and release of the pro-inflammatory cytokine IL-18 (Schroder and Tschopp 2010; Strowig et al. 2012), and accordingly in our work, nigericin treatment of LPS-primed macrophages resulted in a release of IL-1β and IL-18. These two inflammasome-derived cytokines, IL-1β and IL-18, are differentially induced *in vivo* and may even drive different disease symptoms (Brydges et al. 2013). IL-18 has been specifically implicated in the development of age-related macular degeneration, ischemic acute renal failure and NLRC4-associated hyperinflammation (Canna et al. 2017; Doyle et al. 2014; Melnikov et al. 2001). Furthermore, in mouse models of autoinflammatory cryopyrin-associated periodic syndromes, IL-1β and IL-18 drive pathological conditions at different stages of the disease process (Brydges et al. 2013). However, *in vitro* both cytokines are usually detected in macrophage supernatants after NLRP3 inflammasome activation, that is, after ATP, nigericin treatment or hypotonicity stimulation, but levels of IL-18 are usually around 10 times lower than IL-1β (Compan et al. 2012; Doyle et al. 2014; Perregaux et al. 2000), consistent with our findings. Melittin failed to induce IL-18 release, and this could be attributed to low caspase-1 activation induced by melittin treatment, which may be enough to induce IL-1β, but not IL-18 release. We surmise that lack of caspase-1 activity amplification due to macrophage cell

death directly induced by melittin, may explain this observation **(Dick et al. 2016).** Overall, melittin induced a differential release of IL-1β over IL-18, and this could be important for the resulting response to bee venom. In fact, inflammatory response associated with bee venom has been found to be dependent on IL-1 receptor signalling **(Palm and Medzhitov 2013)**.

P2X7 receptor induces mitochondrial failure in monocytes and compromise NLRP3 inflammasome activation in human sepsis.

Sepsis remains the leading cause of deaths in critical care units **(Herrán-Monge et al. 2017)**, our study supports that during the initial inflammatory response of sepsis, septic patients with late-mortality present an early immunoparalysis that affects the priming and activation of the NLRP3 inflammasome. Early immunocompromised septic patients presented a high mortality, which was evidenced by a longer state of severity. However, NLRP3 inflammasome immunoparalysis and high-mortality during sepsis was not identified with the different biochemical or clinical scores to assess sepsis severity at early phase, but were evidenced by an impaired ability to release IL-1β after *ex vivo* stimulation of blood leukocytes with ATP. The expression of P2X7R in monocytes from immunocompromised septic patients correlated with mitochondrial dysfunction, and not with the NLRP3-dependent secretion of IL-1β. Mechanistically, P2X7R activation in monocytes resulted in a fast depolarization of mitochondria membrane potential that impaired subsequent NLRP3 inflammasome priming and activation. Sepsis initially develops a systemic acute inflammatory response driven by the exacerbated production of proinflammatory cytokines in response to infection, and several cytokines, as IL-6 or IL-18, have been proposed as potential biomarkers for septic patients **(Cinel and Opal 2009; Eidt et al. 2016; Gogos et al. 2000; Harbarth et al. 2001; Mera et al. 2010)**. Our study demonstrates that at day 1, the inflammasome-related cytokines IL-1β and IL-18, the alarmin HMGB1 and circulating aggregates of ASC are elevated in the blood of septic patients of intra-abdominal origin, in agreement with previous publications that found elevated inflammasome gene expression in monocytes and circulating IL-18 cytokine during sepsis **(Eidt et al. 2016)**. However, while IL-18 concentration has been positively associated to mortality in septic patients **(Eidt et al.**

2016), the circulating IL-18 measured from our cohort of septic patients do not correlate with mortality. This difference could be due to the inclusion of different ranges of etiologies on the septic patients enrolled in other studies **(Eidt et al. 2016)**, compared to our study that focus in a well-defined population of intra-abdominal septic patients. Despite all current efforts to identify novel biomarkers for sepsis, so far, any cytokine has replaced the use of acute phase proteins, like PCT, as the most used clinical markers for septic patients **(Balci et al. 2003; Harbarth et al. 2001)**. The development of standardized protocols for critical care and the support of vital organs have reduced early deaths in sepsis; however sepsis remains the leading cause of mortality in the critical ill patient **(Herrán-Monge et al. 2017)**. The elevated risk of secondary infections in sepsis correlates with late-deaths and is thought to be related with the immunosuppressive state of blood leukocytes in these patients (Boomer et al. 2012; Fattahi and Ward 2017; Richard S Hotchkiss, Monneret, and Payen 2013). This immunosuppression is hardly mimicked in animal models of sepsis were animals usually die at early times as a consequence of the initial exacerbated inflammatory response **(Rittirsch, Hoesel, and Ward 2006; Ward 2012)**. Immunosuppression during sepsis affects the innate and adaptive immune system, with impairment of monocyte function, increase lymphocyte apoptosis and decrease lymphocyte proliferation (Boomer et al. 2012; Richard S Hotchkiss, Monneret, and Payen 2013). Different studies have demonstrated that human blood monocytes from septic patients displayed an altered response to bacterial endotoxin challenge *ex vivo* **(Boomer et al. 2012; Cheng et al. 2016; van Deuren et al. 1998; Döcke et al. 1997; Ertel et al. 1995; Munoz et al. 1991; Shalova et al. 2015)**, and only one study addressed the activation of the inflammasome in septic patients **(Giamarellos-Bourboulis et al. 2011)**. Here we confirm that activation of the NLRP3 inflammasome is compromised in septic patients, but importantly, individual and not averaged data analysis allowed us to discriminate a group of septic patients with a significant impairment of the NLRP3 inflammasome activation at day 1. This group of patients accumulated the majority of late-deaths. Calculated cut-off values for different cytokines and NLRP3 inflammasome activation markers in monocytes allowed us to precisely define this group of immunocompromised septic patients with related high late-mortality. The different works studying monocyte deactivation in sepsis present their results as an average value compared to the average value of a control group and the majority analyse functional assays for cytokine release in a smaller number of patients (form n= 4 to 15) than our study (n= 23) **(Cheng et al. 2016; van Deuren et al. 1998; Döcke et al. 1997; Ertel et al. 1995; Shalova et al. 2015)**. Our averaged data for IL-1β and IL-6 release is also reduced compared to

control groups; however this was not the case for the release of HMBG1, TNF-α, or IL-8 that had not significant differences in septic patients when compared to control groups. Analysing the individual variability of cytokine release from septic patients we were able to early stratify the patients into a group of septic patients that present normal non-significant differences from healthy controls and another that was unable to activate the NLRP3 inflammasome. This distinction among immunocompromised septic patients has been also detected by the reduced expression of HLA-DR in monocytes (Döcke et al. 1997). We found an increase of caspase-1 activation in monocytes during the first 24h after patient enrolment in our study, which impaired with the low caspase-1 activation in autoinflammatory diseases. Nevertheless, LPS-primed monocytes from CAPS, APLAID, FMF and PAAND patients showed an exacerbated IL-1β production. Our result confirms previous evidences indicating the inflammasome activation dysregulation that lifts the pro-inflammatory cytokines secretion (Brydges et al. 2013). In addition, impairment of NLRP3 inflammasome activation was found as early as within the first 24h, when inflammatory markers were elevated in the blood, including IL-1β and IL-18, suggesting that inflammasome activation is compromised at very early times after infection, supporting previous data demonstrating that release of IL-1β is compromised on *ex vivo* LPS stimulated PBMCs collected after 2h of intravenous LPS infusion in healthy volunteers (Giamarellos-Bourboulis et al. 2011) and that within 24h of onset of human sepsis there is a decrease of inflammasome gene expression in monocytes (Fahy et al. 2008). We also found suppression of NLRP3 inflammasome lasted at least during the first five days of sepsis onset, but this suppression was transient and reversed upon patient recovery. Endotoxin tolerance of monocytes has been suggested as predictive tool of nosocomial infection and death in sepsis, but not as an early marker to guide therapy of the patients (Galbraith et al. 2016), and this study support that NLRP3 inflammasome activation could be an early marker (within the first 24 h) to identify patients at high risk of late (10–30 days) mortality. This work advances towards a personalized biomarker-guided therapy that could be early started in septic patients with immunocompromised NLRP3 inflammasome, being an important advance for the diagnostic and treatment of sepsis (Richard S Hotchkiss, Monneret, and Payen 2013), supporting early studies that found impaired production of IL-6 and TNF-α was persistent on time in patients that die (Munoz et al. 1991). Importantly, our study represents an unbiased finding, that after identifying septic patients with a significant NLRP3 inflammasome-suppression, they accumulated the majority of deaths, and

