THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Comparative analysis of NLRP3 inflammasome-mediated IL-1β production in different types of human monocyte-derived macrophages

by

Marietta Margit Budai

Supervisor: **Szilvia Benkő**, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Contents

1. Introduction

IL-1β is a "master" cytokine regulating a wide variety of physiological and immunological processes. Due to its potent ability, the secretion of the active form of the cytokine is under tight regulation, mediated by multiprotein complexes called inflammasomes. One of the most studied inflammasome complexes is the NLRP3 inflammasome that contains multiple copies of NLRP3 sensor, ASC adaptor, and caspase-1 enzyme. It has been proposed that activation of the NLRP3 inflammasome requires two distinct signals. The first, so called priming signal, is typically induced by Toll-like receptors (TLRs) that triggers the expression of pro-IL-1β and components of the inflammasome (NLRP3), through several signal transduction pathways. The second signal leads to the formation of inflammasome complex, the activation of caspase-1, and the cleavage of inactive pro-IL-1β into a mature cytokine. The second signal may be triggered by a wide variety of molecules that function as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs).

Human macrophages; the most frequently studied models for NLRP3 inflammasome functions, can release IL-1β only when both of these events occur in parallel. Macrophages are very plastic, and may be characterized by different morphological and functional properties. Homeostatic control of human monocyte/macrophage development is mostly dependent on their microenvironment, and influenced by macrophage colony stimulating factor (M-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF). Macrophages are potent inducers and regulators of the immune response. Apart from the different phases of an inflammatory response to infections or skin damage, macrophages take a central role in orchestration of the resolution of inflammation, wound healing, and also in new tissue formation and tissue remodeling.

Here, we report a comparative study focusing on the dynamics and molecular mechanisms of NLRP3 inflammasome priming and activation in LPS-stimulated human blood monocyte-derived GM- or M-macrophages, differentiated in the presence of GM-CSF or M-CSF, respectively. Furthermore, we aimed to study the effect of the immunomodulatory natural compound *Aloe Vera*, which has been used in traditional medicine related to infections and inflammation, as well as wound healing in our system.

2. Theoretical background

2.1. Danger- and pathogen-recognition system

The main function of the immune system is to protect our body from pathogenic organisms. However, even self-derived molecules and cells, or noninfectious foreign substances may elicit immune responses. This defense involves two interrelated systems: innate immunity and adaptive immunity. The innate immune system provides an early response against microbes by several non-specific protective mechanisms, including physical (e.g., skin) and physiological barriers (e.g., pH), as well as humoral or cellular responses [1]. Innate immune cells including monocytes/macrophages, granulocytes, dendritic cells and NK cells detect pathogens with pattern-recognition receptors (PRRs) that recognize molecular structures that are broadly shared by pathogens, known as pathogen-associated molecular patterns (PAMPs) [2]. However, different types of microbes express different types of PAMPs. These structures include nucleic acids that are unique to microbes (e.g. dsRNA, unmethylated CpG DNA sequences), in addition to complex cell wall lipids and carbohydrates (e.g. LPS, lipoteichoic acid, mannan) that are synthesized by microbes but not by mammalian cells. Some PRRs are also able to respond to self-derived, endogenous molecules (e.g. ATP, heat shock protein, ROS). These so-called danger- (or damage-) associated molecular patterns (DAMPs) originate from multiple sources, including damaged and dying cells which are triggered by external factors or diseases such as trauma or infection [3, 4]. Besides immune cells, non-immune cells such as epithelial cells, endothelial cells and fibroblasts also contribute to the inflammatory processes. Acute inflammation developed by the cooperation of immune and non-immune cells has been considered as the first line of host defense against microbial or non-microbial insults [5]. Activation of the PRRs in these cells ultimately leads to the production of cytokines that drive pathogen-derived or sterile inflammatory responses.

