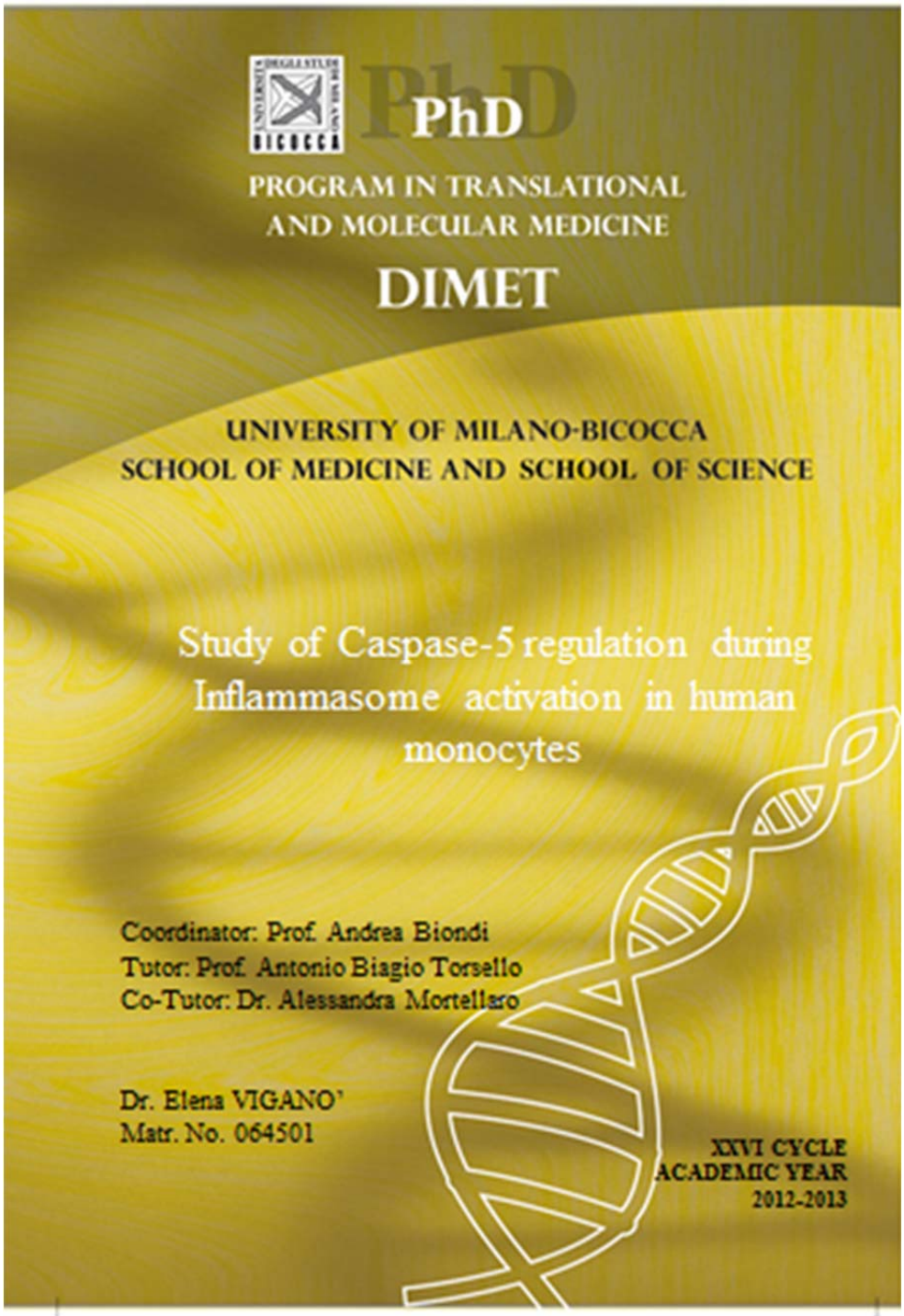
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SCHOOL OF MEDICINE AND SCHOOL OF SCIENCE
Study of Caspase-5 regulation during Inflammasome activation in human monocytes
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Chapter 1.

Introduction

The human immune system consists of two distinct arms that act in harmony to respond against stress signals and pathogens: the innate and the adaptive immunity. The innate immune system is evolutionary more conserved and it plays a primary role in the rapid recognition and elimination of invading microorganisms, through different processes such as phagocytosis and induction of inflammation. In the meanwhile, it is also necessary for the priming and activation of the more recent evolved immune system, the adaptive immune system, which provides long-term protection against specific antigens.

The innate immune system is composed of germline-encoded pattern recognition receptors (PRRs) that recognize and respond to the presence of pathogen- and/or danger-associated molecular patterns (PAMPs and DAMPs, respectively). PRRs are expressed by many cell types, such as monocytes, macrophages, dendritic cells, neutrophils and epithelial cells, and are front line of defense against infection[1].

Sensing of DAMPs/PAMPs by PRRs leads to transcriptional and post-translational changes in the cells that cause the release of cytokines and chemokines into the extracellular milieu. The presence of these molecules induces the recruitment of leukocytes and phagocytes to the site of infection or injury to eliminate the threat and repair the damage.

Our innate immune system has evolved at least four major PRR families: toll-like receptors (TLRs), the C-type lectin receptors

(CTLs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and the nucleotide-binding domain leuchine-rich repeats (NLRs). TLRs and CTLs are membrane-bound receptors which scan the extracellular milieu and endosome compartments; on the contrary, RLRs and NLRs are cytosolic proteins that act as intracellular sensors.

TLRs

Toll-like receptors (TLRs) belong to the PRR superfamily and recognize PAMPs and DAMPs. They are highly evolutionary conserved in flies, plants and mammals. Their discovery started with the identification of a role of Toll, a receptor expressed in Drosophila melanogaster, during fungal infection[2]. Nowadays, ten TLRs (TLR 1-10) in human and twelve (TLR 1-9, 11-13) in mice have been identified and they are broadly expressed in the spleen, peripheral blood leukocytes, gastrointestinal tract and lung[3]. TLRs are integral membrane glycoproteins that can be present in the extracellular membrane (murine TLR 1, 2, 4-6, 11) or in the endosomes (murine TLR 3, 7-9, 13). They have a conserved cytoplasmic domain, necessary for the interaction with adaptor molecules, and a variable extracellular domain containing leucine reach repeat (LRR) motifs specialized for the recognition

of the ligand[4]. After the recognition and engagement with the ligand, the TLRs undergo dimerization (homodimers or heterodimers) which induce the necessary conformational changes that allow the interaction with adaptor molecules to induce the downstream signaling. TLRs activation leads to the activation of the mitogen-activated protein kinases (MAPKs), JUN N-terminal kinase (JNK) and p38, as well as the activation of transcription factors, such as nuclear factor-κB (NF-κB), the inter-feron-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1). TLRs signaling consist of two pathways: myeloid differentiation primary-response protein 88 (MyD88)-dependent and –independent (or TIR-domain-containing adaptor protein inducing IFN-β (TRIF)-dependent) pathways. The MyD88-dependent pathway is common to all TLRs, except TLR3, and it leads mainly to the nuclear translocation of NF-κB, CREB, AP1 whereas TRIF-dependent pathway induces Type I interferons through IRF3/7[5] (Figure 1).

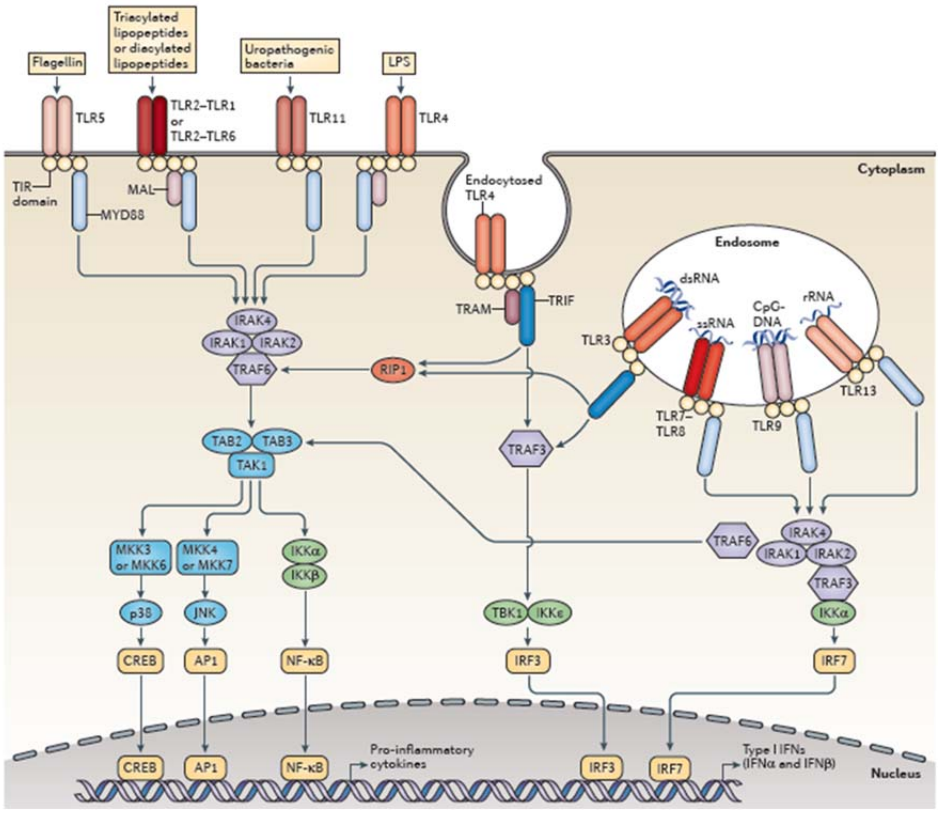

Figure 1. TLR pathways.

Adopted from Luke A. J. O'Neill, Douglas Golenbock and Andrew G. Bowie. Nat Rev Immunol. (2013)[5].

TLR4

TLR4 is a protein that is encoded by the gene *TLR4* located on human chromosome 9. TLR4 is the signaling receptor for lipopolysaccharide (LPS), the major component of the cell wall of

Gram-negative bacteria; in fact, mice lacking TLR4 were not able to respond to LPS stimulation anymore[7]. Moreover, evidences suggested that TLR4 can recognize different stimuli such as fusion protein from respiratory syncytial virus and heat shock proteins[4]. However, TLR4 alone does not have the ability to bind directly to LPS and the recognition of LPS by TLR4 is mediated by co-receptor myeloid differentiation factor 2 (MD2)[8]. Furthermore, lipoprotein binding protein (LBP) together with soluble or transmembrane CD14 is necessary to sense very low amounts of LPS[9,10].

TLR4 is located in the cell surface but it is also internalized to the endosomes after its engagement. Interestingly, it is the only TLR that can lead to the activation of both MyD88-dependent and -independent signaling pathways leading to the expression of cytokines, such as TNFα, IL-1β, IL-6 and IFNs. In particular, the recruitment of CD14 to the endosome has been shown to be important for triggering of the TRIF pathway[11] (Figure 1).

Lipopolysaccharide (LPS) structure

LPS, also known as endotoxin, is an amphiphilic molecule that exists within the outer membrane of Gram-negative bacteria. LPS is essential for both structural and functional integrity of the Gram-negative membrane where it acts as a protective barrier that is permeable only to hydrophilic, low molecular weight molecules.

LPS is generally composed of three main regions: lipid A, core polysaccharide and *O*-polysaccharide[12] (Figure 2). Lipid A is the hydrophobic component of the molecule. It consists typically of a phosphorylated N-acetylglucosamine dimer with six or seven fatty acids attached. The structure of lipid A is quite conserved among Gram-negative bacteria. However, the nature of fatty acids and their degree of acylation can be variable and it contributes to the heterogeneity of these molecules. Interestingly, different species of lipid A can have different functions (agonistic or antagonistic) also based on the host cells applied[13]. Indeed, intact lipid A has been shown to induce little production of cytokines in human monocytes[14] and its precursor, lipid IVa, acts as antagonist of human, but not mouse TLR-4/MD2[15]. Attached to lipid A is the core polysaccharide that appears to be less variable within any *genera*. In particular, the inner core, the one in proximity to lipid A, usually consists of 3-deoxy-D-manno-oct-2-ulopyranosonic acids (KDO) whereas the outer core is generally more variable and is composed of hexose sugars (i.e. glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine). The O-polysaccharide is attached to the outer core and is a polymer of repeating saccharide subunits. Composition and length of this hydrophilic domain greatly vary among species leading to big number of serotypes for particular Gram-negative species[12,13].

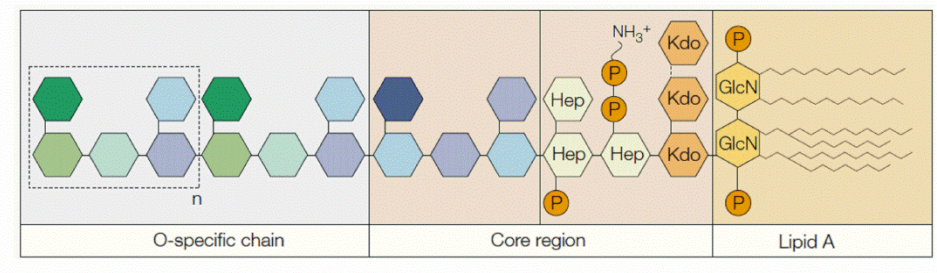

Figure 2. Structure of LPS.

Adapted from Beutler B. and Rietschel E.T., Nat Rev Immunol. (2003)[16].

NLRs

The human and mouse NLRs family consist of 22 and 34 members, respectively. Structurally, NLRs are multi-domain proteins which display a tripartite structural organization consisting of C-terminal LRR, a central nucleotide binding domain (NBD also referred as NACHT domain) and an N-terminal effector domain that initiates signaling.

The LRR domain is a structural motif of 20-30 amino acids with a characteristic sequence pattern rich in leucine, a hydrophobic amino acid that makes LRR domain suitable for protein-protein interactions. On the base of its architecture, LRR domain has been proposed to be implicated in auto-regulation and ligand sensing[17].

NACHT domains are thought to oligomerize upon activation in presence of adenosine triphosphate (ATP)[18]. N-terminal effector domain mediates signal transduction to downstream target. Four different N-terminal domains were described so far: acidic activation domain, baculovirus inhibitory repeat (BIR)-like domain; caspase-recruitment domain (CARD) and pyrin domain (PYD); based on the molecular structure of N-terminal domain, NLRs are divided in four main subclasses: NLRAs, NLRBs, NLRCs and NLRPs containing acidic, BIR, CARD and PYD domains respectively[19]. Within NLRs family, currently only NLRP1[20], NLRP3[21], NLRC4[22], NLRP6[23] and NLRP12[24] have been shown to be capable of forming *in vitro* multi-protein complexes, named inflammasomes.

Inflammasome

The term inflammasome comes from the union between the word “inflammation” and the suffix “some”. “Some”, from the Greek word *soma,* means body and it is frequently used in biology to describe molecular complexes. This name was coined to represent both structure and function of this complex. The inflammasome is a high molecular weight multi-protein complex that induces inflammation through the cleavage and release of

caspase-1-dependent interleukin (IL), IL-1β and IL-18. In general, after activation, the NLRs oligomerize through their nucleotide NBD and recruit procaspase-1 through a CARD-CARD interaction (such as for NLRC4). However, NLRP3 does not have any CARD domain and requires the adaptor apoptosis-associated speck-like protein containing CARD (ASC) to recruit caspase-1 (Figure 3). Indeed, ASC is an intracellular protein that contains both a PYD and a CARD domain to interact with NLRPs and procaspase-1, respectively. Once procaspase-1 is recruited to the multiprotein inflammasome complex, it undergoes proximity-induced autoproteolitic cleavage (autoactivation): procaspase-1 is cleaved initially into p10 and p35 and subsequently p35 is further processed into p20 and p10 subunits. An active caspase-1 tetramer is composed of two p20 subunits and two p10 subunits, which promotes pyroptosis and cytokine release[25].

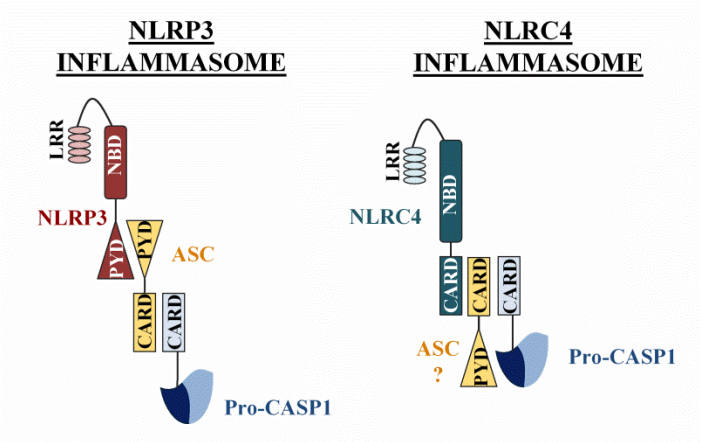

Figure 3. NLRP3 and NLRC4 inflammasome representation

Abbreviations: ASC, apoptosis-associated speck-like protein; CARD, caspase-recruitment domain; CASP, caspase; LRR, leucine rich repeat; NBD, nucleotide binding domain; NLRC4, Nod-like receptor (NLR) family, CARD domain containing 4; NLRP3, NLR family, PYRIN domain-containing-3; PYD, pyrin domain.

Inflammasome activation promotes pyroptosis and cytokine release

Pyroptosis

Pyroptosis (from Greek terms "*pyro*" and "*ptosis*" which mean fire and to fall, respectively) is a particular type of cell death that was described firstly in 1992[26]. Pyroptosis is defined as a caspase-1 dependent cell death that occurs both in myeloid[27] and non-myeloid[28] cells upon infection. It is characterized by plasma membrane disruption that induces in turn water influx and subsequent cytoplasmic swelling and osmotic lysis[29]. Pyroptosis is a pro-inflammatory type of cells death as it results in the release of the cytosolic contents into the extracellular milieu. Indeed, the presence of cytokines (i.e. IL-1α), DAMPs (i.e. high mobility group box 1 (HMGB1)) and microbial antigens in the extracellular space can intensify innate and adaptive immune responses.

IL-1α as a pyroptotic marker

The gene encoding for IL-1α is located on the human chromosome 2. Together with IL-1β and IL-18 (described more in detail below), IL-1α belongs to IL-1 family of cytokines. It has a high tridimensional structure similarity with IL-1β that explains the ability of these two cytokines to bind and act through the same receptor, the IL-1 receptor type I (IL-1RI)[30,31]. Its secretion does not involve endoplasmic reticulum (RE) and the Golgi apparatus, suggesting the involvement of an unconventional pathway. Monocytes and macrophages are the major producers of IL-1 (both IL-1α and IL-1β) cytokines[32]. These cytokines are expressed in the cells as precursors (pro-IL-1α and pro-IL-1β, p31 kDa and p37 KDa, respectively) which undergo cleavage. Interestingly, both precursor and cleaved form of IL-1α exert a biological activity binding to IL-1RI[33]. IL-1α can have a different localization pattern within the cells. It localizes as pro-IL1α on the plasma membranes of several type of cells (e.g. monocytes and B cells) where it seems to be involved in cell-cell paracrine signaling[34]. Because of the presence of a nuclear localization sequence (NLS) in its N-terminal, IL-1α can translocate into the nucleus where it can influence the transcription of pro-inflammatory genes[35]. In addition, IL-1α is also present in the cytoplasm where it can be cleaved into its mature form by calpain II, a membrane-associated, calcium-dependent cysteine protease[36]. The

mechanism leading to the release of mature IL-1α from human monocytes is not yet demonstrated[37]. IL-1α is rarely detected in serum or body fluids in humans[38] but primary cells contain constitutive levels of pro-IL-1α[39] that can be released by necrotic and pyroptotic cells[40].