differentiate from previous work that have failed to identify a predictive gene signature, blood-cytokine/s or clinical marker/s associated to the deaths in sepsis by segregating patients into survival vs non-survival groups and analysing averaged data (Fahy et al. 2008; Munoz et al. 1991). Blood monocytes are important cells in the development of an immunocompromised long-term state in sepsis, since they do not only acquire a deactivation phenotype but are also involved in suppressing lymphocyte function (Albertsmeier et al. 2017; Biswas and Lopez-collazo 2009). Defects on the metabolism of septic monocytes have been found responsible for the immunocompromised state of these cells, including important paralysis of mitochondria with a parallel reduction of oxygen consumption (Arts et al. 2016; Cheng et al. 2016; Japiassú et al. 2011). Furthermore, mitochondria of myeloid cells suffer adaptations in the respiratory chain upon sensing bacteria (Garaude et al. 2016) and HIF-1α appears as a key factor for the metabolic reprogramming of myeloid cells during inflammation and sepsis (Corcoran et al. 2016; Mills et al. 2016; Shalova et al. 2015). Here we confirm that monocytes from septic patients present dysfunctional mitochondria evidenced by a depolarization of the mitochondrial plasma membrane, but this defect on mitochondria was not correlated with an increase of mortality of septic patients. Although previous studies have found an association between mitochondrial failure in non-myeloid cells with organ failure and poor outcome of septic patients (Brealey et al. 2002; Singer 2014). It is known that the purinergic P2X7R is a potent stimulus for the activation of Akt and HIF-1α in different non-myeloid cells (Amoroso et al. 2012; Amoroso et al. 2015; Hirayama et al. 2015; Di Virgilio et al. 2017). P2X7R also promotes aerobic glycolysis via the induction of protein kinase M2 (Amoroso et al. 2012). We found elevated P2X7R in the surface of monocytes from septic patients when compared to monocytes from control groups, but P2X7R expression failed to associate with mortality or immunosuppression. P2X7R is a well-known activator of the NLRP3 inflammasome (Di Virgilio et al. 2017), and accordingly we found a positive correlation with the expression of surface P2X7R in monocytes and the concentration of IL-1β release after LPS and ATP treatment in the control group and also in non-immunocompromised septic patients. However, in immunocompromised septic patients, P2X7R expression does not correlate with the release of IL-1β, but it correlates with an increase of mitochondria dysfunction in the monocytes. In recombinant HEK293 cells, activation of the P2X7R induces a fast mitochondrial depolarization (Mackenzie et al. 2005) and here we found that P2X7R activation in myeloid cells similarly induces mitochondrial dysfunction. This effect was

independent on LPS-priming and the NLRP3 inflammasome, ruling out possible mitochondrial damage as a consequence of caspase-1 activation (Yu et al. 2014). When P2X7R was activated in resting monocytes and macrophages, it decreases the response of these cells to further stimulation with NLRP3 activation, inducing a deactivation state similar to the one found in immunocompromised septic patients. In this situation, P2X7R activation prevents, and not induces (Di Virgilio et al. 2017) NLRP3 inflammasome activation. Similarly, it is known that in M2 macrophages ATP is able to prevent NLRP3 inflammasome activation, but this effect was independent on P2X7R (Pelegrin and Surprenant 2009). Cecal-ligation and puncture septic model in P2X7R deficient mice has result in contradictory results, describing an increase of survival in the knock-out mice (Santana et al. 2015) or increase of mortality in the knock-out (Csoka et al. 2015). Recently, another work has also shown higher mortality of P2X7R deficient mice in a model of sepsis induced by α-hemolysin (Greve et al. 2017). When P2X7R deficiency leads to a higher mortality, the production of pro-inflammatory cytokines measured in the blood were exacerbated (Csoka et al. 2015; Greve et al. 2017), supporting the results of our work where P2X7R activation, under certain situations of severe infection, could inhibit cytokine production. Our data demonstrate that P2X7R activation could be either linked to an inhibition (activated before LPS-priming) or potentiation (activated after LPS-priming) of cytokine production in myeloid cells. This result together with the fact that P2X7R modulates cell metabolism (Amoroso et al. 2012; Amoroso et al. 2015; Hirayama et al. 2015; Di Virgilio et al. 2017), could explain the contradictory results in the different models of sepsis, as different metabolic state of the animals used could lead to differential modulation of the immune response by P2X7R. Therefore, an increase of extracellular ATP concentration due to complications in the surgery or as consequence of different treatments (Martins et al. 2009) that could activate P2X7R before or during the early phase of an infection, could contribute to the development of immunosuppression by decreasing NLRP3 inflammasome priming and activation. In our study, not only monocytes from septic patients experimented changes in P2X7R expression. We also found an elevated number of lymphocytes that expressed P2X7R on cell surface. However, P2X7 percentage in T cells, B cells and NK cells was maintained during the first 5 days of patient enrolment in our study. Increase of P2X7R was overwhelming even in T cells populations with very small number of cells, such as Treg, supporting a previous report that has been demonstrated the high sensitivity of Treg cells to extracellular ATP mediated by P2X7R signalling (Aswad, Kawamura, and Dennert 2005). In addition, it is well known that Treg cells increase and play

an important role inhibiting the inflammatory response during the immunosuppressive phase of sepsis **(Wisnoski et al. 2007)**. In contraposition, we found a lower percentage of Treg cells in the studied cohort of septic patients. However, these changes could be explained by the total number of Treg cells in each sample. Wisnoski explained on his work that the increase of Treg cells could be a relative result of the number of total Th cells, due to differentially to our study, Wisnoski and collaborators showed a decrease of Th cells in sepsis. Furthermore, we observed changes in the different PBMCs populations in septic patients that maintained during the first 5 days. The percentage of Th cells increased, B cells did not experimented changes, meanwhile the percentage of Tc cells and NK cells decreased. Other studies have shown different changes in the different PBMCs populations. Some works revealed that B cells and Th cells suffer apoptosis during sepsis, giving place to an increase of cytotoxic cells such as NK cells and Tc cells **(R S Hotchkiss et al. 2001)**. In contraposition, our study used a cohort of abdominal origin septic patients, meanwhile other studies did not segregate sepsis origin, and in other types of sepsis it is difficult to asses the initial day of the sepsis **(R S Hotchkiss et al. 2001)**. Other condition is the moment of PBMCs extraction within the disease development. Hotchkiss and colleagues studied septic samples in a late state of the disease, taking the samples rapidly post-mortem **(R S Hotchkiss et al. 2001)**, meanwhile our study is mainly focussed on the early times, the first 24h or 5 days since the patient is enrolled in our study. Other works has shown that Th cells starts to decrease only in survivors septic patients after 6 days of sepsis development respect control groups **(Wu et al. 2013)**. This finding is supported with other studies that indicate high levels of apoptosis in the late state of sepsis that decrease the number of lymphocyties **(Crowell, Soybel, and Lang 2017)**. We then found an increase of P2X7R surface expression in the different populations of PBMCs, but this increase was not found in healthy controls. We also found that the amount of IL-6 in the plasma of all septic patients increased in comparison with controls where the concentration of IL-6 is low. This gives us the clue that our cohort of septic patients present an hyperinflammatory response **(Richard S Hotchkiss and Karl 2003)**, since IL-6 is a main driver of acute phase protein production by the liver **(Pepys and Hirschfield 2003)**. However, IL-6 also appear to influence in the immune response by recruiting and activating individual leukocyte subpopulations **(Fischer et al. 1997)**. According with this, the high amounts of IL-6 in plasma could induce an increase of the surface expression of P2X7R on the leukocytes. However, treatment of PBMCs with recombinant IL-6 *in vitro* did not increased P2X7R surface expression in monocytes, but LPS was the only treatment able to increase P2X7R in monocytes. Suggesting that bacterial