Based on their localization, PRRs can be classified into three major groups. Members of soluble receptors (such as pentraxins, collectins, ficolins and complement proteins) are present in the blood and in extracellular fluids, and are responsible for recognizing non-self or altered-self molecular patterns [6-9]. These soluble receptors provide early defense against pathogens, as they act as opsonins promoting inflammatory responses and may also directly kill microbes. The cell-associated PRRs are expressed either as membrane-bound receptors or as cytoplasmic receptors. The Toll-like receptors (TLRs), the C-type lectins (CLRs) and the

scavenger receptors are localized in the plasma membrane to survey the extracellular milieu. Nevertheless, some TLRs (TLR3, 7, 8 and 9) are found in endosomal membranes, where they detect different nucleic acid ligands or unmethylated CpG motifs originating from microbes that are phagocytosed by the host cells. The cytoplasmic receptors; including the retinoic acid inducible gene I (RIG-I) - like receptors (RLRs), the Absent in melanoma (AIM)-like receptors (ALRs), and the nucleotide-binding and oligomerization domain (NOD) - like receptors (NLRs), sense the intracellular space of the host cell [10-13]. The importance of PRRs is also highlighted by the fact that scientists who discovered and described the TLRs have been awarded a Nobel Prize in 2011.

When pathogens succeed in breaching the innate immune system, the adaptive immune system is activated as a second line of defense. The highly specialized T- and B cells of adaptive immunity are able to recognize a wide range of pathogens, and create immunological memory that leads to an enhanced response in case of subsequent encounters.

2.2. Nod-like receptors

In the early 2000s, the existence of a novel gene family has been described. The family was initially termed the CATERPILLER gene family, after which, it was renamed the Nucleotide-binding and oligomerization domain-like receptor (NLR) in 2006 [14, 15]. Proteins encoded by these genes are marked by the presence of a specific amino-terminal domain and a conserved nucleotide-binding domain (NBD), which is followed by the C-terminal leucine-rich repeat (LRR) domains. This type of domain organization was first discovered in 1994 in proteins that represent the subfamily of disease resistance proteins or R proteins in plants, and mediate host responses to a wide range of pathogens [16]. Subsequently, in 1997, the first mammalian pattern recognition receptors, the TLRs were described as membrane-integrated proteins containing an extracellular LRR domain [17]. The characteristic domain structure predicted the pivotal roles of this new, evolutionarily conserved family of NLRs in innate immunity as part of the first line of defense against pathogens.

NLR proteins recognize a specific repertoire of PAMP or DAMP molecules [18, 19]. In humans, there are 22 known NLRs that are divided into five subfamilies according to the type of their N-terminal domain: NLRAs containing an acidic transactivation domain, NLRBs with a baculovirus inhibitor repeat (BIR) domain, NLRCs possessing a caspase-activation and

recruitment domain (CARD), and NLRPs having a pyrin domain (PYD). in addition to NLRX containing a CARD-related X effector domain (Table 1) [20].

It is generally characteristic for NLR proteins to contain an N-terminal protein-protein interaction domain required for oligomerization, a central NACHT domain (or NBD - nucleotide-binding domain) necessary for self-oligomerization and activation, and a C-terminal leucine-rich repeat (LRR) that confers ligand recognition. However, in the absence of ligand stimulation, LRR may act as a repressor of NLR signaling by masking the N-terminal domain. Upon sensing the ligand, the auto-inhibitory LRR undergoes conformational changes that allow the NLR to oligomerize or interact with adaptor proteins [21, 22]. Interestingly, NLRs show remarkable differences in the length of their LRR region. NLRC5 for example contains 27 LRRs, making this protein the largest in the NLR family [23]. On the other hand, no LRR domain is observed in NLRP10, which may indicate a role for this protein as a signaling adaptor rather than an NLR sensor [24, 25].

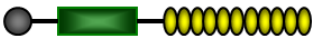
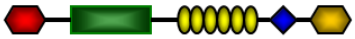
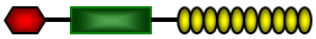
In general, the basic expression level of NLRs is low or under the detection limit, but in most cases, their expression is highly inducible. The inducibility of their expression provides an important level for the regulation of their function. The final outcome of NLR activation is the formation of diverse multiprotein complexes that have the ability to modulate several biological processes. Based on their primary or best-characterized functions, we can divide the receptor family into three subgroups. Members of the most characterized subgroup are able to form inflammasome complexes that have an indispensable role in the maturation of IL-1β and IL-18 pro-inflammatory cytokines (NLRC4, NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12) [26-32]. The function of another subgroup is associated with positive or negative regulation of inflammatory signaling cascades (NOD1, NOD2, NLRC3, NLRC5, NLRP2, NLRP4, NLRP6, NLRP7, NLRP10, NLRP12 and NLRX1). Interestingly, it seems that the third subgroup has an important role in reproduction and embryonic development (NLRP4, NLRP5, NLRP8, NLRP9, NLRP14), which indicates that the function of NLRs is not restricted only to immune functions [33].