Inflammasome related cytokines: IL-1β and IL-18

Proteins that are released in the extracellular milieu, such as cytokines (e.g. IL-6), usually contain a signal peptide that guides them towards the ER where the translocation apparatus is present. From the RE the secreted proteins are transported to the Golgi apparatus first and then to the extracellular compartment through a vesicular network (classical secretory pathway). However, proteins lacking the signal peptide can be secreted from the cells via an unconventional RE-Golgi apparatus-independent pathway[41]. IL-1β and IL-18 (together with IL-1α described above) do not possess the signal peptide; therefore cells use an unconventional route to secrete them. Unlike IL-1α, IL-1β and IL-18 require caspase-1-dependet cleavage to be released. Indeed, macrophages from caspase-1 deficient mice failed to secrete both inflammasome-dependent cytokines[42]. Several models have been proposed to describe this unconventional secretion[41]. However, the exact molecular mechanism remains unclear.

IL-1β

The gene encoding for IL-1β is located on the human chromosome 2. Monocytes, macrophages and dendritic cells are the primary source for IL-1β, but lymphocytes and Natural killer (NK) cells can also produce the cytokine too.

IL-1β is an important pro-inflammatory cytokine. Its release induces expression of adhesion molecules on epithelial cells that mediates the transmigration of immune-competent cells to the site of infection or injury. It has also been shown that IL-1β induces the synthesis of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS) leading to the production of inflammatory mediators, such as prostaglandin-E2 (PGE2), platelet activating factor (PAF) and nitric oxide (NO)[37]. IL-1β plays also a role in the regulation of sleep, appetite, body temperature and threshold of pain[37].

The activity of IL-1β is tightly regulated at different levels including: expression, maturation and secretion. Indeed, its coding region contains an instability element that maintains low expression level of IL-1β[43]. Therefore, the transcription of IL-1β gene requires a first step (called “priming”) usually mediated by TLR ligands (i.e. LPS) or TNFα to induce IL-1β precursor. As described previously, IL-1β activation and release is controlled by caspase-1-dependent inflammasome activation. Furthermore,

when IL-1β is secreted its activity is also regulated by endogenous soluble IL-1 receptor antagonist (IL-1RA), which binds the cell surface of IL-1R avoiding the binding of IL-1β with its receptor[44].

IL-18

IL-18 is synthetized as a precursor (pro-IL-18, 24KDa) that, differently from pro-IL-1β, is constitutively and broadly expressed both in human and in mouse[45,46]. However, pro-IL-18 needs to be cleaved to its active form (IL-18, 17KDa) to be secreted and to bind to its receptor, the IL-18 receptor (IL-18R). IL-18 activity is regulated by an affinity-dependent IL-18 binding protein (IL-18BP)[47] constitutively secreted at high levels in the serum and synovial fluid of healthy humans[48]. Imbalance between IL-18 and IL-18BP (decrease IL-18BP, increase of IL-18) is often associated to an increased severity of different disorders, such as obesity, type II diabetes, cardiovascular diseases and Alzheimer's disease[49].

NLRP3 Inflammasome (canonical pathway)

NLRP3 inflammasome is the best studied inflammasome so far and is composed of NLRP3, ASC and procaspase-1. The LRRs domain in the NLRP3 protein, in concert with two chaperones (SGT1 and HSP90)[50], is suggested to maintain the protein in an auto-repressed state. Upon activation, conformational changes in NLRP3 allow the exposure of NBD and PYD domains; NLRP3 is then enabled to oligomerize with homotypic NBD domains, interact with ASC through PYD domain, which in turn recruits caspase-1. Based on previous studies, it was proposed that LRR domain of NLRP3 binds and senses its specific activators. However, there is no evidence that NLRP3 binds directly to any of its known activators and it is unlikely to be true. Indeed, NLRP3 is activated by a wide range of signals, which could not be recognized by the same LRRs structure. The activators can have different natures: microbial or sterile (exogenous or endogenous) (Table 1).

NLRP3 Activators		
Sterile (DAMPs)		**Microbial (MAMPs)**
Endogenous	**Exogenous**	
Amyloid β ATP Cholesterol Glucose Hyaluronan ROS MSU	Alum Asbestos Silica	Bacteria (whole) Double strand DNA Double strand RNA Fungi Pore forming toxins Virus

Table 1. NLRP3 activators**.**

Abbreviation: ATP, adenosine triphosphate; ROS, reactive oxygen species; MSU, monosodium urate.

The different structure of the NLRP3 agonists (Table 1) suggests the presence of a common intermediate downstream molecule that elicits the formation of a functional inflammasome. Three mechanisms have been proposed for inflammasome activation so far: potassium (K^+) efflux, generation of reactive oxygen species (ROS) and phago-lysosomal destabilization.

Models for NLRP3 inflammasome activation

- *K+ efflux*: K^+ is one of the most abundant ions present in the cytoplasm of the cells. Disruption of plasma membrane integrity induces the release of K^+ ions. The increase of the extracellular K^+ concentration as well as the incubation of cells with glibenclimide, a K^+ channel inhibitor, inhibits the formation of an active inflammasome *in vitro*[51,52]. Two well established stimuli that cause K^+ efflux are: ATP that activates P2X7 receptor cation channel, and nigericine, a pathogen-derived pore forming toxin[53]. Therefore, NLRP3 inflammasome has been proposed to be a sensor for cells integrity and, recently, Muňoz-Parillo and colleagues have highlighted the important role of K^+ as a common modulator of NLRP3 activation[54].

- *ROS*: The redox state of the cells is another indicator of cell viability. Several NLRP3 activators (i.e. MSU) induce an increase in ROS production and elevated levels of ROS have been shown to activate NLRP3 inflammasome. Originally, NADPH oxidase was proposed as the main source of ROS production responsible for the inflammasome activation. However, studies using cells deficient in NADPH oxidase components showed no defective inflammasome activation[55]. Subsequently, it was

reported that the increase of mitochondrial ROS, a consequence of dysregulation of mitochondrial function, leads to NLRP3 activation. Moreover, NLRP3 inflammasome has been shown to co-localize with the mitochondria after activation[56]. The redox state is also controlled by the presence of antioxidant systems (e.g. thioredoxin, glutathione synthase) and a role of antioxidant response during NLRP3 activation has been proposed. Indeed, the induction of ROS-scavenger antioxidant machineries prevent IL-1β release[57] and their expression level inversely correlates with NLRP3 activation[58]. However, the precise function of redox state during activation of NLRP3 inflammasome is still controversial.

- *Phago-lysosomal destabilization*: Both pathogens, that actively escape the phagosome, and crystal particles (i.e. silica) could damage the phago-lysosome membrane leading to the release of its content to the cytoplasm. Indeed, the inhibition of cathepsin B, a lysosomal enzyme, impaired the NLRP3 activation induced by silica[55]. However, cells deficient for cathepsin B do not show any alteration of inflammasome activation[59], suggesting the possible involvement of redundant cathepsins.

More recently, other mechanisms seem to be involved in inflammasome-mediated caspase-1 activation. Indeed, a non-canonical pathway has been described in which caspase-11, another inflammatory caspase, plays a central role in cytokine release and induction of pyroptosis[60].

Caspase-11 and non-canonical Inflammasome

Caspase-11 and its expression

Murine caspase-11 belongs to the inflammatory caspase-1 subfamily of proteases[61] and it shares 46% of amino acid identity with murine caspase-1. It is synthetized as 43 and 38kDa precursors (procaspase-11) and, in contrast to caspase-1, its expression is not constitutive, but requires an inflammatory stimulus. In particular, stimulation with the whole Gram-negative bacteria (i.e. *Vibrio cholera*, flagellin-deficient *Salmonella enterica* serovar typhimurium (*Δflag Salmonella infection*), *Escherichia coli* (*E. coli*), enterohemorrhagic *E. coli* (EHEC), *Legionella pneumophila*, *Citrobacter rodentium*) has been shown to induce procaspase-11 expression in murine macrophages[62-65]. The peculiarity of Gram-negative bacteria is the presence of LPS on their outer membrane. Indeed, LPS induces procaspase-11

expression in splenocytes[66] and macrophages[61,66]. The contribution of downstream players of TLR4 pathway (Figure 1), leading to caspase-11 expression induction, has been assessed. Myd88$^{-/-}$ and Trif$^{-/-}$ macrophages show a delay in procaspase-11 expression after Δflag Salmonella infection[63,65]. On the contrary, other independent groups have demonstrated that procaspase-11 up-regulation is reduced in Trif$^{-/-}$ macrophages infected with *C. rodentium*[67] and *E. coli*[64,67] keeping the role of TRIF still under debate. TRIF-mediated signaling leads to the activation of IRF3-IRF7 transcription factor complex which, in turn, elicits the expression of type I interferons (IFNs), IFN-α/β (Figure 4). Role of IFN-α/β in procaspase-11 expression still remains controversial. Indeed, Rathinam *et al.* reported that macrophages lacking interferon type I receptor (Ifnar$^{-/-}$) infected with EHEC have less procaspase-11 expression compared to wild-type macrophages and the expression is restored when high doses of IFN-β is added to the *in vitro* culture[64]. In contrast, a separate study reported that Ifnar$^{-/-}$ macrophages do not show any reduction in procaspase-11 expression after Salmonella infection and procaspase-11 induction does not increased after the addition of exogenous IFN-β[63].

Caspase-11 and its regulation/cleavage

Caspases are a family of endoproteases which their activation is tightly regulated. Caspases are synthetized as a zymogen (or pro-enzyme), an inactive precursor that needs to be hydrolyzed in order to expose their active site and to be functional. Active caspase is often composed of a dimer (i.e. caspase-1). Procaspase-11 has been shown to be cleaved into a 26-30kDa protein in order to be active[63,64]. Furthermore, TRIF-type I IFNs pathway is essential for the processing of caspase-11. Macrophages lacking TRIF and IFNAR display a reduction in active caspase-11 after Gram negative bacterial infection[63,64,67]. Taking this into account, together with the data regarding the regulation of procaspase-11 expression (described above), two models of activation have been proposed: auto-activation model[64] and receptor/scaffold-mediated activation model[63] (Figure 5). Rathinam and colleagues suggested that induction of caspase-11 expression through TRIF-IFN type I signaling is necessary and sufficient for the caspase-11 processing and activation (auto-activation model)[64]. The second model is proposed by Broz *et al.* where they observed that TRIF deficiency and IFNAR is dispensable for caspase-11 expression but, on the other hand, is required for caspase-11 activation after Salmonella infection. Based on results obtained from TRIF- and IFNAR-deficient macrophages, Broz *et al.* proposed that a still unknown scaffold

protein whose expression is regulated by type I IFNs via the TRIF-pathway, mediates the activation of caspase-11 (receptor/scaffold-mediated activation model)[63] (Figure 5).

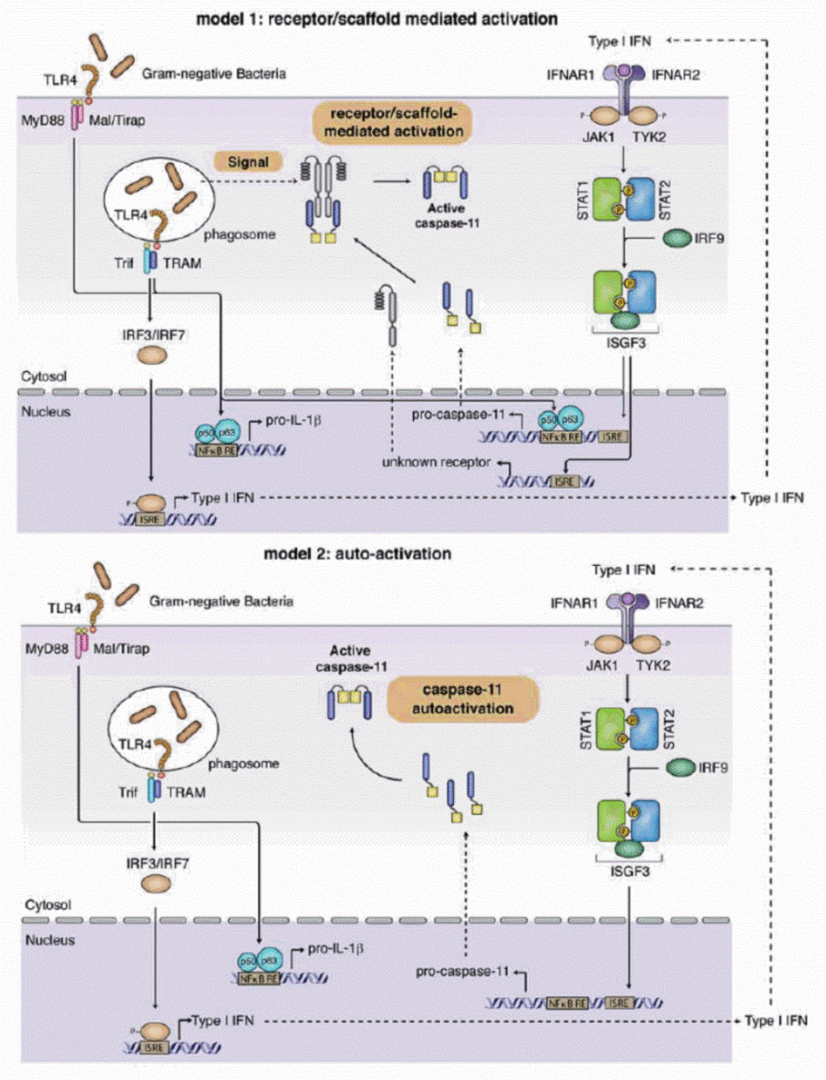

Figure 5. Models for caspase-11 activation.

Modified from Broz P. and Monack D.M. Plos Pathogens (2013)[68].

Initially, it was thought that caspase-11 activation requires the presence of infection, as LPS or IFN-β alone could not induce caspase-11 activation in macrophages[63]. However, this assumption has been disproved by the study of Kayagaki *et al.*[69]. Indeed, it demonstrates that the presence of LPS alone in the cytoplasm of murine macrophages is sufficient for caspase-11 activation in absence of infection[69]. Previous work from the same authors showed non-canonical inflammasome activation through stimulation with cholera toxin B (CTB) together with LPS. Initially, CTB was identified as one of the triggers of caspase-11 activation[62], but only recently the mechanism has been clarified. CTB itself does not trigger caspase-11 processing but facilitates the entry of LPS (serotype O111:B4) into the cytoplasm and the genuine activator of caspase-11 seems to be cytoplasmic LPS (Figure 6).

Sensing of LPS by a still unknown cytoplasmic receptor seems to be the genuine trigger of caspase-11 activation. Indeed, when LPS is forced to enter into the cell, in absence of any other trigger, it induces the activation of non-canonical inflammasome[69,70]. However, the cleavage of caspase-11 after transfection of LPS has not been shown; the relevance of LPS-mediated activation and the non-canonical pathway has been tested using caspase-11-deficient macrophages.

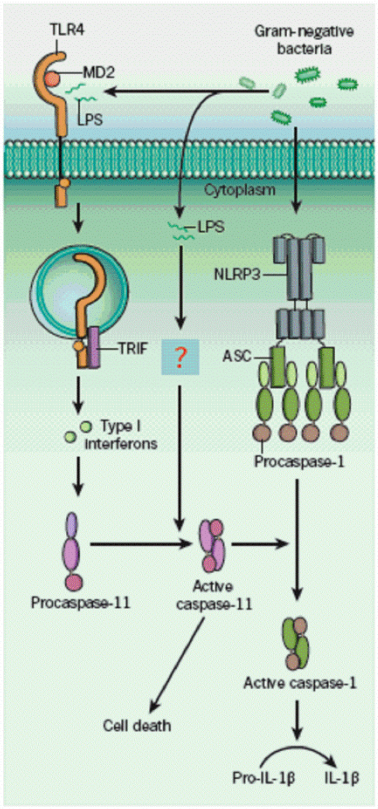

Figure 6. LPS-dependent caspase-11 activation
Adapted from Rathinam V. A. K and Fitzgerald K. A. Nature (2013)[71].

Furthermore, in agreement with these data, recent work clarifies the role played by Type I IFNs during the activation of non-canonical inflammasome induced by intracellular Gram-negative bacteria. Type I IFNs are required to induce the expression of small interferon-inducible GTPases; these proteins are necessary for lysing the pathogen-containing vacuole, which in turn allows

the release of the bacteria into the cytoplasm where LPS can be sensed[72].

Caspase-11 substrate(s)

Caspases are cysteine proteases that play critical roles during apoptosis (apoptotic caspases) and proteolitic activation of cytokines (inflammatory caspases). Inflammatory caspases are encoded by three genes, *caspase-1, -11, -12* in mouse (*caspase-1, -4, -5, -12* in human) and are located in tandem in a cluster on the chromosome 9 (chromosome 11 in human)[73] (Figure 7).

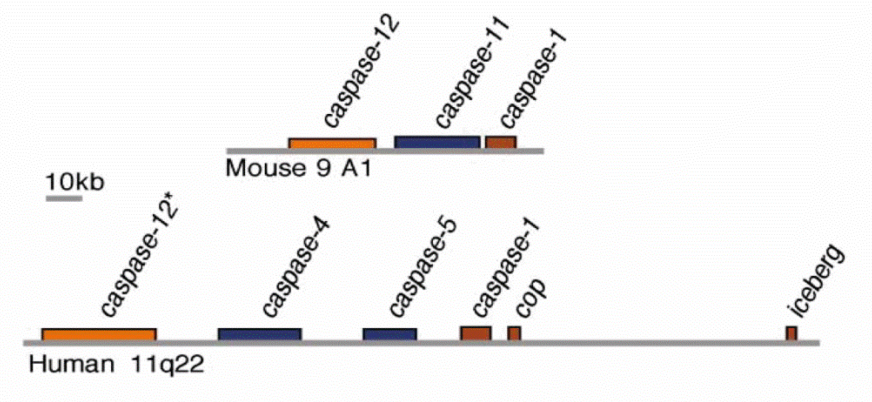

Figure 7. Chromosomal organization of inflammatory caspases. Adapted from Martinon F. and Tschopp J. Cell (2004)[73].

The substrates of the caspases are usually recognized through a conserved amino acid sequence and the cleavage takes place at the C-terminal of an aspartic acid residue. However, the substrates of caspase-11 are still unknown. The crystallography analysis of the protein revealed strong differences between caspase-11 and caspase-1[61], suggesting that caspase-11 does not directly cleave caspase-1 substrates, such as pro-IL-1β and pro-IL-18. Indeed, caspase-11 cleaves pro-IL-1β very poorly and caspase-11 overexpression does not induce any IL-1β cleavage and release when expressed in HEK293t cells. Nevertheless, *in vitro* results indicated the ability of caspase-11 to potentiate the cleavage of caspase-1. Indeed, co-expression of procaspase-11 alongside procaspase-1 potentiated the cleavage of pro-IL-1β compared to cells in which only caspase-1 was overexpressed[61,62]. Furthermore, macrophages lacking caspase-11 were not able to process caspase-1 in its active form when stimulated with non-canonical stimuli[62,65,69,74].

Caspase-11 function

The role played by caspase-11 in inflammasome activation began to be investigated only very recently after caspase-1$^{-/-}$ mice were discovered to be a double knock-out for both caspase-1 and -11[63].

Indeed, *Casp1*$^{-/-}$ mouse strains (now known as caspase-1/11 double knock-out) were all generated using 129 embryonic stem cells which carry a 5bp deletion in caspase-11 causing the lacking of its catalytic activity. Caspase-11 is located in near proximity of caspase-1 locus (Figure 7); therefore, these two mutations could not segregate during backcrossing. The availability of caspase-11 single knock-out allowed the investigation of the role played by caspase-11 during cytokine release and pyroptosis.