products as PAMPs, and not cytokines, are able to increase the expression of P2X7R in human PBMCs. This effect could be explained by the ability of cells to respond at ATP after LPS priming **(Di Virgilio et al. 2017)**. In definitive, the axis P2X7R/NLRP3 inflammasome in human sepsis is immunocompromised in the monocytes of some patients that present high mortality; this could help to stratify septic patients at very early times.

Macrophages adhesion is regulated by the activation of P2X7R.

P2X7R is enrolled in different functions on monocytes and macrophages, as is cell adhesion. Studies in rats have found that P2X7R interacts with a wide variety of proteins, among them we find the laminin recognition integrin α3, the membrane spanning proteins integrin β2 and some cytoskeleton proteins such as non-muscle myosin, α-actinin and β-actin **(Kim et al. 2001)(Wiley)**. In our study, we found that P2X7R-activation was associated to a lack of cell adhesion. Cell adhesion was lost when the concentration of ATP was increased, with concentrations similar to the EC_{50} for P2X7R **(Chessell et al. 1998).** Surprisingly, P2X7R-dependent cell detachment was observed at 2 minutes after ATP incubation, suggesting that this is a very fast mechanism. Previous reports have revealed that extracellular ATP transient application activates P2X7R between 1 to 4 min that induces non-apoptotic changes independent on LPS priming such as microblebbing **(Hanley et al. 2012).** This microblebbing could be related with loss of cell adhesion that we present in our study due to the changes in membrane permeability during blebbing process. Detection of extracellular IL-1β released by LPS-primed macrophages was found after 15 to 30 min of ATP incubation **(Grahames et al. 1999).** Caspase-1- dependent cell death can be observed starting 30 min of extracellular ATP presence **(Le Feuvre et al. 2002)**. Our study reveals that the morphologic changes necessary for cell detachment induced by P2X7R activation on macrophages occurs earlier than pro-inflammatory cytokine cleavage induced by purinergic signalling. This early effect on cell detachment was not dependent on ions movement across P2X7R ion channel (K^+ efflux or increase of intracellular Ca^{2+} or Na^+). Therefore, the main signalling pathways associated to the ionic flux across P2X7R **(Pelegrin and Surprenant 2006; Gudipaty et al. 2003)** are not involved in cell detachment. On the other hand, several studies have revealed that P2X7R induces a fast shedding of integrins and other adhesion molecules via Ca^{2+} dependent, or independent pathways **(Sengstake, Boneberg, and Illges**

2006; Sánchez-Nogueiro, Marín-García, and Miras-Portugal 2005; B. Gu, Bendall, and Wiley 1998; Wiley et al. 2011). P2X7R is able to decrease the interaction between lymphocytes by CD21 and CD62L shedding (Sengstake, Boneberg, and Illges 2006), the adhesion molecule CD44 in macrophages (Lin et al. 2012), or shedding of vascular cells adhesion molecules (VCAMs) in neutrophils (Mishra et al. 2016). This shedding of adhesion molecules induced by P2X7R is mediated by MMPs activation (B. Gu, Bendall, and Wiley 1998; Hinkle et al. 2006; Brennaman, Moss, and Maness 2014). Moreover, other studies have shown that after P2X7R activation there is a release of MMP-9 (B. J. Gu and Wiley 2006). Our work revealed the presence of extracellular β1 integrin after ATP activation of P2X7R in murine BMDMs, suggesting that P2X7R controls the lack of adhesion molecules and therefore decreases cell adhesion. This finding is similar to previous studies that demonstrated the shedding of β1 integrin mediated by P2X7R activation in BMDM cells (Furlan-freguia et al. 2011). Non-primed macrophages adhesion was significantly decreased upon P2X7R activation when cells were culture in a type I collagen matrix, but not to laminin or fibronectin. Type I collagen binds to α1β1 and α2β1 integrins present in macrophages (Jokinen et al. 2004; Vandenberg et al. 1991). Macrophages can bind to laminin through α3β1 and α6β1 receptors (Fujiwara et al. 2001) and fibronectin through several β1 integrins between the most important are α4β1 and α5β1 (Guan and Hynes 1990; Clements et al. 1994; Pierschbacher and Ruoslahti 1984). Nevertheless, macrophages can bind to laminin through α6β4 integrin (Lee et al. 1992) and fibronectin through αv class receptors with higher affinity than α5β1, which also are receptors for vitronectin, such as αvβ3 (Charo et al. 1990; Blystone et al. 1994; Bharadwaj et al. 2017) and αvβ5 (Cheresh et al. 1989). Our work also evidenced β1 shedding. These findings support a model where in non-primed macrophages treated with ATP, β1 shedding will induce lack of adhesion to collagen but not to laminin or fibronectin.

It is well known that LPS-activation augments cell adhesion of monocytes and macrophages (Hmama et al. 1999; Kounalakis and Corbett 2006). By monitoring cell adhesion by conductance on BMDMs, we could observe an earlier increase of macrophages cell adhesion induced by LPS, that could be due to increase of macrophage spreading (Williams and Ridley 2000). In contraposition, we found similar macrophage adhesion to different ECMs after LPS-activation. However, we found a specific increase of macrophage adhesion to fibronectin upon LPS-activation. We found that ATP treatment of LPS-primed

BMDMs induced a significant decrease of cell adhesion when compared to non-primed macrophages. This effect could be explained because upon LPS-priming, P2X7R will induce pyroptosis **(Fink and Cookson 2006)**, and pyroptosis will result in a lack of cellular adhesion **(de Vasconcelos et al. 2018)**. In fact, the timing of cell adhesion upon ATP addition in those experiments is compatible with pyroptosis as high levels of IL-1β and LDH were found in the cellular supernatants as markers of pyroptosis **(Pelegrin, Barroso-gutierrez, and Surprenant 2008; Wewers and Sarkar 2009; Baroja-Mazo et al. 2014; C de Torre-Minguela et al. 2016)**. Our results also indicate that BMDMs presented a decrease cellular adhesion to collagen, laminin and fibronectin when they were primed with LPS and then treated with ATP. Therefore, pyroptosis execution could interspecifically induce cell detachment to different substrates, revealing cell death and membrane permeabilization-dependent cell detachment as has recently suggested **(de Vasconcelos et al. 2018)**.