Beside the formation of multiprotein complexes, intracellular localization of NLRs is also emerging as a critical determinant of their function. Indeed, the ability to shuttle from the cytosol to different compartments of the cell appears to be a common feature of NLR family members. For example, both NOD1 and NOD2 were found to be associated with the plasma membrane, which interfered with their capacity to mediate NF-kB activation [34]. Furthermore, NLRC5 has the ability to shuttle from the cytosol to the nucleus [23] and regulate the transcription of MHC class I genes as a specific transactivator [35], or modulate

antiviral responses [36, 37]. Similarly, NLRX1 was also identified as a mitochondria-localized NLR, a location that appears to be a requisite for its function as regulator of Type I IFNs or ROS production [38, 39].

Although the functions of several NLRs have not been reported yet, the already characterized members of this family have been linked to multiple diseases and auto-inflammatory disorders, highlighting the importance of Nod-like receptors. For instance, mutations in NOD1 and NOD2 have been associated with inflammatory bowel disorders; such as ulcerative colitis or Crohn's disease, which suggests that these NLRs play an important role in maintaining gut homeostasis. Furthermore, NOD2 is also implicated in Blau syndrome, which is characterized by skin rashes, uveitis and recurrent arthritis [40]. Interestingly, a number of NLRs have been found to be involved in certain neurological diseases. Like NLRX1, NLRP12 and NLRP3 play a role in the development of multiple sclerosis. Beside NLRP1 and NLRP10, NLRP3 is also implicated in Alzheimer's disease [41-43]. Genomic mutation in CIITA has been shown to be highly recurrent in bare lymphocyte syndrome and classical Hodgkin lymphoma [44]. Furthermore, the role of NOD1, NOD2 and NLRP3 has been described in the pathogenesis of metabolic disorders that has not been previously considered as inflammatory disorders, such as type 2 diabetes, metabolic syndrome or atherosclerosis [45-47]. Interestingly, both pro- and anti-tumor effects of NLRs have been described, as several reports showed their role in the initiation, progression and regression of various cancers. Hu et al. showed that the regulation of inflammation-induced tumorigenesis in the colon is mediated by NLRC4 and caspase-1 [48]. In contrast, enhanced tumorigenesis was observed in NLRP6, NLRP1 or NLRP12 deficient mice models [49-51]. Additionally, genome-wide sequencing studies have identified a number of further risk in NLR genes that may be associated with certain diseases, including allergic rhinitis, asthma, cardiovascular diseases, vitiligo or bone density loss [52-54].

The various reports about NLR biology show that many studies have aimed to highlight the functions of NLRs in health and disease, to provide new insights into therapeutic strategies for the management of inflammatory pathologies. In spite of all these reports, NLR family is still less defined, and lack significant mechanistic and functional insights, which necessitates more studies to better understand this PRR subgroup.

NLR subfamily	*Name*	*Function*	*Domains*
NLRA	CIITA	MHCII regulation	
NLRB	NAIP	Control of Legionella Pneumophilia infection	
NLRC	NOD1 (NLRC1)	NFκB activation, Autophagy	
	NOD2 (NLRC2)	NFκB activation, Autophagy	
	NLRC3	Negative regulation of NFκB	
	NLRC4	Inflammasome formation	
	NLRC5	MHC-I, NFκB, Type-I IFN regulation	
NLRP	NLRP1	Inflammasome formation	
	NLRP2	Inflammasome formation	
	NLRP3	Inflammasome formation	
	NLRP4	Embriogenesis, Type-I IFN regulation, autophagy	
	NLRP5	Embriogenesis	
	NLRP6	Inflammasome formation	
	NLRP7	Inflammasome formation	
	NLRP8	Unknown, Reproduction?, Fenotypic variation?	
	NLRP9	Unknown, Reproduction?, Fenotypic variation?	
	NLRP10	Negative regulation of NFκB	
	NLRP11	Unknown, role in s-JIA?	
	NLRP12	Inflammasome formation, Negative regulation of NFκB	
	NLRP13	Unknown	
	NLRP14	Spermatogenesis	
NLRX	NLRX1	NFκB, Type I IFN regulation, ROS production, Autophagy	

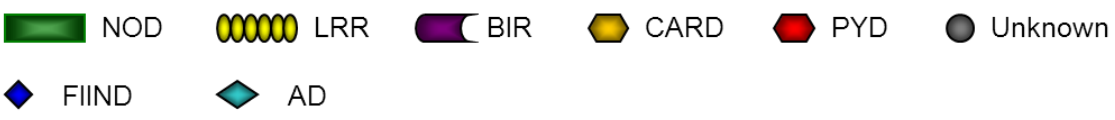


Table 1. Classification, protein structure, and function of the NOD-like receptor family.