Cytokine release and caspase-11

LPS-primed macrophages derived from a genuine *Casp11*$^{-/-}$ mouse strain (generated from C57B6 ES cells) were unable to process caspase-1 and secrete IL-1β and IL-18 in response to non-canonical stimuli (cholera toxin B (CTB), *E. coli*, *C. rodentium*, *V. cholera*, intracellular LPS)[62,64,69] independently from TLR4, MD2, CD14, TRIF and Type I IFNs[69]. Indeed, Pam3CSK4 (TLR2 ligand)–primed bone-marrow derived macrophages obtained from *Tlr4*$^{-/-}$, *Md2*$^{-/-}$, *Cd14*$^{-/-}$, *Trif*$^{-/-}$ and *Ifnar*$^{-/-}$ secrete the same levels of IL-1β as wild type control upon transfection of LPS[69].

In addition to IL-1β and IL-18, caspase-11 regulates the secretion of IL-1α in a NLRP3 independent manner[64]. IL-1α release was suppressed in *Casp11*$^{-/-}$ or in *Casp1/Casp11*$^{-/-}$ double deficient macrophages, but not in *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ macrophages, upon non-canonical stimuli (CTB, *E. coli*). However, IL-1α remains fully

dependent on caspase-1 when canonical stimuli (ATP, *C. difficile* toxin B) are employed[64].

Pyroptosis and caspase-11

Caspases play a crucial role during programmed cell death (also known as apoptosis)[75] and early studies on caspase-11 attributed a possible role of caspase-11 in the regulation of cell death. It has been shown that caspase-3 and -7 (effector apoptotic caspases) can be activated through caspase-11[76]. Moreover, overexpression of caspase-11 *per se* induces cell death in splenocytes upon LPS stimulation[77]. Recently, a role of caspase-11 during pyroptosis (described above) was described[62,69]. HMGB1, a danger signal which is released in association with pyroptosis, is reduced in supernatants obtained from caspase11-deficient macrophages stimulated with non-canonical activators[62]. Moreover, caspase-11, but not NLRP3, NLRC4, ASC, or caspase-1 is required to induce pyroptosis in response to Gram-negative bacteria[62,69,78].

Caspase-11 and Sepsis

The role of caspase-11 has been also assessed *in vivo* through intravenous injection of high doses of LPS, a murine model of

acute septic shock. *Casp-11*$^{-/-}$ mice are more resistant to LPS challenge compared *Casp-1*$^{-/-}$ mice. Furthermore, the release of caspase-11-dependent cytokine IL-1β and IL-18 is dispensable for LPS-induced mortality. Indeed, no differences were observed in the serum levels of IL-1β and IL-18 between the two strains. Moreover, LPS susceptibility of mice lacking IL-1β, IL-18 or IL1RI was comparable to wild type mice. Notably, a reduction of IL-1α in the serum was observed in *Casp-11*$^{-/-}$ mice[62]. Recently, Kayagaki *et al.* demonstrated that the mortality caused by septic shock is independent of TLR4 and fully dependent on caspase-11[69]. The authors postulate a possible detrimental role of caspase-11-dependent pyroptosis in *in vivo* model of sepsis.

Human caspase 5: expression and function

Caspase-5, also called ICErelIII[79] and TY[80], is an inflammatory caspase whose encoding gene is located on human chromosome-11q22 (Figure 8). *Caspase-5* gene shares very high sequence identity with its neighbor *caspase-4* gene and murine *caspase-11*, suggesting the possibility that *caspase-4* and *caspase-5* genes originated from a single ancestral *caspase-11* gene[81] (Figure 8). In nature, 6 variants of caspase-5 have been identified so far (variant a-f) and they arise from differential skipping exon (variant a-e) or

alternative splicing (variant f)[82] (Figure 9). Within the 6 variants, only 3 (variants a, b and f) possess both pro-domain and catalytic domain. The presence of a CARD domain in the pro-domain gives them the ability to bind to other CARD-containing proteins, such as inflammasomes-associated proteins. Indeed, a strong interaction between caspase-5 and NLRP1 CARD domains has been described[20].

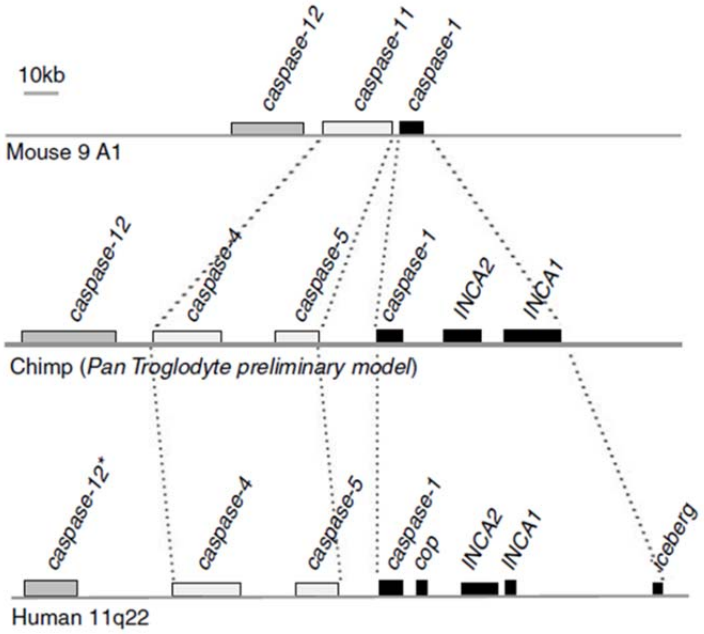

Figure 8. Adopted from Martinon F., Tschopp J. Cell Death Differ (2007)[81].

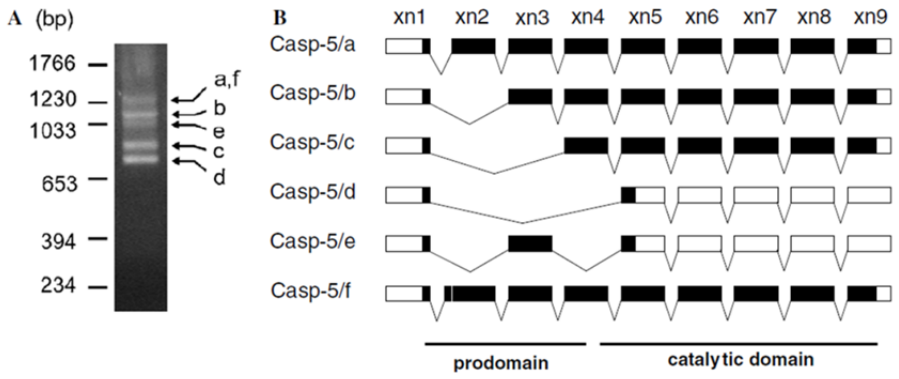

Figure 9. Adapted from Eckhart L. at al., Biochem Biophys Res Commun (2006)[82].

Caspase-5 is expressed in placenta, lung, spleen, small intestine, colon, peripheral blood lymphocytes[83] and psoriatic skin lesion[84]. Interestingly, it is mainly expressed in immune organs and in tissues that are in close contact with pathogens. Infection by both Gram-positive (*Propionibacterium acnes*) or Gram-Negative (*Heamophilus ducrey*) bacteria has been shown to increase caspase-5 expression in monocytes[85] and skin[86], respectively. Furthermore, its expression is induced by pro-inflammatory stimuli, such as LPS, IFN-γ, IL-1β and TNFα[83,87].

However, the mechanism by which caspase-5 is activated and its function is still unclear. Amino acid sequence analysis, comparing the caspase-5 sequence with the well characterized caspase-1

sequence[88,89], revealed high conservation in the residues involved in the catalysis[79]. Furthermore, caspase-5 overexpression leads to the formation of two fragments (p35 and p10)[82] suggesting a possible autocatalytic activity. Active caspase-5 has been detected in LPS-stimulated human retinal pigment epithelial cells[87] and in aortic wall of patients suffering from advanced abdominal aortic aneurysms[90].

Caspase-5 over-expression in different host cell lines induces apoptosis[79,80] due to the ability of caspase-5 to cleave and activate procaspase-3, an effector caspase of the apoptotic cascade[83,91,92]. Furthermore, caspase-5 seems to help the caspase-1 dependent pro-IL-1β cleavage; in fact, pro-IL-1β processing occurs most efficiently when both caspases (caspase-1 and caspase-5) are present and active[20] even though pro-IL-1β is not a substrate of caspase-5[80].

Human monocytes

Monocytes belong to the innate branch of the immune system. They originate from hematopoietic stem cells present into the bone marrow[93] and are subsequently released in the peripheral blood where they comprise 5-10% of the total blood leukocytes in human. However, the monocyte numbers in the blood could be

variable depending on the individual's health. Infection and high fat diet have been reported to induce monocytosis, an increase migration of monocytes from bone marrow into the blood[94,95]. During steady state condition, monocytes circulate for several days in the peripheral blood where they sense the environment. Monocytes are considered as a systemic reservoir of myeloid precursors which give rise to tissue-resident macrophages as well as antigen-presenting dendritic cells[96,97]. Furthermore, they play an important role during homeostasis and infection through their ability to identify and phagocyte apoptotic cells[98] and microorganisms[99], respectively. Indeed, monocytes possess a large panel of scavenger receptors and PRRs, like TLRs, which allow them to recognize DAMPs/PAMPs, whose recognition leads to the production and secretion of a large amount of effector molecules, such as cytokines, myeloperoxidase and superoxide, involved in the defense against pathogens[95]. For instance, the presence of LPS in the bloodstream is rapidly recognized by monocytes, which in turn induce the release of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-1β, necessary to eradicate the infection.

Human monocytes are a heterogeneous population and are characterized by irregular cell shape with oval-shaped nucleus. They have a high cytoplasm-to-nucleus *ratio* and a granular morphology due to the presence of cytoplasmic vesicles[100]. Morphology was the initial characteristic used to classify

monocytes but, recently, the analysis of cell-surface markers allowed the identification of three major populations: $CD14^{+}CD16^{-}$ (classical monocytes), $CD14^{+}CD16^{+}$ (intermediate monocytes) and $CD14^{low}CD16^{+}$ (non-classical monocytes)[101]. Classical monocytes consist of a bigger population and show a higher cytokines production profile[102], higher chemotactic[103] and phagocytic activities[104]. Non-classical monocytes, which represent only 10% of the total monocytes, are characterized by high expression of major histocompatibility complex (MHC) class II and high production of TNFα following TLR stimulation[105]. Intermediate monocytes are the main producers of IL-10[106].

Aims of the thesis

Monocytes are the resident sentinels in bloodstream which produce high levels of cytokines upon infection/activation. They are capable to synthesize and release cytokines *in vitro,* such as IL-1, TNFα, IL-6, when stimulated with TLR ligands[107].

IL-1β is a well characterized cytokine whose release requires an active inflammasome complex. The activation of the inflammasome is regulated by a two-step pathway: first, the cells have to be primed through the activation of NF-κB, that induces the expression of pro-IL-1β and inflammasome components, such as NLRP3 (step 1); second, an additional stimulus is required, in order to induce the inflammasome complex formation, which in turn leads to the activation of caspase-1 and the subsequent cleavage of pro-IL-1β and pro-IL-18 (step 2)[108]. However, human monocytes do not follow this dual mode of activation but they release IL-1β when stimulated with LPS alone in absence of any proper inflammasome activator[107,109].

The presence of LPS in the circulation is a common feature of sepsis induced by Gram-negative bacteria. Sepsis is defined as a host inflammatory disease leading to organ dysfunction in response to severe infections[110], and monocytes play an essential role in driving the early inflammatory response in sepsis[111].

In this study we aimed to understand the key molecular mechanisms involved in monocyte response to stimulation with

LPS. In particular, we investigated the regulation and function of the inflammatory caspase-5, whose role has not been elucidated in this context yet (Chapter 2).

The Chapter 2 is articulated in rationale of the experimental plan, the results obtained and the methodology of experiments performed.

Finally, the findings are analyzed considering their significance in human diseases and their possible application in translational medicine (Chapter 3).

References

1 Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805-820, doi:10.1016/j.cell.2010.01.022 (2010).

2 Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983 (1996).

3 Zarember, K. A. & Godowski, P. J. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* **168**, 554-561 (2002).

4 Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature reviews. Immunology* **4**, 499-511, doi:10.1038/nri1391 (2004).

5 O'Neill, L. A., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors - redefining innate immunity. *Nature reviews. Immunology* **13**, 453-460, doi:10.1038/nri3446 (2013).

6 Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-2088 (1998).

7 Hoshino, K. *et al.* Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* **162**, 3749-3752 (1999).

8 Shimazu, R. *et al.* MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine* **189**, 1777-1782 (1999).

9 Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433 (1990).

10 Schumann, R. R. *et al.* Structure and function of lipopolysaccharide binding protein. *Science* **249**, 1429-1431 (1990).

11 Zanoni, I. *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**, 868-880, doi:10.1016/j.cell.2011.09.051 (2011).

12 Erridge, C., Bennett-Guerrero, E. & Poxton, I. R. Structure and function of lipopolysaccharides. *Microbes and infection / Institut Pasteur* **4**, 837-851 (2002).
13 Caroff, M., Karibian, D., Cavaillon, J. M. & Haeffner-Cavaillon, N. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes and infection / Institut Pasteur* **4**, 915-926 (2002).
14 Caroff, M., Cavaillon, J. M., Fitting, C. & Haeffner-Cavaillon, N. Inability of pyrogenic, purified Bordetella pertussis lipid A to induce interleukin-1 release by human monocytes. *Infection and immunity* **54**, 465-471 (1986).
15 Saitoh, S. *et al.* Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *International immunology* **16**, 961-969, doi:10.1093/intimm/dxh097 (2004).
16 Beutler, B. & Rietschel, E. T. Innate immune sensing and its roots: the story of endotoxin. *Nature reviews. Immunology* **3**, 169-176, doi:10.1038/nri1004 (2003).
17 Bella, J., Hindle, K. L., McEwan, P. A. & Lovell, S. C. The leucine-rich repeat structure. *Cellular and molecular life sciences : CMLS* **65**, 2307-2333, doi:10.1007/s00018-008-8019-0 (2008).
18 Duncan, J. A. *et al.* Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8041-8046, doi:10.1073/pnas.0611496104 (2007).
19 Bauernfeind, F. *et al.* Inflammasomes: current understanding and open questions. *Cellular and molecular life sciences : CMLS* **68**, 765-783, doi:10.1007/s00018-010-0567-4 (2011).
20 Martinon, F., Burns, K. & Tschopp, J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* **10**, 417-426 (2002).
21 Agostini, L. *et al.* NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**, 319-325 (2004).
22 Kofoed, E. M. & Vance, R. E. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* **477**, 592-595, doi:10.1038/nature10394 (2011).

23 Grenier, J. M. *et al.* Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. *FEBS letters* **530**, 73-78 (2002).
24 Wang, L. *et al.* PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *The Journal of biological chemistry* **277**, 29874-29880, doi:10.1074/jbc.M203915200 (2002).
25 Rathinam, V. A., Vanaja, S. K. & Fitzgerald, K. A. Regulation of inflammasome signaling. *Nature immunology* **13**, 333-342, doi:10.1038/ni.2237 (2012).
26 Zychlinsky, A., Prevost, M. C. & Sansonetti, P. J. Shigella flexneri induces apoptosis in infected macrophages. *Nature* **358**, 167-169, doi:10.1038/358167a0 (1992).
27 Bergsbaken, T., Fink, S. L. & Cookson, B. T. Pyroptosis: host cell death and inflammation. *Nature reviews. Microbiology* **7**, 99-109, doi:10.1038/nrmicro2070 (2009).
28 Doitsh, G. *et al.* Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature* **505**, 509-514, doi:10.1038/nature12940 (2014).
29 Fernandes-Alnemri, T. *et al.* The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell death and differentiation* **14**, 1590-1604, doi:10.1038/sj.cdd.4402194 (2007).
30 Priestle, J. P., Schar, H. P. & Grutter, M. G. Crystallographic refinement of interleukin 1 beta at 2.0 A resolution. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9667-9671 (1989).
31 Graves, B. J. *et al.* Structure of interleukin 1 alpha at 2.7-A resolution. *Biochemistry* **29**, 2679-2684 (1990).
32 Dinarello, C. A. The interleukin-1 family: 10 years of discovery. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **8**, 1314-1325 (1994).
33 Mosley, B. *et al.* The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *The Journal of biological chemistry* **262**, 2941-2944 (1987).
34 Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M. & Lovett, D. H. The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal

propiece. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 7245-7249 (1993).
35 Kawaguchi, Y. *et al.* Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14501-14506, doi:10.1073/pnas.0603545103 (2006).
36 Carruth, L. M., Demczuk, S. & Mizel, S. B. Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. *The Journal of biological chemistry* **266**, 12162-12167 (1991).
37 Dinarello, C. A. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* **27**, 519-550, doi:10.1146/annurev.immunol.021908.132612 (2009).
38 Gabay, C., Lamacchia, C. & Palmer, G. IL-1 pathways in inflammation and human diseases. *Nature reviews. Rheumatology* **6**, 232-241, doi:10.1038/nrrheum.2010.4 (2010).
39 Hacham, M., Argov, S., White, R. M., Segal, S. & Apte, R. N. Different patterns of interleukin-1alpha and interleukin-1beta expression in organs of normal young and old mice. *European cytokine network* **13**, 55-65 (2002).
40 Chen, C. J. *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nature medicine* **13**, 851-856, doi:10.1038/nm1603 (2007).
41 Nickel, W. & Rabouille, C. Mechanisms of regulated unconventional protein secretion. *Nature reviews. Molecular cell biology* **10**, 148-155, doi:10.1038/nrm2617 (2009).
42 Keller, M., Ruegg, A., Werner, S. & Beer, H. D. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* **132**, 818-831, doi:10.1016/j.cell.2007.12.040 (2008).
43 Bufler, P., Gamboni-Robertson, F., Azam, T., Kim, S. H. & Dinarello, C. A. Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. *The Biochemical journal* **381**, 503-510, doi:10.1042/BJ20040217 (2004).
44 Arend, W. P. The balance between IL-1 and IL-1Ra in disease. *Cytokine & growth factor reviews* **13**, 323-340 (2002).
45 Tone, M., Thompson, S. A., Tone, Y., Fairchild, P. J. & Waldmann, H. Regulation of IL-18 (IFN-gamma-inducing factor) gene expression. *J Immunol* **159**, 6156-6163 (1997).

46 Puren, A. J., Fantuzzi, G. & Dinarello, C. A. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2256-2261 (1999).
47 Novick, D. *et al.* A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18. *Cytokine* **14**, 334-342, doi:10.1006/cyto.2001.0914 (2001).
48 Shao, X. T. *et al.* Expression of interleukin-18, IL-18BP, and IL-18R in serum, synovial fluid, and synovial tissue in patients with rheumatoid arthritis. *Clinical and experimental medicine* **9**, 215-221, doi:10.1007/s10238-009-0036-2 (2009).
49 Dinarello, C. A., Novick, D., Kim, S. & Kaplanski, G. Interleukin-18 and IL-18 Binding Protein. *Frontiers in immunology* **4**, 289, doi:10.3389/fimmu.2013.00289 (2013).
50 Kadota, Y., Shirasu, K. & Guerois, R. NLR sensors meet at the SGT1-HSP90 crossroad. *Trends in biochemical sciences* **35**, 199-207, doi:10.1016/j.tibs.2009.12.005 (2010).
51 Petrilli, V. *et al.* Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell death and differentiation* **14**, 1583-1589, doi:10.1038/sj.cdd.4402195 (2007).
52 Lamkanfi, M. *et al.* Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *The Journal of cell biology* **187**, 61-70, doi:10.1083/jcb.200903124 (2009).
53 Perregaux, D. & Gabel, C. A. Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *The Journal of biological chemistry* **269**, 15195-15203 (1994).
54 Munoz-Planillo, R. *et al.* K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* **38**, 1142-1153, doi:10.1016/j.immuni.2013.05.016 (2013).
55 Hornung, V. *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature immunology* **9**, 847-856, doi:10.1038/ni.1631 (2008).