In summary, our work identified that P2X7R activation specifically induce a rapid macrophage detachment to collagen by β1 integrin shedding, and this detachment was found to different cellular substrates upon bacterial priming via pyroptotic cell death. Our study opens new approaches to understand the participation of P2X7R in the regulation of cell adhesion and migration of immune cells to the infection site. In fact, we also found an upregulation of P2X7R expression in monocytes and other leukocytes from septic patients, being this increase correlating to the immunocompromised state of the patient. In non-immunocompromised patients it positively correlate with IL-1b release and in immunocompromised patients with mitochondrial dysfunction. Restoration of P2X7R expression levels and NLRP3 inflammasome activation on monocytes could be a good indicator to discharge patients from intensive care units. Therapies aiming to decrease extracellular ATP or blocking the P2X7R at early time points (i.e. during abdominal surgery) or when immunoparalysis of the NRLP3 inflammasome is detected, could be beneficial to individualize therapies for septic patients aimed to decrease immunosuppression and improve the outcomes of the syndrome. Finally, the NLRP3 inflammasome could be a good target to treat the inflammation related to venoms, as the AMP melittin induces the canonical NLRP3 inflammasome activation, but overpassing the execution of pyroptosis, expanding the repertoire of NLRP3 associated cell death.

Conclusions

1- The antimicrobial peptide melittin induces the activation of NLRP3 inflammasome and caspase-1 through a decrease of intracellular K^+ concentration.

2- Melittin induces the release of IL1β, but not IL-18 in LPS-primed macrophages.

3- Cell death induced by melittin is independent on NLRP3 inflammasome and caspase-1 activation.

4- Extracellular IL-1β, IL-18 and HMBG1 are elevated during the first 24 hours of sepsis development.

5- Mortality of septic patients is associated with an impairment of NLRP3 inflammasome activation in blood monocytes upon LPS+ATP stimulation.

6- NLRP3 inflammasome immunoparalysis during sepsis is transitory.

7- P2X7 receptor surface expression is upregulated in monocytes from septic patients.

8- The percentage of P2X7 receptor positive lymphoid-origin peripheral blood mononuclear cells from septic patients increase when compared to healthy subjects.

9- P2X7 receptor activation depolarizes mitochondrial membrane potential in myeloid cells.

10- P2X7 receptor activation decrease cell adhesion to different substrates, independently of cationic flux.

11- P2X7 receptor in macrophages induces the release of β1 integrin independently of LPS-priming.

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Publications from this Thesis

1. Published articles:

Martín-Sánchez, Fátima, Juan José Martínez-García, María Muñoz-García, Miriam Martínez-Villanueva, José A Noguera-Velasco, David Andreu, Luís Rivas, and Pablo Pelegrín. 2017. "Lytic Cell Death Induced by Melittin Bypasses Pyroptosis but Induces NLRP3 Inflammasome Activation and IL-1β Release." *Cell Death and Disease* 8 (8)

Mensa-Vilaro, Anna, María Teresa Bosque, Giuliana Magri, Yoshitaka Honda, Helios Martínez-Banaclocha, Marta Casorran-Berges, Jordi Sintes, et al. 2016. "Brief Report: Late-Onset Cryopyrin-Associated Periodic Syndrome Due to Myeloid-Restricted Somatic NLRP3 Mosaicism." *Arthritis and Rheumatology* 68 (12)

Moghaddas, Fiona, Rafael Llamas, Dominic De Nardo, Helios Martinez-Banaclocha, Juan J. Martinez-Garcia, Pablo Mesa-Del-Castillo, Paul J. Baker, et al. 2017. "A Novel Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis Mutation Further Defines 14-3-3 Binding of Pyrin and Distinction to Familial Mediterranean Fever." *Annals of the Rheumatic Diseases* 76 (12): 2085–94. doi:10.1136/annrheumdis-2017-211473.

2. Ahead of publish:

Martínez-garcía, Juan José, Helios Martínez-banaclocha, Diego Angosto-bazarra, Carlos De Torre-minguela, Alberto Baroja-mazo, Laura Martínez-alarcón, Fátima Martín-sánchez, et al. *ND*. P2X7 receptor induces mitochondrial failure in monocytes and compromises NLRP3inflammasome activation during human sepsis

Spanish summary

1. Introducción

El inflamasoma NLRP3 es un complejo multiproteico que se activa en respuesta a patógenos o a estímulos no causan, como las toxinas capaces de permeabilizar la membrana mediante un poro. La ruta no-canónica del inflamasoma NLRP3 está formada por el receptor NLRP3, la proteína ASC, que es la encargada de formar specks de ASC y reclutar caspasa-1. La activación del inflamasoma NLRP3 puede darse lugar por una disminución del potasio intracelular. En esas condiciones, NLRP3 es capaz de oligomerizar a ASC; formando grandes agregados que amplifican la activación de caspasa-1, para que se libere tanto IL-1β como IL-18. Recientemente, se describió que estas citoquinas proinflamatorias, tras ser procesadas por caspasa-1 eran liberadas por gasdermina D, que es capaz de formar un poro en la membrana e inducir piroptosis.

Los péptidos antimicrobianos tienen como función general proteger al organismo contra patógenos, invadiendo su pared celular. Para ello tienen la capacidad de insertarse en la membrana debido a su origen anfipático, y formar un poro que induce una permeabilización celular que podría estar involucrada en la activación del inflamasoma NLRP3.

Por otro lado, el inflamasoma NLRP3 está involucrado en la respuesta inflamatoria inducida por infecciones. Esta respuesta del inflamasoma está estrechamente relacionada con la activación del receptor P2X7 en macrófagos después del reconocimiento de un componente patógeno. Algunos trabajos previos han demostrado que la alteración en del metabolismo, conduce a una inmunosupresión en sepsis.

En ésta Tesis hemos estudiado el efecto del receptor purinérgico P2X7 y el inflamasoma NLRP3 en la respuesta inflamatoria que se desencadena ante péptidos antimicrobianos (específicamente empleando melitina, que es el componente mayoritario del veneno de abeja), y ante infecciones, que pueden dar lugar a una respuesta inflamatoria sistémica o sepsis en el hospedador. En este trabajo estudiamos cómo afectan los péptidos antimicrobianos en la liberación de citoquinas proinflamatorias dependientes de inflamasoma NLRP3 en macrófagos derivados de médula ósea de ratón. Para ello, tratamos

los macrófagos con diferentes péptidos antimicrobianos (melitina, LL-37, temporina A e indolicidina) y comparamos su acción con el efecto de la nigericina como estímulo canónico del inflamasoma NLRP3. Además, se ha caracterizado la función del receptor P2X7 y el inflamasoma NLRP3 en células mononucleares de sangre periférica, así como en el plasma, de pacientes con sepsis abdominal, pacientes sometidos a una cirugía abdominal y donantes sanos. Para ello realizamos estudios *ex vivo* de distintos marcadores celulares, del receptor P2X7 y de liberación de citoquinas proinflamatorias. También se realizaron estudios *in vitro* en macrófagos de médula ósea de ratón para caracterizar como la activación del receptor P2X7 puede modular la respuesta antes o después de estimular las células con LPS, así como su papel en la inmunosupresión transitoria y la disfunción mitocondrial. Por último, pero no menos importante, revelamos como el receptor P2X7 afecta negativamente a la adhesión celular de macrófagos.

2. Objetivos

1- Caracterizar la activación del inflamasoma NLRP3 inducida por péptidos antimicrobianos.

2- Elucidar la respuesta inflamatoria y la función del inflamasoma NLRP3 en monocitos humanos procedentes con sepsis.

3- Caracterizar la expresión y función del receptor P2X7 en células mononucleares de sangre periférica de pacientes con sepsis.

4- Determinar la función del receptor P2X7 en la adhesión celular de macrófagos.