2.2.1. Inflammasomes

Inflammasomes were described in 2002 by the research group of Tschopp as multiprotein complexes containing NLR sensor, ASC adaptor and caspase-1 enzyme [55]. Since then, a wide array of inflammasomes and their adaptor molecules have been discovered in the host's innate immune system in response to various pathogens, providing an extensive detection of a diverse range of PAMPs and DAMPs (Table 2). From the NLR family, seven proteins have been found so far which are able to form inflammasomes, including NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4, as well as the non-NLR protein AIM2 [26-32, 56].

Despite the pivotal roles of inflammasomes in the innate immune response, little is known about the nature of NLRP agonists and the stimuli that trigger their activation. The first identified inflammasome, NLRP1 is responsible for sensing the lethal anthrax toxin secreted by *Bacillus anthracis* [57]. Interestingly, the NOD2 ligand muramyl-dipeptide (MDP), which is a peptidoglycan motif common to all bacteria, was also shown to trigger NLRP1 inflammasome activation [58]. Although NLRP2 was described a long time ago, its role is still poorly understood. NLRP2 inflammasome was found to be activated by LPS as well as by interferons [59], but it is also believed that the activation mechanism is highly dependent on ATP [30]. Furthermore, NLRP2 appears to be a negative regulator of inflammation through the inhibition of NF-κB activation [59, 60]. Besides NLRP2, NLRP6, NLRP7 and NLRP12 were also shown to act as a negative regulator of NF-κB signaling [61-64], however the detailed mechanisms of their action have not been described yet. In addition to the anti-inflammatory role, NLRP6 inflammasome has been implicated in the maintenance of gut homeostasis, as demonstrated by the fact that NLRP6-/- mice are more susceptible to colitis than their wild-type counterparts [65, 66]. Moreover, NLRP6 was reported to regulate intestinal antiviral response through sensing of viral RNA via the RNA helicase Dhx15 [67]. Alongside the roles of NLRP6, NLRP12 was also involved in the process of colonic inflammation, particularly, protecting against progression of acute colitis [68]. Other studies revealed a role for NLRP12 inflammasome in host resistance against *S. typhimurium* and *Y. pestis* [29, 69]. Besides NLRP12, NLRC4 inflammasome is also activated upon *S. typhimurium* infection, and studies demonstrated that NLRC4 senses the flagellin of this organism in the cytosol [70]. However, later works revealed that non-flagellated pathogens such as *S. flexneri* are also capable of activating the NLRC4 inflammasome, though the molecular details of which have not yet been reported [71]. In contrast, the function of NLRP7 inflammasome is still elusive. Conflicting reports describe NLRP7 as either an

inflammasome activator or as an inhibitor of caspase-1-dependent production of IL-1β [72, 73]. Although little is known about the molecular mechanisms responsible for NLRP7 inflammasome activation, studies have demonstrated that NLRP7 assembles to inflammasome in response to microbial acylated lipopeptides [28], and ATP binding by NLRP7 is required for the inflammasome activation [74]. As these reports show, the ligands specifically sensed or bound by NLRs are currently not completely known, yet the important role of inflammasomes in the immune response against a wide spectrum of microorganisms is indisputable.

While inflammasome complexes are able to sense a broad range of activators, these multiprotein complexes share a common effector function. Upon stimulation, they oligomerize and caspase-1 enzyme becomes activated, which subsequently leads to the cleavage of the inactive pro-IL-1β and pro-IL18 to their matured IL-1β and IL-18 forms for secretion.

Caspases are intracellular proteases that mediate programmed cell death, proliferation and inflammation. For several years, caspases were simply divided into "apoptotic" (caspase-2, -3, -6, -7, -8, -9 and -10) and "pro-inflammatory" caspases (caspase-1, -4, -5), and this classification still remains useful to some extent [75]. Caspase-1 was identified in 1989 and named as ICE (IL-1β converting enzyme) due to its ability to process the 33kDa inactive precursor of IL-1β. Today, it is generally accepted that endogenous caspase-1 is not involved in apoptosis [76]. Nevertheless, caspase-1 activation can induce a form of programmed cell death, called pyroptosis. In pyroptosis, caspase-1 activation triggers the association of gasdermin D protein with the plasma membrane where; upon oligomerization, it forms pores [77, 78].