56 Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225, doi:10.1038/nature09663 (2011).
57 Tassi, S. *et al.* Pathogen-induced interleukin-1beta processing and secretion is regulated by a biphasic redox response. *J Immunol* **183**, 1456-1462, doi:10.4049/jimmunol.0900578 (2009).
58 Carta, S. *et al.* The rate of interleukin-1beta secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. *The Journal of biological chemistry* **286**, 27069-27080, doi:10.1074/jbc.M110.203398 (2011).
59 Gross, O. *et al.* Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* **36**, 388-400, doi:10.1016/j.immuni.2012.01.018 (2012).
60 Vigano, E. & Mortellaro, A. Caspase-11: the driving factor for noncanonical inflammasomes. *European journal of immunology* **43**, 2240-2245, doi:10.1002/eji.201343800 (2013).
61 Wang, S. *et al.* Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *The Journal of biological chemistry* **271**, 20580-20587 (1996).
62 Kayagaki, N. *et al.* Non-canonical inflammasome activation targets caspase-11. *Nature* **479**, 117-121, doi:10.1038/nature10558 (2011).
63 Broz, P. *et al.* Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature* **490**, 288-291, doi:10.1038/nature11419 (2012).
64 Rathinam, V. A. *et al.* TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* **150**, 606-619, doi:10.1016/j.cell.2012.07.007 (2012).
65 Case, C. L. *et al.* Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 1851-1856, doi:10.1073/pnas.1211521110 (2013).
66 Wang, S. *et al.* Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* **92**, 501-509 (1998).
67 Gurung, P. *et al.* Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4

(TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. *The Journal of biological chemistry* **287**, 34474-34483, doi:10.1074/jbc.M112.401406 (2012).
68 Broz, P. & Monack, D. M. Noncanonical inflammasomes: caspase-11 activation and effector mechanisms. *PLoS pathogens* **9**, e1003144, doi:10.1371/journal.ppat.1003144 (2013).
69 Kayagaki, N. *et al.* Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246-1249, doi:10.1126/science.1240248 (2013).
70 Hagar, J. A., Powell, D. A., Aachoui, Y., Ernst, R. K. & Miao, E. A. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* **341**, 1250-1253, doi:10.1126/science.1240988 (2013).
71 Rathinam, V. A. & Fitzgerald, K. A. Immunology: Lipopolysaccharide sensing on the inside. *Nature* **501**, 173-175, doi:10.1038/nature12556 (2013).
72 Meunier, E. *et al.* Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* **509**, 366-370, doi:10.1038/nature13157 (2014).
73 Martinon, F. & Tschopp, J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117**, 561-574, doi:10.1016/j.cell.2004.05.004 (2004).
74 Akhter, A. *et al.* Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization. *Immunity* **37**, 35-47, doi:10.1016/j.immuni.2012.05.001 (2012).
75 McIlwain, D. R., Berger, T. & Mak, T. W. Caspase functions in cell death and disease. *Cold Spring Harbor perspectives in biology* **5**, a008656, doi:10.1101/cshperspect.a008656 (2013).
76 Kang, S. J. *et al.* Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *The Journal of cell biology* **149**, 613-622 (2000).
77 Kang, S. J., Wang, S., Kuida, K. & Yuan, J. Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. *Cell death and differentiation* **9**, 1115-1125, doi:10.1038/sj.cdd.4401087 (2002).
78 Aachoui, Y., Sagulenko, V., Miao, E. A. & Stacey, K. J. Inflammasome-mediated pyroptotic and apoptotic cell death, and

defense against infection. *Current opinion in microbiology* **16**, 319-326, doi:10.1016/j.mib.2013.04.004 (2013).
79 Munday, N. A. *et al.* Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. *The Journal of biological chemistry* **270**, 15870-15876 (1995).
80 Faucheu, C., Blanchet, A. M., Collard-Dutilleul, V., Lalanne, J. L. & Diu-Hercend, A. Identification of a cysteine protease closely related to interleukin-1 beta-converting enzyme. *European journal of biochemistry / FEBS* **236**, 207-213 (1996).
81 Martinon, F. & Tschopp, J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell death and differentiation* **14**, 10-22, doi:10.1038/sj.cdd.4402038 (2007).
82 Eckhart, L. *et al.* Identification of a novel exon encoding the amino-terminus of the predominant caspase-5 variants. *Biochemical and biophysical research communications* **348**, 682-688, doi:10.1016/j.bbrc.2006.07.104 (2006).
83 Lin, X. Y., Choi, M. S. & Porter, A. G. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and interferon-gamma. *The Journal of biological chemistry* **275**, 39920-39926, doi:10.1074/jbc.M007255200 (2000).
84 Salskov-Iversen, M. L., Johansen, C., Kragballe, K. & Iversen, L. Caspase-5 expression is upregulated in lesional psoriatic skin. *The Journal of investigative dermatology* **131**, 670-676, doi:10.1038/jid.2010.370 (2011).
85 Qin, M. *et al.* Propionibacterium acnes Induces IL-1beta secretion via the NLRP3 inflammasome in human monocytes. *The Journal of investigative dermatology* **134**, 381-388, doi:10.1038/jid.2013.309 (2014).
86 Li, W., Katz, B. P., Bauer, M. E. & Spinola, S. M. Haemophilus ducreyi infection induces activation of the NLRP3 inflammasome in nonpolarized but not in polarized human macrophages. *Infection and immunity* **81**, 2997-3008, doi:10.1128/IAI.00354-13 (2013).
87 Bian, Z. M. *et al.* Expression and functional roles of caspase-5 in inflammatory responses of human retinal pigment epithelial cells. *Investigative ophthalmology & visual science* **52**, 8646-8656, doi:10.1167/iovs.11-7570 (2011).

88 Wilson, K. P. *et al.* Structure and mechanism of interleukin-1 beta converting enzyme. *Nature* **370**, 270-275, doi:10.1038/370270a0 (1994).
89 Walker, N. P. *et al.* Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. *Cell* **78**, 343-352 (1994).
90 Dihlmann, S. *et al.* Increased expression and activation of Absent in Melanoma 2 inflammasome components in lymphocytic infiltrates of Abdominal Aortic Aneurysms. *Mol Med*, doi:10.2119/molmed.2013.00162 (2014).
91 Kamada, S., Funahashi, Y. & Tsujimoto, Y. Caspase-4 and caspase-5, members of the ICE/CED-3 family of cysteine proteases, are CrmA-inhibitable proteases. *Cell death and differentiation* **4**, 473-478, doi:10.1038/sj.cdd.4400268 (1997).
92 Zhu, Q. *et al.* The role of acid sphingomyelinase and caspase 5 in hypoxia-induced HuR cleavage and subsequent apoptosis in hepatocytes. *Biochimica et biophysica acta* **1821**, 1453-1461, doi:10.1016/j.bbalip.2012.08.005 (2012).
93 Fogg, D. K. *et al.* A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* **311**, 83-87, doi:10.1126/science.1117729 (2006).
94 Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *The Journal of clinical investigation* **117**, 195-205, doi:10.1172/JCI29950 (2007).
95 Serbina, N. V., Jia, T., Hohl, T. M. & Pamer, E. G. Monocyte-mediated defense against microbial pathogens. *Annual review of immunology* **26**, 421-452, doi:10.1146/annurev.immunol.26.021607.090326 (2008).
96 Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M. & Muller, W. A. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* **11**, 753-761 (1999).
97 Krutzik, S. R. *et al.* TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. *Nature medicine* **11**, 653-660, doi:10.1038/nm1246 (2005).
98 Devitt, A. *et al.* Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* **392**, 505-509, doi:10.1038/33169 (1998).

99 Grunwald, U. *et al.* Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. *J Immunol* **157**, 4119-4125 (1996).
100 Auffray, C., Sieweke, M. H. & Geissmann, F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annual review of immunology* **27**, 669-692, doi:10.1146/annurev.immunol.021908.132557 (2009).
101 Yang, J., Zhang, L., Yu, C., Yang, X. F. & Wang, H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker research* **2**, 1, doi:10.1186/2050-7771-2-1 (2014).
102 Schinkel, C., Sendtner, R., Zimmer, S. & Faist, E. Functional analysis of monocyte subsets in surgical sepsis. *The Journal of trauma* **44**, 743-748; discussion 748-749 (1998).
103 Ohura, K., Katona, I. M., Wahl, L. M., Chenoweth, D. E. & Wahl, S. M. Co-expression of chemotactic ligand receptors on human peripheral blood monocytes. *J Immunol* **138**, 2633-2639 (1987).
104 Grage-Griebenow, E. *et al.* Human MO subsets as defined by expression of CD64 and CD16 differ in phagocytic activity and generation of oxygen intermediates. *Immunobiology* **202**, 42-50, doi:10.1016/S0171-2985(00)80051-0 (2000).
105 Belge, K. U. *et al.* The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol* **168**, 3536-3542 (2002).
106 Skrzeczynska-Moncznik, J. *et al.* Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scandinavian journal of immunology* **67**, 152-159, doi:10.1111/j.1365-3083.2007.02051.x (2008).
107 Netea, M. G. *et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113**, 2324-2335, doi:10.1182/blood-2008-03-146720 (2009).
108 Latz, E., Xiao, T. S. & Stutz, A. Activation and regulation of the inflammasomes. *Nature reviews. Immunology* **13**, 397-411, doi:10.1038/nri3452 (2013).
109 Rossol, M. *et al.* Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature communications* **3**, 1329, doi:10.1038/ncomms2339 (2012).

110 Vincent, J. L., Opal, S. M., Marshall, J. C. & Tracey, K. J. Sepsis definitions: time for change. *Lancet* **381**, 774-775, doi:10.1016/S0140-6736(12)61815-7 (2013).
111 Hotchkiss, R. S., Monneret, G. & Payen, D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nature reviews. Immunology* **13**, 862-874, doi:10.1038/nri3552 (2013).

Chapter 2.

Caspase-5 licenses inflammasome activation via Syk/PLCγ pathway

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Submitted

Rationale and Biological question

The NLRP3 inflammasome is a multiprotein complex regulating the secretion of IL-1β and IL-18 in response to a variety of stimuli, including pathogen- and danger-associated molecular patterns (PAMPs and DAMPs)[1]. Upon activation, the apoptosis associated speck-like protein (ASC) adaptor recruits caspase-1 to the NLRP3 inflammasome complex, where it first undergoes auto-cleavage and then processes the inactive precursors of IL-1β and IL-18. Importantly, NLRP3 is not responsive to stimulation unless primed by an NF-κB-triggering signal, such as LPS[2]. Recent studies have identified an alternative mechanism leading to caspase-1 activation in mouse. This "non-canonical" pathway requires the inflammatory caspase-11 participating to the regulation of IL-1α, in addition to IL-1β and IL-18[3,4]. It was also reported that Gram-negative bacteria, but not Gram-positive bacteria, or intracellular LPS are the main triggers of the non-canonical pathway[5,6]. *In vivo* evidences indicate that, the non-canonical pathway seems detrimental in murine models of sepsis[5-7]. However, the existence and the relevance of the non-canonical inflammasome in humans have not been demonstrated yet.

Thus, we investigated whether the inflammatory caspase-4 and caspase-5 - human homologs of murine caspase-11- are induced in human monocytes after TLR4 stimulation by LPS. We found

that caspase-5, but not caspase-4, is activated in LPS-treated monocytes. Importantly, the release of IL-1β and IL-1α depends on caspase-5 through a mechanism that requires SYK, PLCγ and calcium (Ca^{2+}) flux.

Results

LPS activates NLRP3 inflammasome in human monocytes in the absence of a second signal

Human monocytes display an unusually high sensitivity to LPS[8]; in fact, they rapidly released large amounts of inflammatory cytokines, including IL-1α, IL-1β and IL-6 (Fig. 1a) in a time- (Fig. 1b) and dose-dependent manner (Fig. 1c). This result indicates that in monocytes LPS alone serves as both priming (signal 1) and activating signal (signal 2) for the NLRP3 inflammasome activation. Indeed, LPS alone induced the expression of pro-IL-1β and NLRP3 (priming signal) at both mRNA (Fig. 2a) and protein level (Fig. 2b, c), as well as the processing of caspase-1 and pro-IL-1β (activating signal) (Fig. 2b, d) in human monocytes.

Therefore, the contribution of NLRP3 inflammasome to LPS-induced cytokine release by human monocytes was assessed. Silencing of NLRP3 expression using a specific siRNA (Fig. 3a) significantly abolished LPS-mediated caspase-1 processing (Fig. 3b), as well as IL-1β and IL-1α release (Fig. 3c). Consistently, the release of IL-6, a NLRP3 independent cytokine, remained unchanged (Fig. 3c). This mode of NLRP3 activation in monocytes differs significantly from the one in dendritic cells

(DCs). Although LPS alone induced pro-IL-1β expression (Fig 4a) a classical NLRP3 activator, such as ATP, is required to induce NLRP3 activation and IL-1β release in DCs (Fig 4b). ATP stimulation is required to activate caspase-1 (Fig 4a) and the subsequent release of IL-1β (Fig. 4b) in DCs, whereas LPS alone induced only expression of pro-IL-1β (Fig. 4a) and secretion of IL-6 (Fig. 4b). These data indicate that LPS is a genuine trigger of NLRP3 inflammasome activation in monocytes and suggest the presence of a unique NLRP3-mediated pathway in these cells that differs from *in vitro* differentiated DCs.

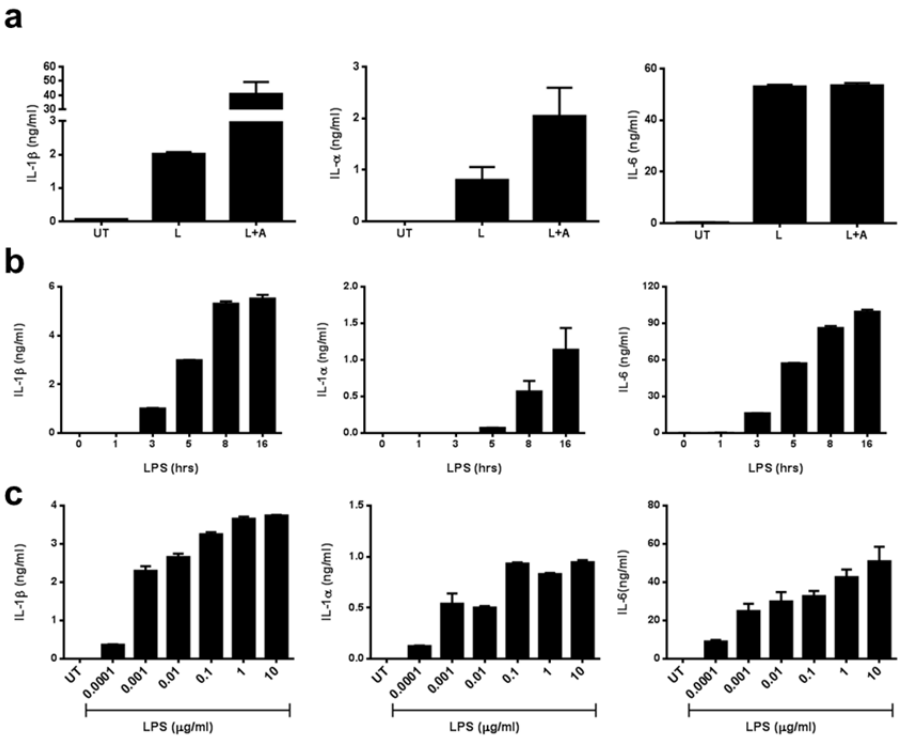


Fig. 1| Cytokine release by human monocytes in response to LPS

a, IL-1α, IL-1β and IL-6 secretion from monocytes cultured in medium alone (UT), LPS alone (L) or in combination with ATP (L+A). **b-c,** release of IL-1β, IL-1α and IL-6 was tested in supernatants obtained from monocytes stimulated with LPS (10 ng/ml) at different time points (**b**) or with different doses of LPS for 5 hours (**c**). Graphs show the mean ± standard deviation of triplicate wells representative of three independent experiments.

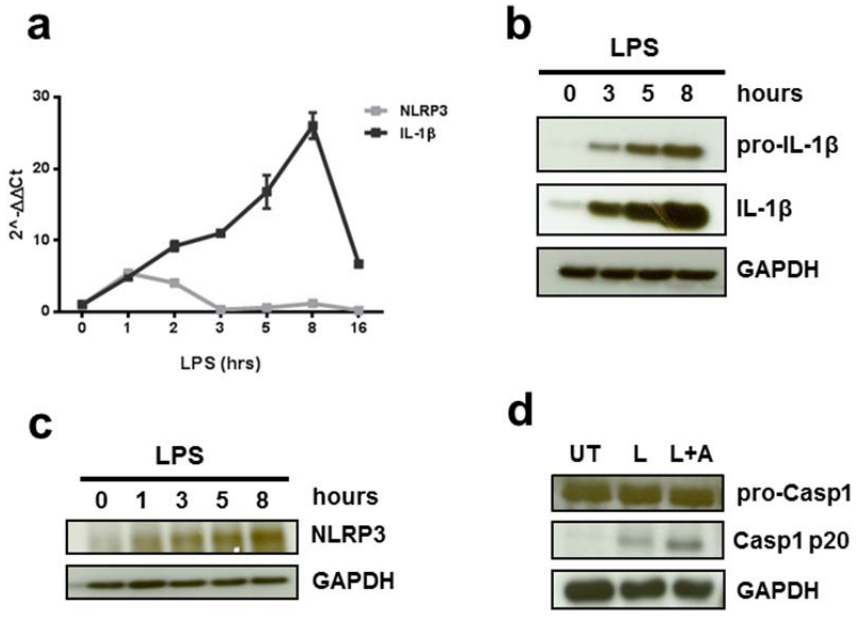


Fig. 2| LPS acts as an inflammasome priming and activator signal in human monocytes

a-c, Expression of NLRP3 and IL-1β were assessed by Real-Time PCR (**a**) and western blot (**b, c**) in monocytes treated with LPS for the indicated time points. **d**, Immunoblot for procaspase-1, caspase-1 p20 and housekeeping gene (GAPDH) in cellular lysates of monocytes cultured in medium alone (UT), LPS alone (L) or in combination with ATP (L+A). Graphs shows the mean ± standard deviation of triplicate wells and data are representative of three independent experiments.