3. Material y métodos

Para realizar los experimentos se utilizaron muestras de sangre humana de donantes sanos, pacientes con sepsis de origen abdominal recogidas durante los primeros 5 días de su inclusión al estudio, pacientes que sufrieron una cirugía abdominal sin desarrollo de sepsis, y pacientes que sufrieron enfermedades autoinflamatorias. De la sangre se obtuvieron las células mononucleares de sangre periférica (PBMCs) que se

cultivaron y se realizaron ensayos *in vitro*. También se utilizaron para realizar los estudios *in vitro* muestras de médula ósea de ratón, de donde se diferenciaron macrófagos derivados de médula ósea (BMDMs). A parte de BMDMs, también se realizaron estudios *in vitro* en la línea celular de monocitos-macrófagos humanos THP-1, y la línea celular embrionaria de riñón humano HEK293 y su variante que expresa de forma estable el receptor P2X7 de rata.

Los estudios *in vitro* consistieron en tratamientos farmacológicos adaptados a cada protocolo para cada estudio: Para estudiar la respuesta del inflamasoma NLRP3 frente a péptidos antimicrobianos, las células fueron incubadas con LPS a una concentración de 1μg/ml durante 4h a 37ºC con un 5% de CO_2, y posteriormente se trataron en las mismas condiciones ambientales con nigericina a 25μM y diferentes concentraciones de melitina como segundos estímulos durante 30 min. Para estudiar la adhesión celular, las células fueron tratadas con LPS como se ha descrito anteriormente sobre células adheridas a la placa de cultivo o adheridas sobre matrices extracelulares (colágeno de tipo I, laminina o fibronectina) y posteriormente se estimularon con ATP (generalmente a 5mM) durante 30 min. Las PBMCs de donantes sanos fueron tratadas con distintas concentraciones de LPS o TNF-α, IL-6, o IFNγ (cada uno a 20ng/ml), 0 10ng/ml de PMA solo o con 500ng/ml de ionomycin, 20μl/ml de anti-CD3/CD28, o PWM a 1μg/ml durante 4, 24 y 48h, para estudiar la expresión del receptor P2X7 una vez las PBMCs están activadas. Las PBMCs de pacientes de enfermedades auto inflamatorias fueron pre-tratadas con 2h de LPS a 1μg/ml y posteriormente tratadas con ATP a 3mM durante 30 min o la toxina b de *Clostridium difficile* (Cdtb) a una concentración de 1μg/ml durante 1h. Para estudiar la disfunción mitocondrial *in vitro*, las BMDMs se trataron con ATP a 3mM o el inhibidor de la actividad mitocondrial antimicina A a 5μM. En estos ensayos se añadieron alternativamente y previo al tratamiento con ATP, antagonistas específicos de P2X7 (A438079) a 100μM, el agente protector del potencial de membrana mitocondrial pirrolidinditiocarbamato (PDTC) a 40μM, y los nano-cuerpos dimerizados con albúmina específicos para P2X7, que potencian (14D5), o inhiben (13A7) la actividad del receptor **(Danquah et al. 2016)**.

Para obtener los resultados de cada uno de los estudios *in vitro* se emplearon diferentes técnicas: La cantidad de IL-6, IL-1β, e IL-18 liberadas por las células se determinó mediante ELISA; para determinar la muerte celular en los ensayos *in vitro*, se cuantificó la presencia de LDH en los sobrenadantes celulares y se relativizó comparándolo con la

cantidad total de LDH intracelular. La adhesión celular de macrófagos al sustrato fue determinada la cantidad de células que quedaban adheridas tras agitar vigorosamente la placa durante XX minutos a XX rpm mediante las técnicas de MTT y Yo-Pro-1, y la detección se realizó en un lector de placas. Los ensayos para determinar el potencial de membrana de las mitocondrias de BMDMs y de PBMCs fueron realizados utilizando la molécula fluorescente JC-10 y detectados en un lector de placas o alternativamente por citometría de flujo.

La cantidad de receptor P2X7 soluble, IL-18, HMGB1 en el plasma de pacientes con sepsis se determinaron mediante ELISA. La la expresión del receptor P2X7, el potencial de membrana mitocondrial, y la actividad de caspasa-1 en PBMCs, así como la determinación de las citoquinas IL-1β, IL-6, IL-8, y agregados de ASC extracelulares en plasmas de pacientes con sepsis y controles fueron medidos por citometría de flujo. Para la determinación de las distintas poblaciones de PBMCs, se realizó una tinción con anticuerpos contra receptores de membrana, marcados con distintos fluorocromos. Se utilizaron anticuerpos contra CD3, CD4 y CD8 para determinar las poblaciones principales de linfocitos T, CD3 y CD19 para determinar los linfocitos B, CD3 y CD16 para determinar las células NK, CD3, CD14 y CD16 para determinar las distintas poblaciones de monocitos CD14-CD16. Alternativamente, se realizaron tinciones para CD4, CD25, y CD127 para determinar la población de células T reguladoras.

4. Resultados y discusión

4.1. El efecto lítico inducido por melitina activa al inflamasoma NLRP3, pero impide la ejecución de la piroptósis.

Los péptidos antimicrobianos se producen por diferentes células de diferentes organismos para como mecanismos de defensa frente a infecciones **(Zasloff 2002)**. En este estudio, examinamos la función de cuatro péptidos antimicrobianos catiónicos de naturaleza anfipática en la activación del inflamasoma NLRP3: a) LL-37, la única cathelicidina humana producida por distintos tipos celulares, incluyendo los monocitos; b) temporina a, producida en la piel de la rana europea *Rana temporaria*; c) indolicidina, un

péptido presente en los gránulos citoplásmicos de los neutrófilos bovinos, y d) melitina, el componente mayoritario del veneno de abeja que presenta una alta capacidad microbicida (Zasloff 2002; Rinaldi et al. 2002; Raghuraman and Chattopadhyay 2007). El efecto lítico de estos péptidos viene dado por su pequeña estructura anfipática y catiónica, que permite desestabilizar distintas membranas lipídicas por diferentes mecanismos (Zasloff 2002). En este trabajo, se identificó que la melitina es el péptido antimicrobiano más activo induciendo la liberación de IL-1β en BMDMs activados con LPS, confirmando un trabajo previo (Palm and Medzhitov 2013). Aunque otros trabajos han revelado que LL-37 es capaz de liberar IL-1β en monocitos humanos (Elssner et al. 2004), nuestros resultados indicaron que LL-37, no era capaz de liberar IL-1β en macrófagos de ratón. Esto puede deberse a las diferencias de las rutas de activación entre ambas especies, ya que existe una gran diferencia en la activación del inflamasoma NLRP3 entre la línea celular humana THP-1 y BMDMs, como por ejemplo tras someterse a soluciones hipotónicas (Compan et al. 2012). Además se sabe que los monocitos y los macrófagos regulan la activación del inflamasoma NLRP3 de una manera diferente (Netea et al. 2009). Mientras que los macrófagos requieren dos estímulos consecutivos para ser activados, los monocitos son capaces de activar NLRP3 con un solo estímulo a través de una ruta alternativa TRIF–RIPK1–FADD–CASP8, tras la señalización por TLR4 (Gaidt et al. 2016). Estas diferencias, pueden explicar porque en trabajos previos, LL-37 activa NLRP3 en monocitos humanos (Elssner et al. 2004), pero no sobre macrófagos de ratón (nuestro trabajo). La melitina es el principal componente del veneno de abeja, y es el componente responsable del veneno de abeja de inducir una reacción alérgica junto con una inflamación local (Müller 2010). Se sabe que el inflamasoma NLRP3 juega un papel esencial en esta respuesta *in vivo* al veneno de abeja (Palm and Medzhitov 2013). En ésta Tesis, se encontró que la melitina activa el inflamasoma NLRP3 a través de un descenso de la concentración de K^+ intracelular, que a su vez es un importante paso para activar el inflamasoma NLRP3 (Muñoz-Planillo et al. 2013; Pétrilli et al. 2007). Ya que NLRP3 activa caspasa-1 a través de la proteína adaptadora ASC (Próchnicki, Mangan, and Latz 2016; de Torre-Minguela, del Castillo, and Pelegrín 2017), en este trabajo, demostramos como melitina es capaz de activar caspasa-1 y liberar IL-1β empleando esta proteína ASC. Esto está además en sintonía con un trabajo previo dónde la activación de caspasa-1 y liberación IL-1β inducida por veneno de abeja requiere de la proteína ASC (Palm and Medzhitov 2013). No obstante, la melitina no produjo un proceso de muerte celular por