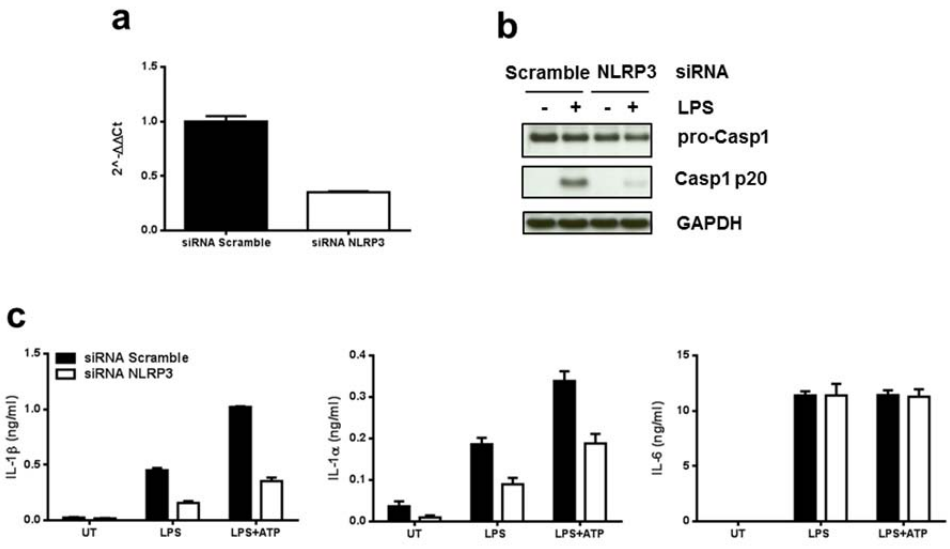


Fig. 3| NLRP3 silencing

a, Efficacy of silencing was measured by Real-Time PCR. **b-c**, caspase-1 cleavage (**b**) and IL-1β, IL-1α and IL-6 secretion by monocytes in response to LPS and LPS/ATP stimulation (**c**) were detected by western blot and ELISA, respectively. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

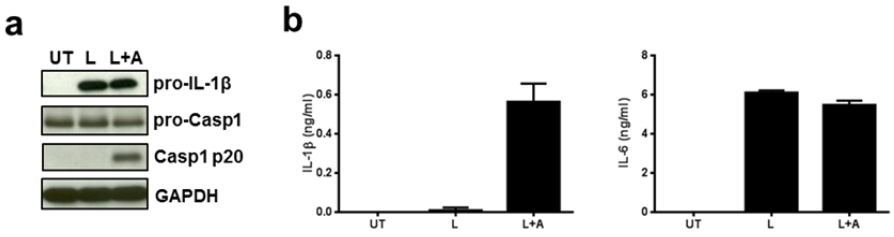


Fig. 4| Human monocytes-derived DCs requires a second signal to activate inflammasome

a, Immunoblot analysis of pro-IL-1β, procaspase-1, caspase-1 p20 and GAPDH loading control in cell lysates. **b**, IL-1β and IL-6 secretion was assessed in DCs cultured in medium alone (UT), LPS alone (L, 1 μg/ml) or in combination with 1 mM of ATP (L+A). Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

LPS induces activation of caspase-5 in human monocytes

Bearing in mind that LPS alone (although delivered intracellularly) can induce the non-canonical inflammasome pathway in murine macrophages[5,6], we hypothesized that other inflammatory caspases, such as human caspase-4 and/or caspase-5 (orthologues of murine caspase-11[9], may be involved in the unique responsiveness to LPS of human monocytes. To test this hypothesis, we firstly evaluated caspase-4 and caspase-5 expression in monocytes, either at the steady state or upon LPS stimulation. LPS boosted transcription of both caspases (Fig. 5a). We also evaluated whether mRNA boost observed resulted in higher procaspase-4 and procaspase-5 protein levels. Western blot analysis revealed that both caspase-4 and caspase-5 precursors were already abundant in unstimulated monocytes, indicating constitutive protein expression and that LPS stimulation did not further boost protein levels (Fig. 5b-d). Importantly, we found that LPS induced the processing of procaspase-5, but not that of procaspase-4 (Fig. 5b-d), as reveled by the appearance of the cleaved caspase-5 fragment. Activated caspase-5 increased in a time- (Fig. 5c) and dose-dependent manner (Fig. 5d). Furthermore, the NLRP3 activator ATP did not increase caspase-5 cleavage significantly compared to LPS alone (Fig. 6a). Differently from monocytes, caspase-5 and caspase-4 cleavage

was not induced in DCs stimulated with LPS alone or in combination with ATP (Fig. 6a). These results suggest that caspase-5 was activated independently of the known canonical inflammasome pathways. As caspase-4 processing is not induced by LPS in monocytes, we mainly focused our attention primarily on mechanism(s) of caspase-5 activation.

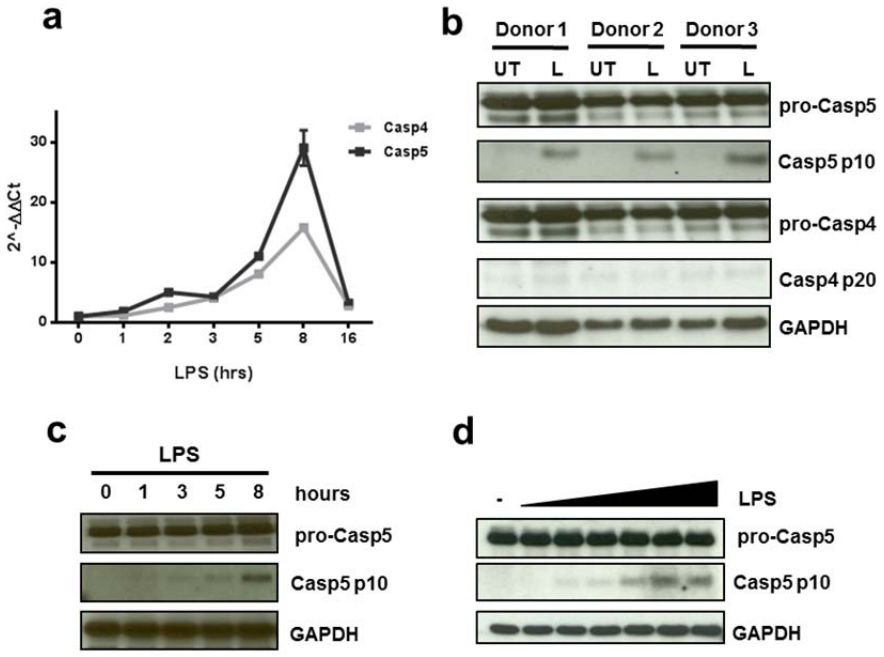


Fig. 5| Caspase-4 and caspase-5 expression and activation in human monocytes

a, Caspase-4 and caspase-5 mRNA expression was assessed in monocytes stimulated with LPS for the indicated time points via Real-Time PCR. **b**, Immunoblot analysis of caspase-4 and caspase-5 in cell lysates from monocytes unstimulated (UT) or stimulated with LPS (L, 10 ng/ml) for 5 hours was assessed. (**c, d**) Level of procaspase-5 and cleaved caspase-5 in monocytes stimulated with LPS for the indicated time points (**c**) or with increased doses of LPS (0.0001, 0.001, 0.01, 0.1, 1, 10, 100 µg/ml) for 5 hours was assessed (**d**).

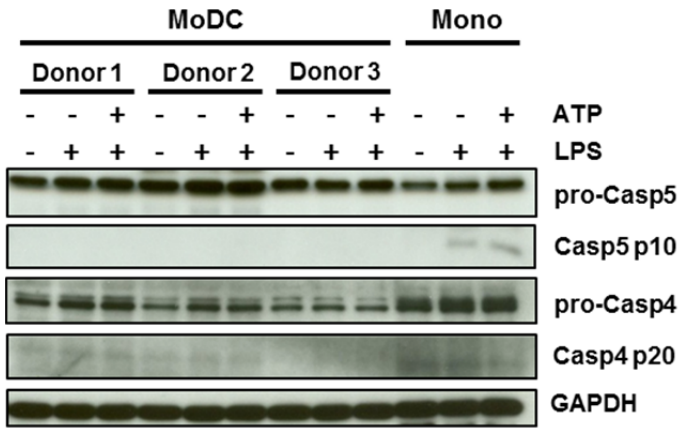


Fig.6| Caspase 5 is specifically cleaved in human monocytes upon LPS stimulation

Immunoblot analysis of caspase-4 and caspase-5 in cell lysates from unstimulated (UT) DCs or after 5 hours stimulation with LPS (L,1 µg/ml) alone or in combination with ATP (1 mM).

Human caspase-5 triggers IL-1α and IL-1β release

Next, we assessed whether caspase-5 plays any role in NLRP3-dependent IL-1α and IL-1β release. To determine whether caspase-5 activity is required for IL-1α/β secretion from monocytes, cells were treated with the caspase-5 inhibitor LEVD for 1 hour prior to LPS stimulation. Suppression of caspase-5 activity significantly inhibited IL-1β secretion from monocytes stimulated with LPS for 5 hours, suggesting that caspase-5 activity is important for IL-1β secretion under these conditions (Fig. 7a). Suppression of release of active IL-1β is similarly achieved when monocytes were pre-treated with the caspase-1 inhibitor ZYVAD (Fig. 7a). Importantly, IL-1α release was not reduced when monocytes were pre-treated with caspase-1 inhibitor (Fig. 7a). Altogether these data indicate that caspase-5, but not caspase-1, is required for the release of IL-1α.

To test whether caspase-5 is required for the proteolytic processing of IL-1α and IL-1β, HEK293t cells were co-transfected with plasmids encoding caspase-5 or caspase-1 with IL-1α and IL-1β in different combinations. IL-1α and IL-1β cleavage was assessed by Western blot. Caspase-1 expression caused processing of IL-1β and IL-1α precursors, whereas caspase-5 did not, indicating that IL-1β and IL-1α are not direct substrates of caspase-5 (Fig. 7b).

To test whether caspase-5 can directly process caspase-1, full-length catalytic mutant (C285A) caspase-1 was incubated with recombinant human caspase-5. The appearance of the active caspase-1 fragment p18/p22 was detected when pro-caspase-1 was incubated with caspase-5. Caspase-1 processing was reduced in presence of caspase-5 inhibitor LEVD (Fig. 7c).

Caspase-5 mediated processing of caspase-1 was further investigated in monocyte pre-treated with caspase-5 inhibitor before LPS stimulation. We found that suppression of caspase-5 activity reduced caspase-1 processing induced by LPS (Fig. 7d, e). Collectively, these data indicate that caspase-5 regulates IL-1β secretion by triggering procaspase-1 processing and, in addition, promotes the release of IL-1α via an unknown mechanism.

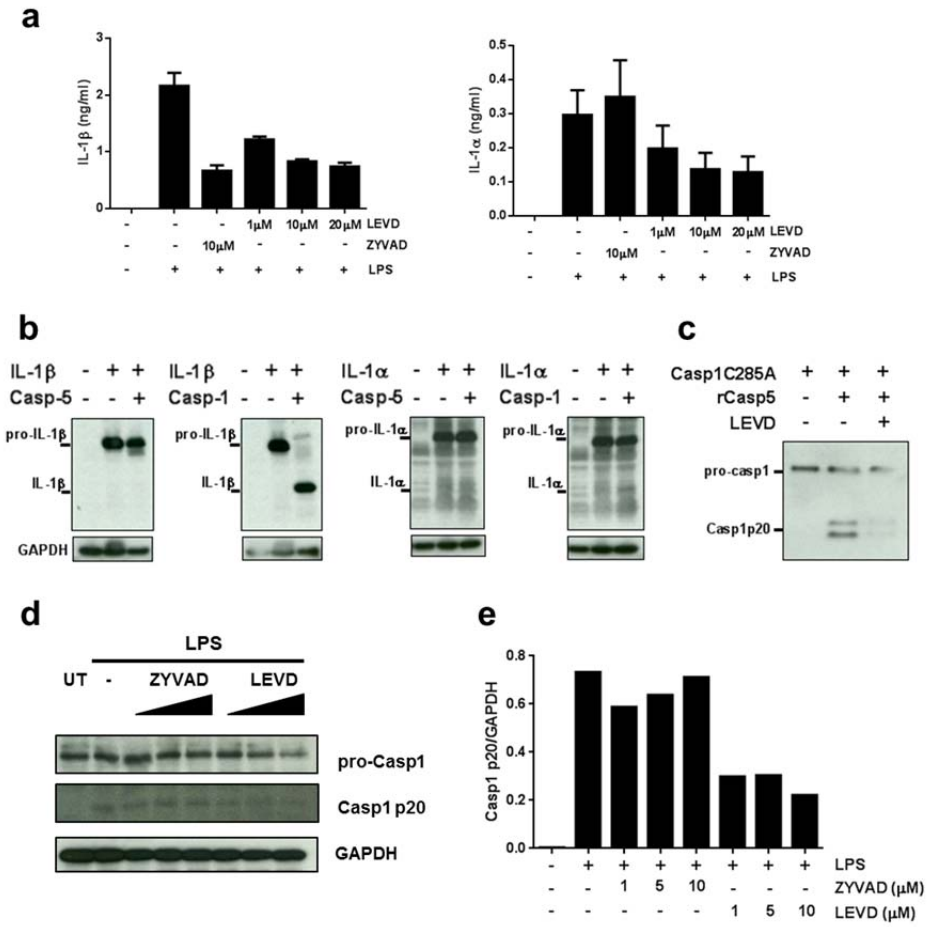


Fig. 7| Caspase-5 induces IL-1β and IL-1α release via a caspase-1-dependent and independent mechanism, respectively.

a, IL-1β and IL-1α secretion by pre-treated with ZYVAD or LEVD and stimulated with LPS (10 ng/ml) for 5 hours was assessed by ELISA. **b,** Expression of IL-1β and IL-1α were analyzed in cell

lysates obtained from HEK293t transfected with pCMV6-pro-IL-1α, pcDNA3-pro-IL-1β, pcDNA3-procaspase-5 and pCI-procaspase-1 as indicated. **c**, purified full-length caspase-1 catalytic mutant (C285A) and recombinant caspase-5 (rCasp5) were incubated for 20 minutes at 37ºC in absence or presence of LEVD (50μM) and caspase-1 cleavage was assessed by immunoblot.

d-e, levels of procaspase-1 and caspase-1 p20 in cell lysates of monocytes pre-treated with ZYVAD and LEVD (1, 5, 10 μM) prior LPS stimulation were assessed by WB (**d**) and densitometry analysis were performed using ImageJ (**e**). Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

Caspase-5 expression and activation is independent of IFN pathway

Murine caspase-11 is not constitutively expressed at steady state in murine macrophages, but it is rapidly induced by type I IFNs[7,10,11]. Since human caspase-5 is homologues of murine caspase-11 and the ability of human monocytes to secrete high levels of IFN-β upon LPS stimulation (Fig. 8a), we investigated whether autocrine IFN-β signalling may regulate caspase-5 activation. High doses of recombinant IFN-β alone or in combination with LPS induced the expression of interferon-stimulated genes (ISGs) (Fig. 8b, c), but did not increase either the expression (Fig. 8b, c) or the processing of caspase-5 (Fig. 8d, e). Moreover, the release of IL-1β and IL-1α was similarly unaffected mirroring caspase-5 activation (Fig. 8f). Therefore, we concluded that IFN-β does not regulate caspase-5 activation and does not further boost caspase-5 activation when incubated together with LPS.

Furthermore, loss-of-function experiments confirmed the data obtained from gain-of-function experiments, in which the neutralization of the type I interferon receptor (IFNAR) effectively impacted ISG expression (Fig. 9a), but neither caspase-5 processing (Fig. 9b) nor IL-1β and IL-1α release (Fig. 9c) were affected. Collectively, our data indicate that procaspase-5 is

constitutively expressed in human monocytes, and neither LPS nor IFN-β boost caspase-5 protein expression.

In contrast to murine caspase-11 regulation, LPS, but not type I IFNs, is required for caspase-5 processing, highlighting a significant divergence between the regulation of the human and murine orthologues caspases.

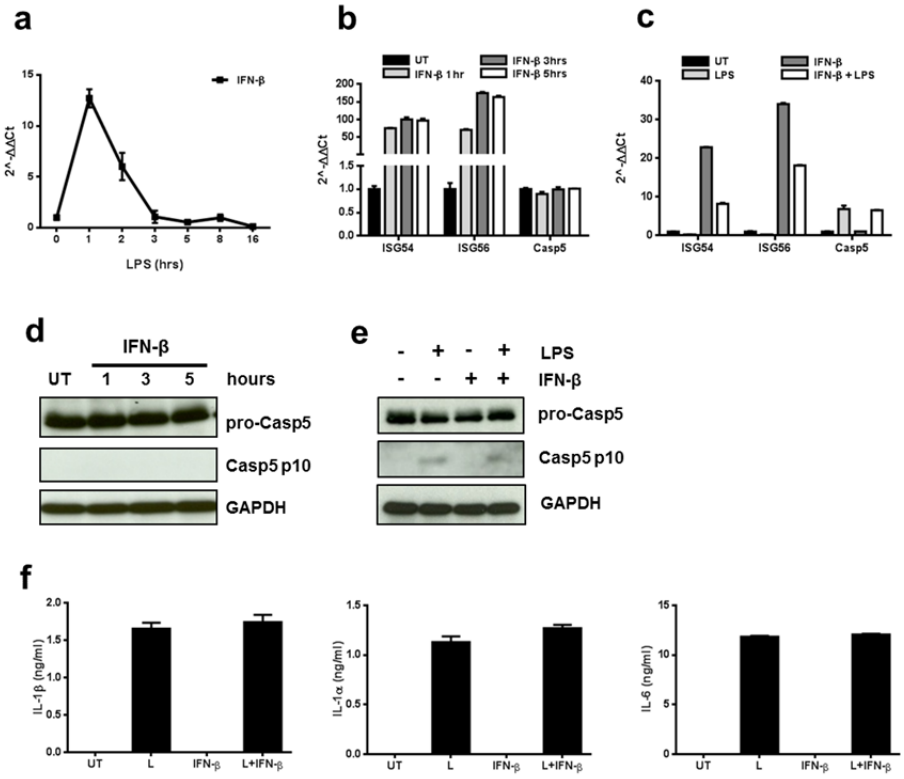


Fig. 8| LPS-dependent caspase-5 activation is independent of IFN-β

a-c, mRNA expression analysis. **a**, IFN-β expression in monocytes stimulated with LPS for the indicated time points. **b-c**, ISG54, ISG56 and caspase-5 expression was assessed in monocytes treated with IFN-β alone for 1, 3 and 5 hours (**b**) or in combination with LPS after 5 hours of culture (**c**). **d**, Procaspase-5 and caspase-5 p10 were immunoblotted in cell extracts from monocytes unstimulated (UT) or treated with IFN-β for the

indicated time points. **e-f**, WB analysis of procaspase-5 and caspase-5 p10 levels (**e**) and ELISA analysis for secreted IL-1β, IL-1α and IL-6 (**f**) in monocytes treated with LPS (L) and/or IFN-β Ia. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

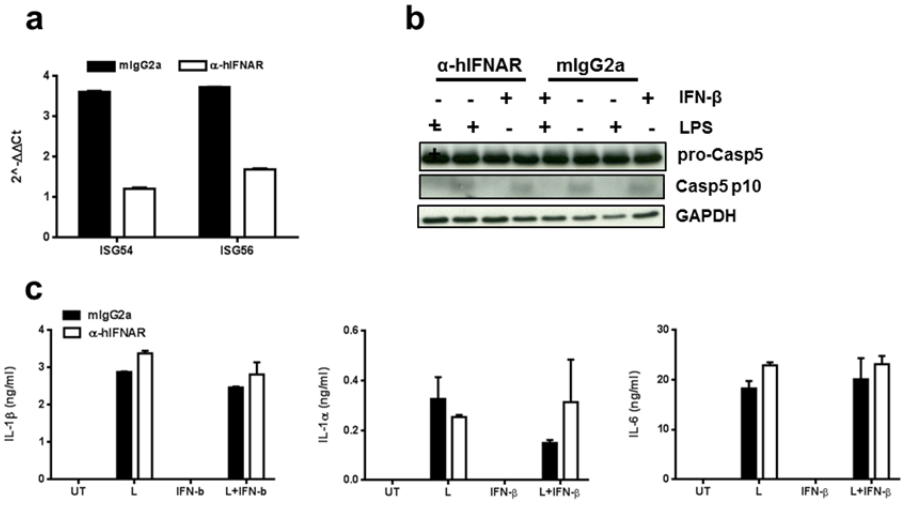


Fig. 9| LPS-dependent caspase-5 activation is independent of Type I IFN receptor

a, ISG54 and ISG56 expression of monocytes pre-treated with anti-human IFNAR (α-hIFNAR) or its isotype control (mIgG2a) and, subsequently stimulated with IFN-β. **b-c**, WB analysis of procaspase-5 and caspase-5 p10 (**b**) and ELISA analysis for secreted IL-1β, IL-1α and IL-6 (**c**) in monocytes treated with LPS (L) and/or IFN-β. The cells were pre-treated for 1 hour with neutralizing α-hIFNAR antibody or isotype control (mIgG2a). Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

Syk regulates caspase-5 activation and IL-1α/β release

Based on low similarities between human caspase-5 and murine caspase-11, we decided to explore molecular mechanisms underlying caspase-5 activation. It was recently demonstrated that in murine DCs LPS engagement of CD14 triggers Syk, which in turn causes endocytosis of CD14/TLR4 complex[12]. Moreover, intracellular detection of LPS triggers caspase-11[5,6]. Therefore, we hypothesized that Syk-mediated endocytosis may lead to caspase-5 activation in human monocytes. Western blot analysis showed that Syk is rapidly phosphorylated in monocytes upon LPS stimulation (Fig. 10a), and pharmacological inhibition of Syk suppressed LPS-induced endocytosis of the CD14/TLR4 complex (Fig. 10b, c). Consistent with our hypothesis, caspase-5 cleavage (Fig. 11a) was reduced by Syk inhibition. Indeed, the secretion of IL-1β and IL-1α, but not the one of IL-6, (Fig. 11b) was also affected by Syk inhibition. Similarly, blocking the endocytic pathway, using dynasore (dynamin inhibitor), suppressed CD14 re-localization to the early endosome (Fig. 12a, b) preventing both caspase-5 activation (Fig 12c) and IL-1β and IL-1α release (Fig. 12b). These findings prove that Syk-mediated LPS endocytosis is essential to promote caspase-5-dependent inflammasome activation.

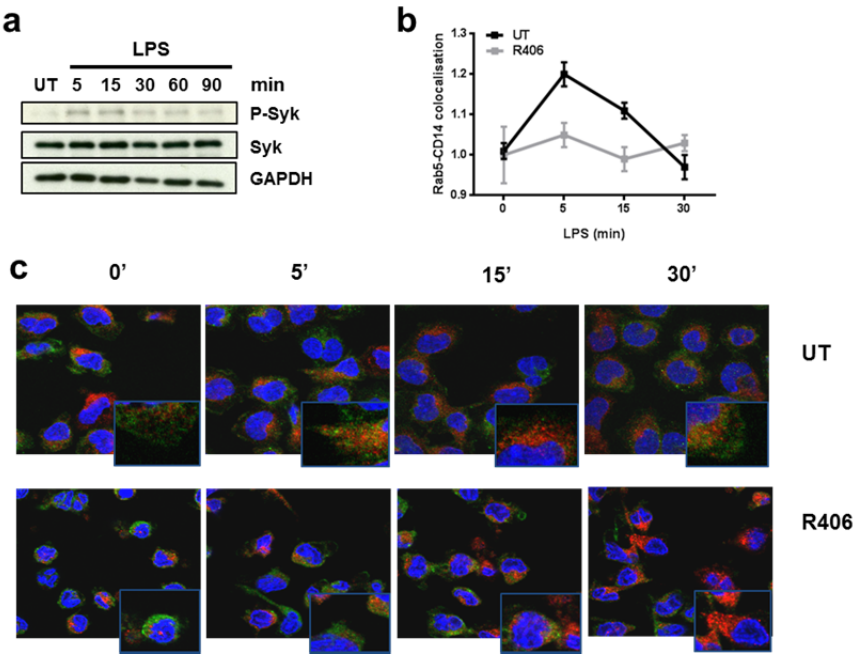


Fig. 10| LPS-mediated Syk-dependent CD14 internalization

a, WB analysis of extracts from monocytes untreated (UT) and treated with LPS for the indicated time points was performed using anti-phospho-Syk (Tyr 525-526) and total Syk antibodies. **b-c**, Monocytes were pre-treated with R406 then stimulated with LPS for indicated period of time: LPS-dependent endocytosis of CD14-TLR4 is illustrated by confocal imaging of CD14/Rab5 expression (**c**) and co-localization of CD14 and Rab5 (**b**).

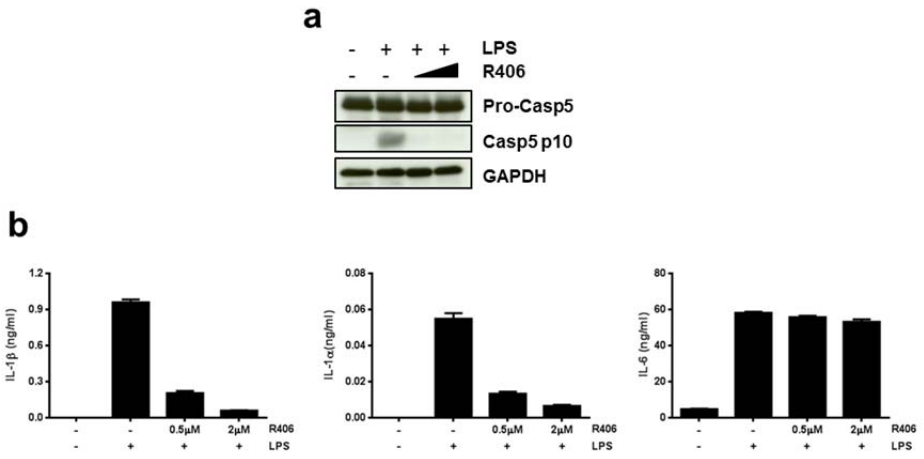


Fig. 11| Syk-mediated caspase-5 activation

a-b, WB analysis for procaspase-5, caspase-5 p10 (**a**) and cytokines secretion (**b**) were assessed in monocytes pre-treated with R406 (0.5 μM and 2 μM, 1 hour) and subsequently stimulated with LPS (10 ng/ml, 5 hours). Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

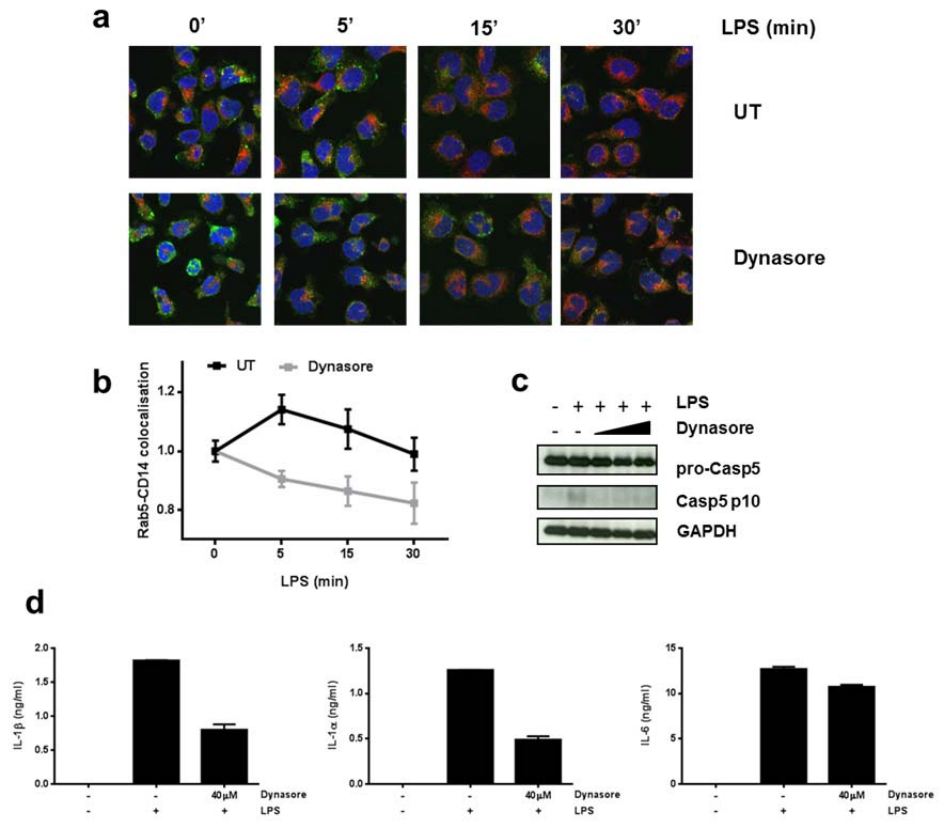


Fig. 12| Endocytosis-mediated caspase-5 activation

a, Monocytes pre-treated with dynasore and then stimulated with LPS for indicated period of time. LPS-dependent endocytosis of CD14-TLR4 was visualized by confocal microscopy for CD14/Rab5 colocalization. **b,** CD14/Rab5 colocalization was evaluated using Pearson's correlation coefficients calculated by Imaris software. **c,** levels of procaspase-5 and caspase-5 p10 were assessed in lysates collected from monocytes pre-treated

with dynasore (20, 40, 80 μM) and stimulated with LPS (10 ng/ml) for 5 hours. **d**, IL-1β, IL-1α and IL-6 were tested in supernatants collected from monocytes pre-treated for 1 hour with dynasore and stimulated with LPS for 5 hours. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

Caspase-5 is activated by Syk-mediated intracellular Ca^{2+} flux

Tyrosine kinase Syk operates downstream of lectin receptors, where it promotes pro-IL-1β synthesis and production of ROS, which in turn leads to NLRP3 inflammasome activation and release of mature IL-1β[13]. We asked whether Syk activation leads to ROS production in monocytes and whether Syk-dependent ROS may have any role in LPS-driven caspase-5 activation. Monocytes exposed to LPS generated ROS in a time-dependent manner and inhibition of Syk activity suppressed this ROS production (Fig. 13a). As expected, DPI-mediated inhibition of ROS-generating NADPH oxidase suppressed IL-1β release (Fig. 13b), but, caspase-5 activation (Fig 13c) and IL-1α secretion (Fig 13b) were unaffected by this treatment. Therefore, both caspase-5 activation and IL-1α release are independent of ROS production in monocytes.

This distinction between caspase-5/IL-1α axis and IL-1β is a reminiscent of similar observations reported in the mouse, whereby IL-1β release may or may not depend on caspase-11 on the base of experimental conditions and stimulation[4]. Conversely, IL-1α secretion has more specifically been linked to caspase-11 activation[3].

Syk is also essential for PLCγ activation and Ca^{2+} mobilization[14,15]. Therefore, we investigated whether Syk-dependent Ca^{2+} plays a role in LPS-driven caspase-5 activation. The exposure of monocytes to LPS generated Ca^{2+} influx, which was suppressed when either Syk (R406) or PLCγ (U73122) inhibitors were employed (Fig. 14). Furthermore, Ca^{2+} mobilization were impaired when monocytes were pre-treated with a cell permeable intracellular Ca^{2+} chelator (BAPTA/AM) or an extracellular Ca^{2+} chelator (EGTA) (Fig. 14).

To assess the role of LPS-induced Ca^{2+} as a possible second messenger for caspase-5 activation, monocytes were pre-treated with U73122, BAPTA/AM and EGTA followed by LPS stimulation, and caspase-5 cleavage and cytokine secretion were evaluated. When monocytes were pre-treated with U73122, BAPTA/AM or EGTA a clear reduction in LPS-induced caspase-5 activation was observed (Fig. 15a). Consistently, we found that both IL-1α and IL-1β release was dependent on PLCγ (Fig. 15b). However, the abrogation of Ca^{2+} mobilization reduced IL-1α, but not IL-1β, release (Fig. 15b) suggesting a specific role of Ca^{2+} for IL-1α secretion. Therefore, we hypothesized that this apparent inconsistency in cytokine release observed in monocytes pre-treated with PLCγ inhibitor or Ca^{2+} chelators may be due to a role of PLCγ in the generation of ROS. Indeed, PLCγ inhibition reduced ROS production induced by LPS (Fig. 16).

As previously reported, ROS production does not contribute to caspase-5 cleavage (Fig. 13c) and IL-1α release but effectively induces IL-1β secretion (Fig 13b). Here we showed that PLCγ induces IL-1β release through ROS production. Meanwhile, PLCγ regulates the secretion of IL-1α and the activation of caspase-5 via Ca^{2+} mobilization.

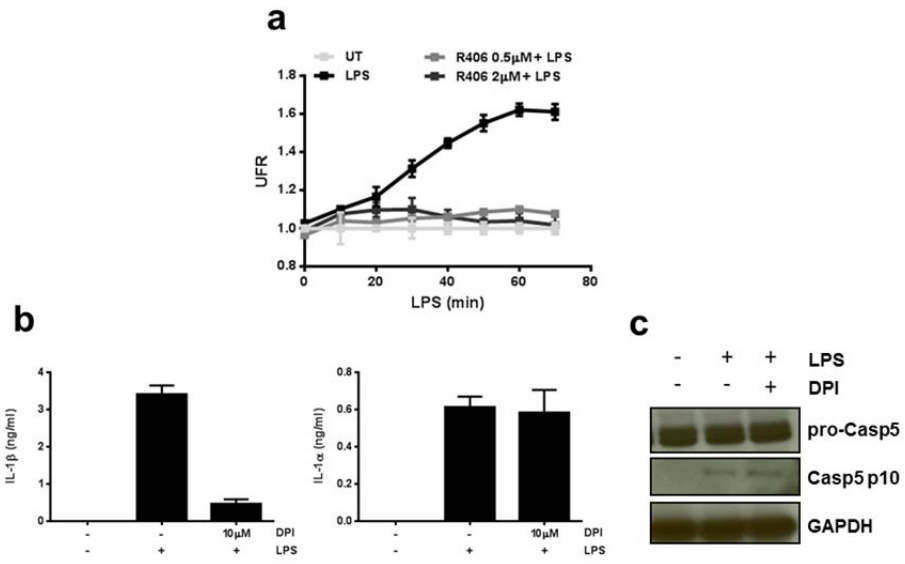


Fig. 13| ROS-independent caspase-5 activation

a, ROS measurement in monocytes pre-treated with R406 and stimulated with LPS. **b-c**, IL-1α and IL-1β release (**b**) and levels of procaspase-5 and cleaved caspase-5 (**c**) were determined in supernatants and cellular lysates, respectively, obtained from monocytes pre-treated with DPI and stimulated with LPS (10 ng/ml) for 5 hours. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

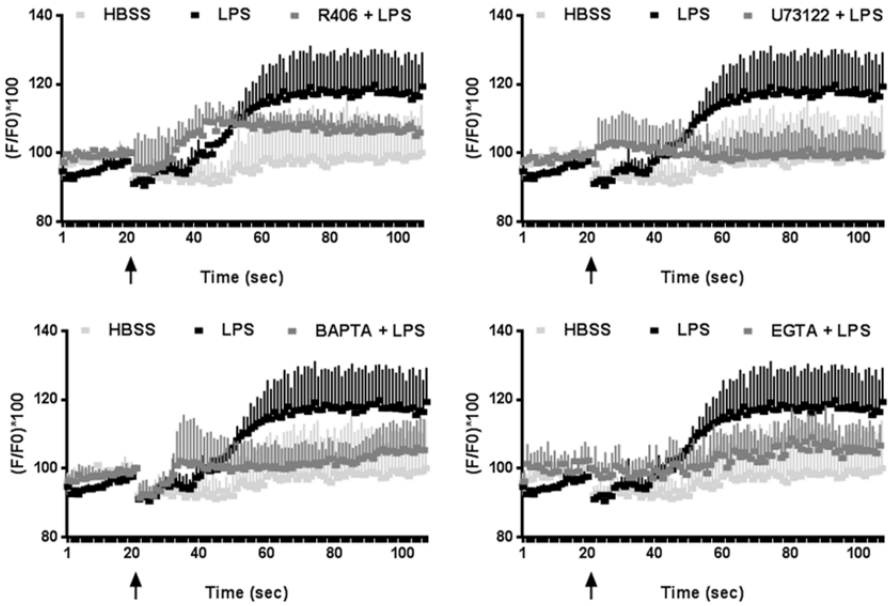


Fig. 14| LPS induces Ca^{2+} influx in human monocytes

Ca^{2+} mobilization from monocytes after LPS stimulation. Cells were pre-treated with R406 (0.5 μM), U73122 (1 μM), BAPTA (10 μM) and EGTA (2 mM) and subsequently stimulated with LPS (1 μg/ml). Arrows represent the moment when monocytes were stimulated with LPS. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

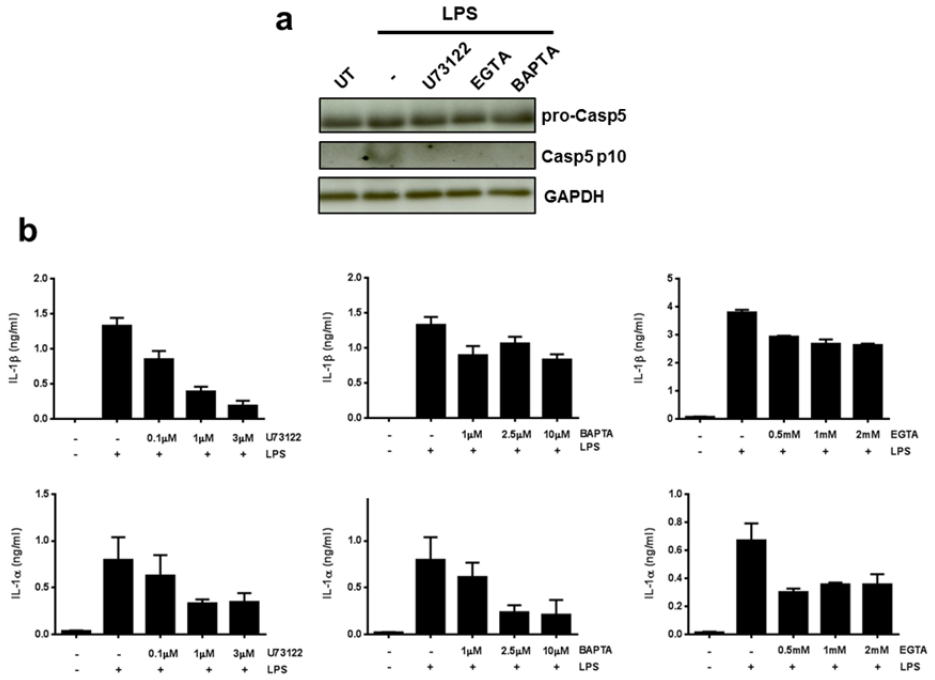


Fig. 15| Caspase 5 activation and IL-1α release is triggered by Ca^{2+} influx through Syk/PLCγ axis.

a, Caspase-5 activation was analyzed in monocytes pre-treated with U73122 (3 μM), EGTA (2 mM), BAPTA (10 μM) and subsequently stimulated with LPS (10 ng/ml) for 5 hours. **c,** IL-1α and IL-1β release from monocytes pre-treated with U73122, BAPTA and EGTA and then stimulated with LPS (10 ng/ml) for 5 hours. Graphs show the mean ± standard deviation of triplicate wells and are representative of three independent experiments.

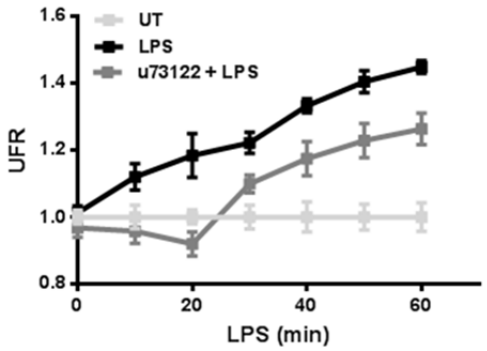


Fig. 16| PLCγ inhibitor interferes with ROS production.

ROS measurement induced by LPS (10 ng/ml) stimulation in monocytes pre-treated with U73122 (3 μM). Graph shows the mean ± standard deviation of triplicate wells and is representative of three independent experiments.