piroptosis dependiente de la activación de caspasa-1. La ausencia de muerte piroptótica tras la activación del inflamasoma NLRP3 a través de melitina puede estar asociada a la desestabilización de la membrana plasmática inducida directamente por este péptido antimicrobiano, que ocurre de forma rápida y en paralelo a la activación de NLRP3. La melitina es capaz de inducir muerte celular por la formación de poros en la membrana plasmática de células eucariotas, dando lugar a una lisis celular osmótica (**van den Bogaart et al. 2008; Subbalakshmi, Krishnakumari, and Nagaraj 1996; Raghuraman and Chattopadhyay 2007**). En éste trabajo revelamos que en macrófagos la melitina induce una desestabilización de la membrana plasmática de forma inmediata, que probablemente sea la responsable de disminuir el K^+ intracelular. Sin embargo, mientras que esta activación del inflamasoma NLRP3 mediada por melitina fue suficiente para activar caspasa-1 e inducir la secreción de IL-1β, no fue suficiente como para que caspasa-1 activara el procesamiento y liberación de IL-18. Por tanto, el efecto lítico producido por melitina es independiente del proceso piroptótico, ya que la membrana plasmática está ya permeabilizada cuando caspasa-1 procesara gasdermina D. En otros trabajos se ha observado que el péptido LL-37 es capaz de inhibir la piroptosis después de la activación de NLRP3 (**Hu et al. 2014**), indicando que la inhibición de la piroptosis podrá tratarse de un mecanismo característico de los péptidos antimicrobianos. Tras la activación de caspasa-1, junto con el procesamiento y la liberación de IL-1β, hay un procesamiento y liberación de IL-18 (**Schroder and Tschopp 2010; Strowig et al. 2012**). No obstante, en distintas condiciones patológicas, ambas citoquinas desarrollan distintas condiciones patológicas en diferentes enfermedades o en distintas etapas de una enfermedad (**Brydges et al. 2013**). Sin embargo, *in vitro*, ambas citoquinas son liberadas tras la activación del inflamasoma NLRP3, bien por ATP o por nigericina, siendo normalmente los niveles de IL-18 diez veces menores que los niveles de IL-1β en cultivos *in vitro* (**Compan et al. 2012; Doyle et al. 2014; Perregaux et al. 2000**). En éste estudió revelamos que la melitina es capaz de inducir liberación de IL-1β, pero no de IL-18. Este efecto se puede atribuir a una activación reducida de caspasa-1 inducida por el tratamiento de melitina y su efecto lítico. Ésta activación reducida de caspasa-1 puede deberse a una pérdida de la amplificación de la activación de caspasa-1 y reflejo de que tampoco se ejecuta la muerte celular por piroptosis (**Dick et al. 2016**). En resumen, la melitina ejerce un efecto diferencial sobre la liberación de IL-1β e IL-18, pudiendo ser importante para la respuesta contra péptidos antimicrobianos. De hecho,

existen evidencias que revelan que la respuesta inflamatoria inducida por melitina es dependiente de la señalización por el receptor de IL-1 **(Palm and Medzhitov 2013)**.

4.2. El receptor P2X7 induce una alteración de la función mitocondrial en monocitos, que compromete la activación del inflamasoma NLRP3 en sepsis.

La sepsis es la mayor causa de muerte en las unidades de cuidados intensivos **(Herrán-Monge et al. 2017)**. Nuestro trabajo sugiere que, durante la respuesta inflamatoria inducida por sepsis, los pacientes sépticos con una mortalidad tardía tienen una inmunoparálisis que afecta a la activación del inflamasoma NLRP3. Los pacientes sépticos inmunocomprometidos presentaron una alta mortalidad que fue evidenciada por una elevada gravedad continuada en el tiempo, sin embargo, aquellos pacientes que se recuperaron, su gravedad inicial disminuyó con el tiempo. Además, la inmunoparálisis del inflamasoma NLRP3 y la alta mortalidad en sepsis no se pudo identificar por otros parámetros bioquímicos e índices clínicos para evaluar la gravedad de la sepsis a día 1 tras el diagnóstico de sepsis, pero si se pudo identificar mediante la estimulación *ex vivo* de PBMCs con LPS + ATP cuantificando la liberación de IL-1β. La expresión en superficie del receptor P2X7 aumenta en los monocitos de pacientes con sepsis y en los pacientes no immunocomprometidos, a mayor cantidad de P2X7, más liberación de IL-1β tras estimular con LPS + ATP. No obstante, se observó que la expresión del receptor P2X7 en monocitos en los pacientes inmunocomprometidos correlaciona con la disfunción mitocondrial, pero no con la activación del inflamasoma NLRP3 y la liberación de IL-1β. La activación del receptor P2X7 en monocitos y macrófagos resultó en una rápida despolarización del potencial de membrana mitocondrial. Proceso que afectó la activación del inflamasoma NLRP3. Inicialmente, la cohorte de pacientes sépticos que estudiamos presentaban una respuesta inflamatoria sistémica aguda que detectamos como una "tormenta de citoquinas", presentándose de manera exacerbada y de forma sistémica distintas citoquinas proinflamatorias como la IL-6 e IL-18, así como distintas proteínas de la fase aguda, que han sido propuestos en distintos estudios como biomarcadores para la gravedad en sepsis **(Cinel and Opal 2009; Eidt et al. 2016; Gogos et al. 2000; Harbarth et al. 2001; Mera et al. 2010)**. Nuestro estudio demostró que durante las primeras 24h del desarrollo de la sepsis, las citoquinas dependientes de inflamasoma IL-1β e IL-18, la alarmina HMGB1, y los

agregados circulantes de ASC están elevados en la sangre de pacientes sépticos de origen abdominal. Sin embargo, mientras que la concentración de IL-18 ha sido positivamente correlacionada con la mortalidad en pacientes sépticos (Eidt et al. 2016), en nuestro estudio, la concentración de IL-18 en plasma de pacientes sépticos no correlacionó con la mortalidad. Ésta diferencia podría ser debida a que los pacientes de nuestro estudio fueron recopilados estrictamente con el criterio de sepsis de origen abdominal, mientras que los pacientes en otros estudios recopilaron sepsis de distintas etiologías (Eidt et al. 2016). Distintos estudios han demostrado que los monocitos humanos de pacientes sépticos presentan una respuesta alterada frente a endotoxina bacteriana (Boomer et al. 2012; Cheng et al. 2016; van Deuren et al. 1998; Döcke et al. 1997; Ertel et al. 1995; Munoz et al. 1991; Shalova et al. 2015), de estos estudios solo uno de ellos estudió la activación del inflamasoma NLRP3 en pacientes sépticos, encontrando una disminución generalizada en el inflamasoma (Giamarellos-Bourboulis et al. 2011). Nuestro estudio confirma en una cohorte independiente de pacientes sépticos que la media de la activación del inflamasoma NLRP3 está disminuida cuando se compara con grupos control. No obstante, pudimos segregar un grupo de pacientes con una inhibición de la actividad del inflamasoma NLRP3 muy acusada frente a otro grupo con una activación normal del inflamasoma. El grupo de pacientes con una actividad muy baja del inflamasoma presentó la mayoría de muertes a largo plazo (> 9 días). Por el contrario, el grupo de pacientes sépticos que no presentó diferencias significativas en la activación del inflamasoma NLRP3 con respecto a los grupos control, solo registró una muerte temprana a día 1, pudiéndose deber a la respuesta proinflamatoria.