Discussion

In the current project we aimed to investigate the molecular mechanisms underlying LPS-mediated inflammasome activation in human monocytes.

Monocytes constitute a large fraction (~10%) of circulating PBMCs. Distinct from macrophages and DCs, which reside in peripheral tissues, they constitute a reservoir of circulating myeloid progenitors in the bloodstream. The majority of the literature on inflammasome has focused mainly on macrophages, and occasionally on DCs, while the contribution of monocytes has not been broadly investigated. The reason is mainly technical; inasmuch monocytes spontaneously differentiate towards the macrophage lineage upon culture *in vitro* making difficult to perform any experimental manipulations. Nonetheless, monocytes play a crucial role in inflammasome-mediated physiopathology. Indeed, in case of systemic infectious diseases, such as sepsis, circulated monocytes play a predominant role in recognizing the causative agent of infection as well as in the subsequent induction of inflammation and recruitment of adaptive immune cells.

Monocytes represent an exception to the two signals model of NLRP3 inflammasome activation, according to which priming induced by an NF-kB-activating agent (such as LPS) precedes activation by ATP, crystals, toxins or other NLRP3-specific stimuli (danger signals). Conversely, monocytes promptly release IL-1β

in response to LPS alone, and adding a second NLRP3-triggering stimulus enhances cytokine secretion.

Based on this peculiar mode of inflammasome activation in monocytes we decided to investigate deeply the mechanism(s) of activation of inflammasome in this cell type.

It has been previously proposed that caspase-1 is constitutive active in human monocytes[8] explaining why IL-1β is released by LPS without a proper NLRP3 activating stimulus. However, in our hands, caspase-1 is not cleaved in resting monocytes, as much as in DCs and macrophages.

Recently, an alternative mechanism of NLRP3 inflammasome activation driven by murine caspase-11 has been proposed[4-6]. Particularly, caspase-11 mediates the non-canonical activation of the NLRP3 inflammasome when LPS is present into the cytoplasm[5,6]. Therefore, we hypothesized that other inflammatory caspases, such as caspase-4 and caspase-5, may be the direct link between LPS pathway and NLRP3 inflammasome activation in monocytes.

The expression of murine procaspase-11 has been reported to be inducible in macrophages[10,16-19]. On the contrary, inflammatory caspases, caspase-4 and caspase-5 (human orthologues of murine caspase-11), are regulated differently from what have been described for caspase-11. Indeed, both procaspase-4 and procaspase-5 are constitutive expressed in human monocytes and DCs. Moreover, LPS induces the cleavage of caspase-5, but

not caspase-4, in monocytes. Importantly, caspase-5 activation by LPS occurs only in monocytes but not in DCs. Differently from experiments reported in murine macrophages, the cleavage of caspase-5 is observed by simply adding LPS to the cell culture medium, without forcing the delivery of LPS into the cytoplasm by using transfection reagents or cholera toxin B[3,5,6]. Furthermore, when caspase-5 activity is inhibited, the release of IL-1α and IL-1β induced by LPS is reduced accordingly. In line with these findings, caspase-5 inhibition reduces caspase-1 activation. Therefore, caspase-5, activated by LPS, contributes to caspase-1 activation observed in monocytes.

LPS is naturally endocytosed along with TLR4/CD14 upon receptor-ligand interaction[12], but this is not sufficient to initiate the non-canonical pathway in murine macrophages. Conversely, human monocytes stimulated with LPS activate NLRP3 inflammasome via the endocytosis pathway downstream TLR4. Indeed, inhibition of endocytosis pathway prevents both caspase-5 cleavage and IL-1α/β release.

Then, we characterized the molecular mechanism downstream LPS recognition leading to caspase-5 activation, identifying the Syk-PLCγ-Ca^{2+} axis as essential for the cleavage of caspase-5 and the release of IL-1α (Supplementary Figure 1).

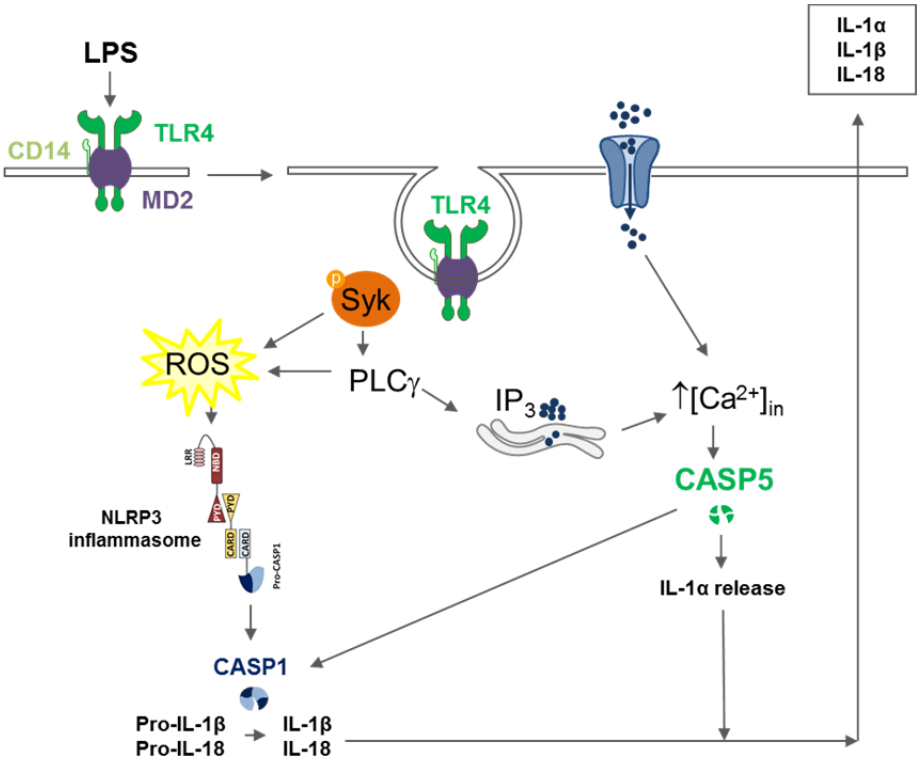
LPS induces a strong release of type I IFN mediated by the activation of TRIF pathway. Type I IFNs have been already indicated as essential factor for caspase-11 functionality, although

with some disagreement on their precise role[1,7,10,20]. However, IFN-β was surprisingly dispensable in monocytes. These findings further distinguish the pathway upstream the inflammasome activation present in monocytes compared to the non-canonical inflammasome activation already described in murine macrophages.

While IL-1β and IL-18 release are the most studied read-outs for inflammasome activation, NLRP3 also mediates pyroptosis and IL-1α secretion. It has been shown that caspase-11 can contribute to all those events, either in association with, or independently of, caspase-1[6]. Here, we found that caspase-5 plays a role both in IL-1β and IL-1α release in response to LPS in monocytes. However, no lactate dehydrogenase (LDH) release (a pyroptotic marker) was detected in supernatant obtained from LPS-stimulated monocytes (data not shown). Moreover, the release of IL-1α by monocytes is caspase-1-independent[21,22] but caspase-5 dependent.

In summary, we provided evidences of new role of caspase-5 during LPS-mediated release of IL-1β and IL-1α in human monocytes (Supplementary Figure 1).

Our results suggest caspase-5 as a novel immune-regulatory player during pro-inflammatory stage of sepsis and its potential application as a therapeutic target needs to be further investigated.



Supplementary Figure 1. Graphical scheme of the proposed pathway downstream LPS in human monocytes.

Interaction between LPS and its TLR4-MD2-CD14 receptor complex induces LPS internalization by endocytosis and phosphorylation of Syk. Activation of Syk results in an increase in ROS production and Ca^{2+} influx. ROS induces NLRP3-mediated caspase-1 activation which in turn leads to IL-1β/IL-18 release.

Meanwhile, the increase of Ca^{2+} in the intracellular space leads to caspase-5 activation and consequent release of IL-1α and IL-1β in the extracellular milieu.

Abbreviation: ASC, apoptosis-associated speck-like protein; Ca^{2+}, calcium; CARD, caspase-recruitment domain; Casp, caspase; IL, interleukin; IP_3, inositol triphosphate; LPS, lipopolysaccharide; MD2, myeloid differentiation factor 2; NLRP3, Nod-like receptor family, pyrin domain-containing-3; PYD, pyrin domain; PLCγ, phospholipase C gamma; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; TLR4, Toll-like receptor 4.

Material and Methods

Monocyte isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the Blood Bank of National University Hospital (NUH, Singapore; NUS-IRB 12-044E) using Ficoll-Hypaque density gradient centrifugation. Monocyte isolation was performed by negative selection using the Monocytes isolation Kit II (Miltenyi Biotech) according to the manufacturer's instructions. Purity was measured by FACS (>90% $CD14^+$) using a PE-labeled anti-human CD14 antibody (clone 61D3, eBioscience).

Monocytes were plated in non-treated 96-well plates (Corning, 1.5 x 10^5/well) or 24-well plates (Corning, 1 x 10^6 cells/well) in RPMI 1640-Glutamax medium (Gibco) supplemented with 3% human serum (Gemini Bio Product), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (all from Gibco). Monocytes were stimulated with *E. coli* LPS (serotype O55:B5, Enzo Lifesciences, 10 ng/ml) and/or human recombinant IFN-β (IFN-β) (PBL InterferonSource, 250 U/ml) for 5 hours, unless otherwise indicated. Inhibitors, such as Z-YVAD (Enzo Lifesciences), LEVD (Enzo Lifesciences), diphenyleneiodonium (DPI) (Enzo Lifesciences), R406 (Selleck), U73122 (Calbiochem), dynasore

(Sigma-Aldrich) were added 1 hour before LPS treatment, while ATP (Sigma-Aldrich, 1 mM) was added 30 minutes before the end of the culture.

Dendritic cells differentiation

$CD14^+$ monocytes (>95% pure) isolated by positive selection using the $CD14^+$ monocyte isolation kit (Miltenyi Biotec) were cultured in treated 6-well plates (Thermo scientific, 2 x 10^6/well) in RPMI 1640-Glutamax medium, supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, MEM non-essential amino acids (MEM-NEA) (all from Gibco) in the presence of 50 ng/ml rh-GM-CSF (Miltenyi Biotec) and 10 ng/ml rh-IL-4 (R&D Systems). On day 3, cytokine-supplemented medium was refreshed. On day 7, adherent cells were collected. Differentiated DCs were plated in non-treated 96-well plates (Corning) at 1.5x10^5/well in complete medium. DCs were stimulated with LPS (1 µg/ml) for 5 hours, followed by ATP (1 mM) for 30 minutes.

Real-time quantitative PCR

10^6 cells were collected in 800µl of Trizol (Invitrogen) in 1.5 ml eppendorf tube and RNA was isolated using the RNeasy kit (Qiagen) and treated with DNAse I (Promega) according to the manufacturer's instructions. Quantitative PCR was performed in triplicate using the GoTaq qPCR Master Mix (Promega) and the validated SYBR Green primer pairs listed in Supplementary table 1. Amplification was performed using a 7500 Real-Time PCR system (Applied Biosystems) and relative expression levels were calculated through the ΔΔ*Ct* method, using GAPDH as a housekeeping gene and the untreated (UT) or the scramble samples as calibrators.

Gene	**Primers (5'→3')**		**Size**
CASP4	Forward	AAGAGAAGCAACGTATGGCAGGAC	145
	Reverse	GGACAAAGCTTGAGGGCATCTGTA	
CASP5	Forward	GGTGAAAAACATGGGGAACTC	141
	Reverse	TGAAGAACAGAAAGCAATGAAGT	
IL-1β	Forward	CCAGTGAAATGATGGCTTATTAC	151
	Reverse	CTGTAGTGGTGGTCGGAGATT	
IFN-β	Forward	GAGAACCTCCTGGCTAATGTCTATCA	102
	Reverse	GCTCATGAGTTTTCCCCTGGTG	

ISG54	Forward	GGAGGGAGAAAACTCCTTGGA	100
	Reverse	GGCCAGTAGGTTGCACATTGT	
ISG56	Forward	TCAGGTCAAGGATAGTCTGGAG	147
	Reverse	AGGTTGTGTATTCCCACACTGTA	
NLRP3	Forward	TGGCTGTAACATTCGGAGATTG	138
	Reverse	GAAGTCACCGAGGGCGTTGT	
GAPDH	Forward	CCACATCGCTCAGACACCAT	114
	Reverse	GGCAACAATATCCACTTTACCAGAGT	

Supplementary table 1. Primers list

Western blotting

Total cell lysates from 1-2x10^6 cells were prepared using Laemmli buffer containing 5% β-mercaptoethanol and boiled at 95ºC for 15 minutes. Lysate concentrations were determined using Nanodrop ND1000 (Thermo Scientific). Equal amounts of total proteins (20-50 µg) were separated in SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories). Membranes were blocked with 5% milk powder in phosphate-buffered saline (PBS)/0.1% Tween 20 (Sigma-Aldrich) (PBST) for 1 hour at room temperature, followed by incubation overnight at 4ºC with the following primary

antibodies diluted in 5% BSA/PBST: anti-NLRP3 (#AG-20B-0014-C100, Adipogen), anti-IL-1α (#MAB200, R&D) anti-IL-1β (#2022), anti-total Syk (#2712), anti-caspase-1 (#4199), anti-caspase-4 (#4450), anti-caspase-5 (#4429) (Cell Signaling) and anti-GAPDH (#MAB374, Millipore). For the detection of phospho-Syk (#2710S), blocking and incubation with primary antibody were performed in 5% BSA dissolved in Tris-buffered saline/0.1% Tween 20 (TBST). Densitometry analysis was performed using ImageJ software and the data were normalized against GAPDH.

Cytokine measurements

IL-1β, IL-6 (Biolegend) and IL-1α (R&D using Duoset kit) levels were determined by ELISA in cell-free supernatants.

Small interfering RNA-mediated knockdown

Monocytes (5-10x10^6) were nucleofected with 300 nM siRNA according to the manufacturer's instruction (Amaxa Nucleofector Technology, program Y001). Transfected monocytes were incubated for 36 hours with 20 ng/ml IFN-γ and then stimulated with LPS alone (10 ng/ml for 8 hours) or together with ATP (1 mM

for 30 minutes). The following validated siRNA (AITbiotech) was used: NLRP3, 5'-ACCGCGGUGUACGUCUUCUUCCUUU-3'[23].

Transient transfection in HEK293t cells

HEK293t cells were plated $0.25x10^6$ in tissue-culture treated 6-well-plates (Thermo Scientific). Cells were transfected with the following plasmids (1.3 µg/each) using Lipofectamine 2000 (Invitrogen): pCMV6-pro-IL-1α (#RC202084, Cayman), pcDNA3-pro-IL-1β (courtesy of Dr. Bruno Reversade), pcDNA3-procaspase-5 (courtesy of Dr. Shu-ichi Matsuzawa) and pCI-procaspase-1 (#41552, Addgene). Cell lysates were collected 24 hours after transfection and IL-1α/IL-1β expression were assessed by Western blotting.

Type I-IFN receptor neutralization

Human monocytes were plated in non-treated 12-well (10^6 cells/well) or 96-well (1.5×10^5 cells/well) plates (Corning) to collect lysates or RNA, respectively. Cells were pre-treated for 1 hour with 5 µg/ml neutralizing anti-human interferon α/β receptor chain 2 (α-hIFNAR, clone MMHAR-2, PBL InterferonSource) or its

isotype control (mouse IgG2a, Biolegend) (5 µg/ml). Then, cells were stimulated with LPS (10 ng/ml) or IFN-β (250 U/ml) for 5 hours.

Production and purification of full-length caspase-1 catalytic mutant (C285A) in *E. coli*

Plasmid containing HIS-tagged full-length caspase-1 catalytic mutant (C285A) (courtesy of Stefan Riedl) was transformed into *E. coli* strain BL21 (DE3) (Stratagene). Transformed bacteria were growth in Luria Broth (LB) media containing ampicillin (Sigma, 100 µg/ml). Caspase-1 C285A expression was induced by 1-isopropyl-β-D-thiogalactopyranoside (IPTG) (Gold Biotech, 0.5 mM) added to the growing culture in log phase (OD_{600} = 0.7) for 3 hours at 30ºC by vigorous shaking (275 rpm). Bacteria were lysed in 50 mM Tris-HCl 100 mM NaCl (pH 8.0). Lysates were sonicated and centrifuged for 30 minutes at 18.000 x *g* at 4ºC. Soluble proteins contained in the supernatant were subjected to purification using nickel-chelating sepharose HIS-TRAP HP column (GE Healthcare) and eluted using imidazole (Sigma, 200 mM) using a peristaltic pomp (speed 1ml/minute).

Caspase cleavage assay

Purified caspase-1 catalytic mutant (C285A) was incubated together with recombinant caspase-5 (rCasp-5) (Merck Millipore) (100 U) for 20 minutes at 37ºC. To block the activity of caspase-5, rCasp5 was pre-incubated with LEVD (50 uM) for 15 minutes at 37ºC.

ROS measurement

Monocytes (1.5 x 10^6 cells/well) were plated in black non-treated 96-well plates (Thermo Scientific) and rested at 37°C for at least 2 hours. Cells were stimulated with LPS (10 ng/ml). The cell-permeant H_2DCF-DA probe (10 μM) was immediately added and fluorescence was measured every 10 minutes using a microplate fluorimeter (TECAN) (excitation, 480 nm; emission, 530 nm). Monocytes were pre-incubated for 1 hour with the Syk inhibitor R406 (0.5-2 μM) or PLCγ inhibitor U73122 (3 μM) where indicated. Values were normalized to the average of the untreated control. Each condition was performed in triplicates.

Immunofluorescence and confocal microscopy

Monocytes (0.35 x 10^5 cells/well) plated in µ-Slide (Ibidi) were incubated with R406 (2 µM) or Dynasore (40 µM) for 30 minutes at 37ºC, and then stimulated with LPS (10 ng/ml) for 5, 15 or 30 minutes. Cells were fixed with 2% paraformaldehyde (Polysciences) and permeabilized with 0.1% saponin in the presence of 0.2% gelatin and 5 mg/ml BSA. Cells were immunostained with CD14-FITC (BD Bioscences) and Rab5 (Cell signaling) and imaged on a FV1000 confocal microscope (Olympus) with a 200X objective. Colocalization was evaluated using Pearson's correlation coefficients calculated by the Imaris software (Bitplane).

Intracellular Ca^{2+} mobilization assay

Monocytes (1.5 x 10^5/well) were plated in black non-treated 96 well-plates (Thermo scientific) and rested overnight at 37ºC. Cells were incubated for 45 minutes in the dark with 100 µL of Hanks' balanced salt solution (HBSS) containing 20 mM HEPES, 2.5 mM probenecid and Fluo4-NW (Invitrogen). Fluorescence was monitored for 70 seconds after LPS stimulation (1 µg/ml) using a Victor4 plate reader (Perkin Elmer) (excitation, 485 nm; emission,

535 nm). When indicated, inhibitors (0.5 μM R406, 3 μM U73122, 10 μM BAPTA/AM) were added 1 hour before Fluo4-NW and EGTA (2 mM) was added during the last 45 minutes of incubation together with Fluo4-NW. All the experiments were performed at 37ºC. Values (F) were normalized against the baseline acquired at the time of LPS stimulation (F0).

References

1 Latz, E., Xiao, T. S. & Stutz, A. Activation and regulation of the inflammasomes. *Nature reviews. Immunology* **13**, 397-411, doi:10.1038/nri3452 (2013).

2 Bauernfeind, F. G. *et al.* Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* **183**, 787-791, doi:10.4049/jimmunol.0901363 (2009).