En nuestro trabajo también identificamos un incremento de caspasa-1 activa en los monocitos durante las primeras 24h del desarrollo de la sepsis, en comparación con la ausencia de activación de caspasa-1 en los grupos control. De forma sorprendente, cuando se comparó con la activación de caspasa-1 en pacientes que sufrían distintos síndromes auto-inflamatorios, estos monocitos no presentaron activación de caspasa-1. Esto puede deberse a que la mayoría de pacientes examinados estaban con terapia anti-IL-1 y durante fases no inflamatorias de la enfermedad. No obstante, cuando los monocitos de pacientes con síndromes auto-inflamatorios se activaron con LPS, entonces sí que aumentaron la activación de caspasa-1 cuando se compararon con monocitos activados de donantes sanos. Nuestro resultado confirma las evidencias previas que indican que la desregulación de la activación del inflamasoma induce una elevación de la secreción de citoquinas pro-

inflamatorias **(Brydges et al. 2013)**. Además, se observó una alteración de la activación del inflamasoma NLRP3 en las primeras 24h, cuando los marcadores proinflamatorios estaban elevados en sangre, incluidas las citoquinas IL-1β e IL-18, sugiriendo que la activación del inflamasoma está comprometida en un estado muy temprano del proceso séptico. Nuestros datos apoyan a trabajos previos en los que la liberación de IL-1β por PBMCs está comprometida *ex vivo* tras la inyección intravenosa de LPS durante 2h en sangre de voluntarios sanos **(Giamarellos-Bourboulis et al. 2011)**. En nuestro trabajo encontramos una supresión del inflamasoma NLRP3 durante los 5 primeros días del desarrollo del proceso séptico. Sin embargo esta inmunosupresión fue transitoria y reversible, ya que tras 3 meses del episodio séptico la activación del inflamasoma se recupera hasta niveles normales. Nuestro trabajo avanza hacia una terapia personalizada, basada en la detección de la liberación de citoquinas por los PBMCs como biomarcadores tempranos que podría predecir aquellos pacientes sépticos inmunocomprometidos para el inflamasoma NLRP3 y con mayor probabilidad de muerte tardías en sepsis. Ésta evidencia apoya algunos trabajos anteriores en los que se detecta una alteración en la liberación de IL-6 y TNF-α, que fue persistente en el tiempo en aquellos pacientes que finalmente fallecieron **(Munoz et al. 1991)**.

Los monocitos de sangre periférica juegan un papel importante en el desarrollo de la fase de inmunosupresión en sepsis, ya que no sólo adquieren un fenotipo inactivo, sino que también tienen la capacidad de suprimir la actividad de los linfocitos **(Albertsmeier et al. 2017; Biswas and Lopez-collazo 2009)**. Estudios previos encontraron defectos en el metabolismo en los monocitos de los pacientes sépticos, dando lugar a un estado de inmunosupresión, incluyendo una parálisis mitocondrial, y una reducción del consumo de oxígeno **(Arts et al. 2016; Cheng et al. 2016; Japiassú et al. 2011)**. Además, se conoce que las mitocondrias de células mieloides sufren adaptaciones en la cadena respiratoria después de un estímulo bacteriano **(Garaude et al. 2016)**, y HIF-1α parece ser un factor clave para la reprogramación metabólica de las células mieloides durante la respuesta inflamatoria en sepsis **(Corcoran et al. 2016; Mills et al. 2016; Shalova et al. 2015)**. En ésta Tesis confirmamos que los monocitos de pacientes sépticos presentan disfunción mitocondrial, identificada por una despolarización de la membrana mitocondrial. No obstante, la disfunción mitocondrial en monocitos no correlacionó con la mortalidad en sepsis, como sí apuntan

otros trabajos **(Brealey et al. 2002; Singer 2014)**. El receptor P2X7 es un potente estímulo de la activación de HIF-1α en diferentes células no-mieloides **(Amoroso et al. 2012; Amoroso et al. 2015; Hirayama et al. 2015; Di Virgilio et al. 2017)**. Además, el receptor P2X7 también promueve la glicolisis aeróbica mediante la expresión de la protein kinasa M2 **(Amoroso et al. 2012)**. En ésta Tesis encontramos un aumento de la expresión del receptor P2X7 en la superficie de la membrana de monocitos de pacientes con sepsis. Sin embargo, éste aumento de la expresión del receptor P2X7 no correlacionó con la mortalidad de la inmunosupresión. Como el receptor P2X7 es un potente activador del inflamasoma NLRP3 **(Di Virgilio et al. 2017)**, encontramos una correlación positiva entre la expresión en la superficie de monocitos de P2X7 con la liberación de IL-1β inducida por LPS + ATP, en los PBMCs de los grupos control, y de pacientes sépticos no inmunocomprometidos. Sin embargo, esa correlación no se encontró en pacientes inmunocomprometidos, aunque en este grupo de pacientes sí correlacionó la expresión de P2X7 con el aumento de la disfunción mitocondrial, de forma independiente a la activación previa por LPS y la activación del inflamasoma NLRP3. Además, encontramos que la estimulación del receptor P2X7 en monocitos y macrófagos previo a la activación por LPS, disminuye la capacidad de estas células ante el LPS, dando lugar a un estado de inmunosupresión similar al de un paciente séptico inmunocomprometido. En esta situación, el receptor P2X7 no induce la activación del inflamasoma NLRP3. De forma similar, se ha descrito previamente que los macrófagos tipo M2 reconocen ATP, pero en esta situación el ATP previene la activación del inflamasoma NLRP3, siendo este efecto independiente del receptor P2X7 **(Pelegrin and Surprenant 2009)**. En esta Tesis se demuestra que la activación del receptor P2X7 en células mieloides puede estar diferencialmente modulando la producción de citoquinas proinflamatorias a una inhibición (células tratadas con ATP antes de activarlas con LPS) o una potenciación (células tratadas con ATP después de activarlas con LPS). De hecho, otros trabajos han revelado que el aumento de ATP extracelular debido a complicaciones quirúrgicas, puede contribuir al desarrollo del estado de inmunosupresión disminuyendo el priming y activación del inflamasoma NLRP3 **(Martins et al. 2009)**.

En ésta Tesis también se estudió la expresión del receptor P2X7 en distintas poblaciones linfoides, y se observó de manera transitoria un aumento del porcentaje de células linfoides que expresan el receptor P2X7 en la superficie celular. Sin embargo, a diferencia de otros trabajos **(Hotchkiss et al. 2001)**, en nuestra cohorte de pacientes, el porcentaje de linfocitos

Th aumentó, los linfocitos B no presentaron cambios, mientras que los porcentajes de linfocitos Tc y células NK disminuyó en pacientes sépticos. Una posible explicación a éstas discrepancias, es que mientras que nuestro trabajo estudia las distintas poblaciones de células durante los 5 primeros días del desarrollo de la sepsis, los otros trabajos presentan las poblaciones de células en un estado de sepsis más tardío, o incluso postmortem **(Hotchkiss et al. 2001)**. Otros estudios revelaron que la cantidad de células Th comienza a disminuir en pacientes supervivientes a partir de los 6 días en comparación con los grupos control **(Wu et al. 2013)**. Adicionalmente, observamos una correlación entre la expresión del receptor P2X7 en monocitos y el aumento del porcentaje de células positivas para P2X7 en pacientes sépticos, pero no en individuos controles. Podría ser que el aumento sistémico de IL-6 observada en los pacientes sépticos, influyera en la activación de las distintas subpoblaciones linfoides que dependen de un progenitor hematopoyético común que expresa el IL-6R **(Fischer et al. 1997)**. De acuerdo con esta evidencia, los elevados valores de IL-6 en plasma pueden elevar la cantidad de células con P2X7 en linfocitos.