3 Kayagaki, N. *et al.* Non-canonical inflammasome activation targets caspase-11. *Nature* **479**, 117-121, doi:10.1038/nature10558 (2011).

4 Vigano, E. & Mortellaro, A. Caspase-11: the driving factor for noncanonical inflammasomes. *European journal of immunology* **43**, 2240-2245, doi:10.1002/eji.201343800 (2013).

5 Hagar, J. A., Powell, D. A., Aachoui, Y., Ernst, R. K. & Miao, E. A. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* **341**, 1250-1253, doi:10.1126/science.1240988 (2013).

6 Kayagaki, N. *et al.* Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246-1249, doi:10.1126/science.1240248 (2013).

7 Broz, P. *et al.* Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature* **490**, 288-291, doi:10.1038/nature11419 (2012).

8 Netea, M. G. *et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113**, 2324-2335, doi:10.1182/blood-2008-03-146720 (2009).

9 Martinon, F. & Tschopp, J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell death and differentiation* **14**, 10-22, doi:10.1038/sj.cdd.4402038 (2007).

10 Rathinam, V. A. *et al.* TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* **150**, 606-619, doi:10.1016/j.cell.2012.07.007 (2012).

11 Schauvliege, R., Vanrobaeys, J., Schotte, P. & Beyaert, R. Caspase-11 gene expression in response to lipopolysaccharide and interferon-gamma requires nuclear factor-kappa B and signal transducer and activator of transcription (STAT) 1. *The Journal of biological chemistry* **277**, 41624-41630, doi:10.1074/jbc.M207852200 (2002).

12 Zanoni, I. *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**, 868-880, doi:10.1016/j.cell.2011.09.051 (2011).

13 Said-Sadier, N., Padilla, E., Langsley, G. & Ojcius, D. M. Aspergillus fumigatus stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PloS one* **5**, e10008, doi:10.1371/journal.pone.0010008 (2010).

14 Law, C. L., Chandran, K. A., Sidorenko, S. P. & Clark, E. A. Phospholipase C-gamma1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Molecular and cellular biology* **16**, 1305-1315 (1996).

15 Rao, S., Liu, X., Freedman, B. D. & Behrens, E. M. Spleen tyrosine kinase (Syk)-dependent calcium signals mediate efficient CpG-induced exocytosis of tumor necrosis factor alpha (TNFalpha) in innate immune cells. *The Journal of biological chemistry* **288**, 12448-12458, doi:10.1074/jbc.M113.454405 (2013).

16 Akhter, A. *et al.* Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization. *Immunity* **37**, 35-47, doi:10.1016/j.immuni.2012.05.001 (2012).

17 Case, C. L. *et al.* Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 1851-1856, doi:10.1073/pnas.1211521110 (2013).

18 Py, B. F. *et al.* Caspase-11 controls interleukin-1beta release through degradation of TRPC1. *Cell reports* **6**, 1122-1128, doi:10.1016/j.celrep.2014.02.015 (2014).
19 Meunier, E. *et al.* Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* **509**, 366-370, doi:10.1038/nature13157 (2014).
20 Sander, L. E. *et al.* Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* **474**, 385-389, doi:10.1038/nature10072 (2011).
21 Freigang, S. *et al.* Fatty acid-induced mitochondrial uncoupling elicits inflammasome-independent IL-1alpha and sterile vascular inflammation in atherosclerosis. *Nature immunology* **14**, 1045-1053, doi:10.1038/ni.2704 (2013).
22 Gross, O. *et al.* Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* **36**, 388-400, doi:10.1016/j.immuni.2012.01.018 (2012).
23 Atianand, M. K. *et al.* Francisella tularensis reveals a disparity between human and mouse NLRP3 inflammasome activation. *The Journal of biological chemistry* **286**, 39033-39042, doi:10.1074/jbc.M111.244079 (2011).

Chapter 3.

Conclusion

Summary, conclusion and future prospective

IL-1α/β are pro-inflammatory cytokines playing an important role in response to infection. Their release is mediated by caspase-1 whose activation is mediated by an active inflammasome complex.

Inflammation is a well-known process that has been linked to the pathogenesis of various diseases, such as cancer[1], infectious disorders (e.g. sepsis)[2] and autoinflammatory disease (e.g. rheumatoid arthritis)[3]. The available treatments for those pathologic conditions are often limiting and, therefore, the development of new therapeutic approaches targeting the host immune response is required.

LPS stimulation induces IL-1β release by human monocytes[4-6]. The exact mechanisms responsible for inflammasome activation and the subsequent release of IL-1β by LPS-stimulated monocytes have not been fully understood.

In the chapter 2 we identified caspase-5 as an important target controlling inflammasome activity induced by LPS in human monocytes. In particular, we showed that caspase-5 is specifically activated upon LPS stimulation in human monocytes and its activation regulates the inflammasome-dependent cytokine release. Indeed, inhibition of caspase-5 reduces secretion of both IL-1α and IL-1β.

In this PhD study, the molecular mechanisms driving caspase-5 activation in human monocytes have been uncovered. Interestingly, we found that the release of IL-1α/IL-1β is dependent on caspase-5. Caspase-5 activation depends on Syk and PLCγ-Ca^{2+} but is independent of ROS production.

The requirement of the tyrosine kinase Syk for inflammasome activation has been recently reported. Syk is involved in inflammasome activation at different level; firstly, it induces ROS production[7] and secondly, it regulates inflammasome aggregation (ASC speck formation) by phosphorylating the inflammasome adaptor ASC, which in turn triggers the activation of caspase-1[8]. Our data provide new insights into the mechanism driven by Syk during the activation of caspase-5 and the subsequent release of IL-1α and IL-1β. IL-1α release turns to be ROS-independent and PLCγ-Ca^{2+}-dependent, whereas IL-1β release depends on PLCγ and ROS.

In this new scenario, we proposed caspase-5 as a new candidate target for future therapeutic strategies aimed at limiting the excessive inflammatory response, which can be deleterious for different pathologic conditions, such as cancer and sepsis. These aspects are discussed in the following paragraphs.

Future prospective: caspase-5 and cancer

Cancer, a disorder characterized by uncontrolled cell growth and proliferation, is one of the major causes of death worldwide. Caspases play a crucial role for the induction of programmed cell death (apoptosis) and, in fact, dysregulation of their activity has been associated with predisposition to develop cancer, such as colorectal cancer[9]. Little is known about the role of caspase-5 during carcinogenesis. Mutations in *CASP5* gene have been associated to colorectal[10-12], endometrial[12,13] and lung cancer[14] as well as in leukemia and lymphoma[15,16]. Furthermore, low *CASP5* expression has been observed in highly metastatic subpopulations of a lung cancer cell line suggesting a possible role of caspase-5 in suppressing the formation of metastases[14]. On the contrary, the presence of high caspase-5 activity has been also associated with cervical malignancy[17].

Inflammation has been reported to contribute to the pathogenesis of cancer[18]. Epidemiological studies reported that 15% of the cancer incidence worldwide is associated with chronic inflammation induced by microbial infection[19] (e.g. papilloma virus and *Helicobacter pylori*, etiologic agents for cervical carcinoma and gastric cancer, respectively). The production of ROS and the release of pro-inflammatory cytokines are two of the mechanisms by which the host controls microbial infections. However, these mechanisms also promote the proliferation of the tumor cells and

the formation of the tumor mass. Indeed, ROS production leads to an oxidative damage, which introduces mutations in the DNA, while inflammatory cytokines provide a survival signal promoting tumor progression.

IL-1β can be produced and released directly by tumor cells[20], as well as by tumor-infiltrating immune cells (e.g. macrophages, DCs). Monocytes are the circulated reservoir of myeloid cells. Monocytes migrate from the bloodstream to other tissues, where they subsequently differentiate into tissue resident macrophages. Macrophages have been found in close proximity or within the tumor mass, for this reason they have been named tumor-associated macrophages (TAMs). TAMs represent the major leukocytes infiltrating the tumor, and their role remains controversial since they have been implicated in both pro-tumoral[21] and anti-tumoral[22].

In our study the activation of caspase-5 has not been detected in both monocytes-derived dendritic cells and macrophages (data not shown) indicating that the caspase-5-mediated release of inflammasome regulated cytokines is specific for monocytes. Although we cannot exclude a possible role of caspase-5 in TAMs; further studies are needed to clarify caspase-5 role during tumor progression.

IL-1α can be also released by necrotic tumor cells and can lead to up-regulate the expression of adhesion molecules, which in turn lead to higher endothelial permeability and extravasation of

cancer cells contributing to metastasis dissemination[23]. Blocking of IL-1R signaling by Anakinra reduces angiogenesis and tumor growth in mice[24]. Recently, a clinical study reported that the administration of a neutralizing antibody against IL-1α in refractory end-stage cancer patients leads to the alleviation of fatigue and pain, as well as increase of appetite and body mass[25].

In our study we showed the functional role of caspase-5 in the release of IL-1β and IL-1α by human monocytes. Caspase-5 activation and the further cytokine release are mediated by Syk, a tyrosine kinase whose inhibitors have already been developed to be used in patients with rheumatoid arthritis[26,27]. However, little is known on the precise role of Syk during tumor progression[28-30].

Therefore, additional studies on caspase-5 are necessary to investigate its potential as a therapeutic or prognostic target during tumor progression.

Future prospective: caspase-5 and sepsis

Sepsis is a complex syndrome causing high mortality rate in most intensive care units. Sepsis is defined as an overwhelming immune response to severe life-threatening infection and it manifests with an abnormal body temperature, tachycardia and tachypnea. Severe sepsis is often associated with damage of several organs (lung, kidney and liver) which quickly cause death. Sepsis should be considered as a heterogeneous disease based on host genetic composition, age and type of pathogen initiating the infection. The hallmark of sepsis is the presence of systemic inflammatory response syndrome (SIRS) in response to invading pathogens, in which TNFα and IL-1 cytokines are the main players. However, in some patients the acute inflammatory response is followed by a compensatory anti-inflammatory response syndrome (CARS) characterized by the induction of anti-inflammatory mechanisms that impede patient immune system to further respond to the infection. For long time, SIRS has been considered to initiate pathophysiology of sepsis, followed by a second phase characterized by CARS. Recently, it has been proposed that these two syndromes occur simultaneously during the development of sepsis. Indeed, LPS injection, often used to mimic sepsis *in vivo*, leads to the induction of both pro- and anti-inflammatory transcripts in leukocytes from healthy patients[31].

Innate immune cells (neutrophils, monocytes and macrophages) play an important role in the systemic inflammatory response which occurs during sepsis. Indeed, they are the main producers of cytokines, such as TNFα, IL-1, IL-6, which are essential to control and eradicate the infection. However, the uncontrolled and prolonged release of pro-inflammatory cytokines (also known as cytokine storm) that takes place during sepsis causes tissue damage and multiple organ failure. Indeed, elevated level of pro-inflammatory cytokines in the serum directly correlates with the rate of mortality in human sepsis[2].

TLRs play a central role in pathogenesis of sepsis. They are required for the induction of the innate immune response to recognize and eradicate the pathogenic insult. Polymorphisms in TLR encoding genes have been associated with impaired susceptibility to infection[32], and, in particular, polymorphisms in TLR4 gene have been associated to sepsis induced by Gram-negative bacteria[33]. However, an exaggerate inflammation induced by an excessive TLR stimulation is deleterious. Numerous drugs suppressing TLR signaling or neutralizing inflammatory cytokines have been developed and used to prevent uncontrolled inflammatory response in sepsis patients. In particular, compounds targeting TLR4, such as E5531 and Eritoran (Eisai Research Institute of Boston, MA, USA) and TAK242 (Takeda Pharmaceutical Co, Japan) have been tested in

clinical trials[34]. Unfortunately none of these agents successfully blocked disease progression and they were not carried further.

In our study we focused our attention on the pro-inflammatory mechanism(s) underlying the release of IL-1 cytokines by isolated human monocytes in response to TLR4 stimulation. We observed that LPS stimulation of monocytes triggers the expression of genes encoding inflammatory mediators, such as pro-inflammatory cytokines and inflammasome components. Importantly we identified caspase-5 as crucial mediator of IL-1β and IL-1α release in human monocytes.

Further studies will be necessary to elucidate the possible role of caspase-5 in patients with sepsis caused by Gram-negative bacteria. Moreover, the possibility to use caspase-5 as a potential target for the development of new therapeutic approaches in various clinical settings remains to be investigated.

References

1 Zitvogel, L., Kepp, O., Galluzzi, L. & Kroemer, G. Inflammasomes in carcinogenesis and anticancer immune responses. *Nature immunology* **13**, 343-351, doi:10.1038/ni.2224 (2012).

2 Matsuda, A. *et al.* Novel therapeutic targets for sepsis: regulation of exaggerated inflammatory responses. *Journal of Nippon Medical School = Nippon Ika Daigaku zasshi* **79**, 4-18 (2012).

3 Shaw, P. J., McDermott, M. F. & Kanneganti, T. D. Inflammasomes and autoimmunity. *Trends in molecular medicine* **17**, 57-64, doi:10.1016/j.molmed.2010.11.001 (2011).

4 Piccini, A. *et al.* ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 8067-8072, doi:10.1073/pnas.0709684105 (2008).

5 Carta, S. *et al.* The rate of interleukin-1beta secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. *The Journal of biological chemistry* **286**, 27069-27080, doi:10.1074/jbc.M110.203398 (2011).

6 Netea, M. G. *et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113**, 2324-2335, doi:10.1182/blood-2008-03-146720 (2009).

7 Said-Sadier, N., Padilla, E., Langsley, G. & Ojcius, D. M. Aspergillus fumigatus stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PloS one* **5**, e10008, doi:10.1371/journal.pone.0010008 (2010).

8 Hara, H. *et al.* Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nature immunology* **14**, 1247-1255, doi:10.1038/ni.2749 (2013).

9 van der Woude, C. J., Kleibeuker, J. H., Jansen, P. L. & Moshage, H. Chronic inflammation, apoptosis and (pre-)malignant lesions in the gastro-intestinal tract. *Apoptosis : an international journal on programmed cell death* **9**, 123-130, doi:10.1023/B:APPT.0000018794.26438.22 (2004).

10 Trojan, J. *et al.* BAX and caspase-5 frameshift mutations and spontaneous apoptosis in colorectal cancer with microsatellite instability. *International journal of colorectal disease* **19**, 538-544, doi:10.1007/s00384-004-0597-1 (2004).
11 Soung, Y. H. *et al.* Mutational analysis of caspase 1, 4, and 5 genes in common human cancers. *Human pathology* **39**, 895-900, doi:10.1016/j.humpath.2007.10.015 (2008).
12 Schwartz, S., Jr. *et al.* Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer research* **59**, 2995-3002 (1999).
13 Kawaguchi, M. *et al.* Analysis of candidate target genes for mononucleotide repeat mutation in microsatellite instability-high (MSI-H) endometrial cancer. *International journal of oncology* **35**, 977-982 (2009).
14 Hosomi, Y. *et al.* Somatic mutation of the Caspase-5 gene in human lung cancer. *International journal of molecular medicine* **12**, 443-446 (2003).
15 Takeuchi, S., Takeuchi, N., Fermin, A. C., Taguchi, H. & Koeffler, H. P. Frameshift mutations in caspase-5 and other target genes in leukemia and lymphoma cell lines having microsatellite instability. *Leukemia research* **27**, 359-361 (2003).
16 Offman, J. *et al.* Repeated sequences in CASPASE-5 and FANCD2 but not NF1 are targets for mutation in microsatellite-unstable acute leukemia/myelodysplastic syndrome. *Molecular cancer research : MCR* **3**, 251-260, doi:10.1158/1541-7786.MCR-04-0182 (2005).
17 Babas, E. *et al.* Indication of participation of caspase-2 and caspase-5 in mechanisms of human cervical malignancy. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society* **20**, 1381-1385, doi:10.1111/IGC.0b013e3181ed7896 (2010).
18 Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883-899, doi:10.1016/j.cell.2010.01.025 (2010).
19 Kuper, H., Adami, H. O. & Trichopoulos, D. Infections as a major preventable cause of human cancer. *Journal of internal medicine* **248**, 171-183 (2000).
20 Okamoto, M. *et al.* Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1

processing and secretion of interleukin-1beta. *The Journal of biological chemistry* **285**, 6477-6488, doi:10.1074/jbc.M109.064907 (2010).

21 Riabov, V. *et al.* Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Frontiers in physiology* **5**, 75, doi:10.3389/fphys.2014.00075 (2014).

22 Bingle, L., Brown, N. J. & Lewis, C. E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of pathology* **196**, 254-265, doi:10.1002/path.1027 (2002).

23 Scherbarth, S. & Orr, F. W. Intravital videomicroscopic evidence for regulation of metastasis by the hepatic microvasculature: effects of interleukin-1alpha on metastasis and the location of B16F1 melanoma cell arrest. *Cancer research* **57**, 4105-4110 (1997).

24 Weinreich, D. M. *et al.* Effect of interleukin 1 receptor antagonist gene transduction on human melanoma xenografts in nude mice. *Cancer research* **63**, 5957-5961 (2003).

25 Hong, D. S. *et al.* MABp1, a first-in-class true human antibody targeting interleukin-1alpha in refractory cancers: an open-label, phase 1 dose-escalation and expansion study. *The lancet oncology* **15**, 656-666, doi:10.1016/S1470-2045(14)70155-X (2014).

26 Genovese, M. C. *et al.* An oral Syk kinase inhibitor in the treatment of rheumatoid arthritis: a three-month randomized, placebo-controlled, phase II study in patients with active rheumatoid arthritis that did not respond to biologic agents. *Arthritis and rheumatism* **63**, 337-345, doi:10.1002/art.30114 (2011).

27 Weinblatt, M. E. *et al.* Effects of fostamatinib (R788), an oral spleen tyrosine kinase inhibitor, on health-related quality of life in patients with active rheumatoid arthritis: analyses of patient-reported outcomes from a randomized, double-blind, placebo-controlled trial. *The Journal of rheumatology* **40**, 369-378, doi:10.3899/jrheum.120923 (2013).

28 Coopman, P. J. *et al.* The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature* **406**, 742-747, doi:10.1038/35021086 (2000).

29 Friedberg, J. W. *et al.* Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin

lymphoma and chronic lymphocytic leukemia. *Blood* **115**, 2578-2585, doi:10.1182/blood-2009-08-236471 (2010).

30 Pritchard, E. M. *et al.* Pharmacokinetics and Efficacy of the Spleen Tyrosine Kinase Inhibitor R406 after Ocular Delivery for Retinoblastoma. *Pharmaceutical research*, doi:10.1007/s11095-014-1399-y (2014).

31 Xiao, W. *et al.* A genomic storm in critically injured humans. *The Journal of experimental medicine* **208**, 2581-2590, doi:10.1084/jem.20111354 (2011).

32 Schroder, N. W. & Schumann, R. R. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *The Lancet infectious diseases* **5**, 156-164, doi:10.1016/S1473-3099(05)01308-3 (2005).

33 Netea, M. G., Wijmenga, C. & O'Neill, L. A. Genetic variation in Toll-like receptors and disease susceptibility. *Nature immunology* **13**, 535-542, doi:10.1038/ni.2284 (2012).

34 Savva, A. & Roger, T. Targeting Toll-Like Receptors: Promising Therapeutic Strategies for the Management of Sepsis-Associated Pathology and Infectious Diseases. *Frontiers in immunology* **4**, 387, doi:10.3389/fimmu.2013.00387 (2013).

Contribution to international publications

Caspase-11: the driving factor for non-canonical inflammasomes.
E. Viganò and A. Mortellaro. Eur. J. Immunol. 2013. 43: 2240–2245

Tyrosine kinases: the molecular switch for inflammasome activation.
F.Laudisi, E. Viganò and A. Mortellaro. Cell Mol Immunol. 2014 Mar:11(2):129-132