4.2. La activación del receptor P2X7 modula la adhesión celular de los macrófagos.

Estudios con el P2X7 de rata han demostrado que el receptor P2X7 interacciona con una gran variedad de proteínas, entre las que podemos encontrar la integrina α3, que reconoce laminina, la integrina β2 y otras proteínas del citoesqueleto como α-actinina y β-actina **(Kim et al. 2001).** En esta Tesis, encontramos que la adhesión celular de macrófagos es dependiente de la activación del receptor P2X7. La adhesión celular disminuyó tras tratar las células con ATP de forma dependiente a la activación del receptor P2X7. La pérdida de adhesión celular resultó ser muy rápida, en un tiempo máximo de dos minutos después de la incubación con ATP. Trabajos anteriores revelan que el ATP extracelular induce una activación transitoria del receptor P2X7 durante los primeros cuatro minutos **(Hanley et al. 2012)**, mientras que la liberación de IL-1β se detecta a partir de los quince minutos desde la presencia de ATP **(Grahames et al. 1999)**. La muerte celular inducida por la activación de P2X7 se observó a partir de los treinta minutos de incubación con ATP **(Le Feuvre et al. 2002)**. Nuestro estudio revela que los cambios necesarios para la disminución de la adhesión inducidos por la activación del receptor P2X7 ocurren de manera muy temprana,

e independiente del flujo de iones a través del receptor P2X7, en contraposición con la liberación de citoquinas pro-inflamatorias y la inducción de la respuesta inflamatoria que es dependiente del descenso de K^+ intracelular permeando a través del poro de P2X7. Estudios previos han demostrado que las células mieloides activadas con LPS aumentan su capacidad de adhesión **(Hmama et al. 1999; Kounalakis and Corbett 2006)**. Por el contrario, en nuestro estudio no observamos una diferencia de adhesión inducida por LPS en las células adheridas a placa de cultivo, ni tampoco en las BMDMs adheridas a colágeno de tipo I y laminina. En cambio, si observamos un aumento significativo de la adhesión celular en BMDMs pre-tratadas con LPS y adheridas a fibronectina. Esta evidencia se podría explicar debido a que algunos trabajos previos han descrito que los macrófagos adheridos a fibronectina, son capaces de liberar al medio extracelular la metaloproteasa de la matriz (MMP)-1 **(Saito et al. 1999)**. En nuestro trabajo encontramos que el tratamiento con ATP sobre BMDMs indujo una disminución de la adhesión celular después de una activación con LPS. Este efecto es debido a que P2X7 es capaz de inducir activación de caspasa-1, la liberación de IL-1β y muerte celular por piroptosis **(Fink and Cookson 2006)(Ferrari et al. 1997)**. Además, encontramos una liberación de LDH extracelular significativa únicamente en tratamientos de LPS+ATP, lo que indica la ejecución de la piroptosis. Por tanto, una vez tratados los macrófagos con LPS, la activación de P2X7 induce una pérdida de la adhesión celular inespecífica tras ejecutarse la piroptosis. Esto queda patente al perder la adhesión de los macrófagos a todos los sustratos estudiados. Por otra parte, vimos que tras estimular P2X7 en macfrófagos sin tratar con LPS, estos solo perdían adherencia al plástico de las placas de cultivo y a colágeno tipo I, pero seguían adheridos a fibronectina y laminina. Estudios previos han revelado que la activación del receptor P2X7 induce la pérdida de integrinas, así como otras moléculas de adhesión, tales como CD21 y CD62L, CD44 en macrófagos o las moléculas de adhesión celular vascular (VCAMs) en neutrófilos **(Sengstake, Boneberg, and Illges 2006; Lin et al. 2012; Mishra et al. 2016)**. Esta liberación es inducida tras activar distintas MMPs (B. Gu, Bendall, and Wiley 1998; Hinkle et al. 2006; Brennaman, Moss, and Maness 2014). Además, otro estudio reveló que la activación del receptor P2X7 induce la liberación de MMP-9 **(B. J. Gu and Wiley 2006)**. En esta Tesis, detectamos la presencia en el medio extracelular de la integrina β1 tras la activación del receptor P2X7, tal y como revelaron otros estudios con anterioridad **(Furlan-freguia et al. 2011)**. La pérdida de integrina β1 resultó ser independiente a la activación previa con LPS. Se sabe que la integrina β1 es esencial para la adhesión de los macrófagos a colágeno de tipo

I, empleando las combinaciones de integrinas α1β1 y α2β1 **(Jokinen et al. 2004; Vandenberg et al. 1991)**. La integrina β1 es también importante para la unión a laminina mediante las integrinas α3β1 y α6β1 **(Fujiwara et al. 2001)**, y a fibronectina mediante las integrinas α4β1 y α5β1 **(Guan and Hynes 1990; Clements et al. 1994; Pierschbacher and Ruoslahti 1984)**. No obstante, a diferencia de la adhesión al colágeno de tipo I, la unión a laminina y fibronectina puede ser mediante integrinas de macrófagos que no están formadas por β1, como la α6β4 en el caso de laminina **(Lee et al. 1992)**, o las integrinas de clase αv en el caso de fibronectina, como αvβ3 o o αvβ5 **(Charo et al. 1990; Blystone et al. 1994; Bharadwaj et al. 2017) (Cheresh et al. 1989)**. Por tanto, en BMDMs sin tratar con LPS, cuando reciben la activación con ATP, que induce la pérdida de β1, sólo se observa una pérdida significativa de adhesión a la matriz de colágeno, debido a que laminina y fibronectina aún pueden estar adheridas a otras integrinas β3 y β5. En resumen, los resultados de este estudio indican que la disminución de la adhesión de macrófagos tras la activación del receptor P2X7 se controla por distintos mecanismos, como la pérdida de la integrina β1 y la ejecución de la piroptosis una vez el macrófago se ha activado con LPS, que sería sinérgico a la pérdida de la integrina β1.

5- Conclusiones

1- El péptido antimicrobiano melitina induce la activación del inflamasoma NLRP3 y caspasa-1 a través de una disminución de la concentración de K^+ intracelular.

2- La melitina induce en macrófagos la liberación de IL-1β, pero no de IL-18.

3- La muerte celular inducida por melitina es independiente de la activación del inflamasoma y de caspasa-1.

4- La concentración de IL-1β, IL-18 y HMBG1 está elevada en plasma de pacientes sépticos durante las primeras 24h desde el desarrollo de la sepsis.

5- La mortalidad en sepsis está asociada a una inmunosupresión del inflamasoma NLRP3.

6- La inmunoparálisis desarrollada durante el proceso séptico es transitoria.

7- La expresión de membrana del receptor P2X7 está aumentada en monocitos de pacientes sépticos.

8- El porcentaje de células mononucleares de sangre periférica de origen linfoide que expresan el receptor P2X7 en la superficie celular está aumentado en pacientes con sepsis.

9- La activación del receptor P2X7 induce una despolarización del potencial de membrana mitocondrial.

10- El receptor P2X7 disminuye la adhesión celular de los macrófagos a distintos substratos celulares de forma independiente del flujo de iones.

11- El receptor P2X7 induce la liberación de la integrina β1 de forma independiente a la activación por LPS.