Caspase-1 is synthesized as a catalytically weak precursor; pro-caspase-1, that is activated through auto-proteolysis induced by protein oligomerization through their CARD domain. Proteolytic processing of a short linker between CARD domain and catalytic domain allows the rearrangement for the catalytic site formation. This is the activation step that is followed by another auto-proteolytic cleavage called "maturation event". Maturation involves the cleavage of the linker between the large (p20) and small (p10) subunits of the catalytic domain. In the absence of an activation process, maturation is unable to generate enzymatic activity. Despite intensive studies, the detailed mechanism of caspase-1 maturation still awaits further clarification. Nevertheless, data suggest that caspase-1 activity is maintained only for minutes, after which the protease is rapidly inactivated [79]. This way of inflammasome activation has been termed the canonical pathway.

Besides canonical IL-1β production, recently, an alternative way of inflammasome activation has been described first in murine system, which promotes the activation of caspase-11 [80, 81]. To distinguish these mechanisms from the caspase-1 – dependent route of inflammasome engagement, the term "non-canonical inflammasome activation" had been introduced. Whereas mice encode caspase-11, humans encode two putative functional orthologs: caspase-4 and caspase-5 [82]. All three inflammatory caspases bind directly to LPS *in vitro* [83]. Recent results also indicate that the precursor of caspase-4 becomes functionally active, and it ultimately mediates the secretion of IL-1β without auto-processing [83, 84]. Unlike caspase-4, caspase-5 undergoes auto-processing upon LPS stimulation, which is necessary for its activation [85]. Early studies suggested that beside caspase-1, caspase-5 is also a component of the NLRP1 inflammasome, and may participate in IL-1β processing [55]. Nevertheless, the mechanisms involved in the activation and function of caspase-5 in the non-canonical pathway are less known. However, recent studies confirm the presence of an alternative NLRP3 pathway in human cells, and suggest that both caspase-5 and caspase-4 act independently and upstream of the inflammasome [86, 87].

The activation of inflammasomes ultimately leads to cytokine release, however, unlike most cytokine, IL-1β and IL-18 lack a signal peptide for protein secretion. These cytokines are released from the cell by a poorly understood pathway, termed "unconventional protein secretion", which occurs independently of the classical ER/Golgi pathway [88, 89]. There are several various observations in the literature that provide possible explanation or speculation on the secretion mechanism of the leaderless IL-1β cytokine: (a) via exocytosis of secretory lysosomes [90], (b) by shedding of microvesicles (100-600nm) or exosomes (50-80nm) [91, 92], and (c) through caspase-dependent formation of gasdermin D pores (10-33 nm) in the plasma membrane (pyroptosis) [77, 78]. Again, despite the intensive studies, the precise mechanism(s) of IL-1β secretion has not yet been clarified.

Due to its diverse and indispensable functions in inflammatory- and immune responses, IL-1β is considered as a master regulatory cytokine. For example, IL-1β induces the production of other inflammatory cytokines and chemokines and it also enhances the expression of adhesion molecules on endothelial cells, which trigger infiltration of inflammatory and immune cells. In addition, IL-1β enhances pain sensitivity, causes fever and vasodilation, thereby orchestrating local and systemic inflammatory responses underlying a broad spectrum of diseases (Familial Mediterranean fever, Adult-onset Still's disease, Chronic neutrophilic respiratory disorders). Since this inflammatory cytokine is implicated in many diseases (Table 2), it became a focus of the pharmaceutical industry. Following

thorough studies, some agents were developed and are currently available to interfere with IL-1β actions. The first successful drug in this class was a recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1Ra) known as Anakinra [93]. Later, two additional IL-1-targeted agents were described with longer half-lives and fewer side effects in contrast to Anakinra; the soluble decoy receptor Rilonacept and the neutralizing monoclonal antibody Canakinumab [94, 95]. Further drug development targeting inflammasome activation is a promising, continuously expanding field. Because of the crucial role of caspase-1 enzyme in IL-1β production, there have also been advances in generating specific and clinically relevant caspase-1 inhibitors; such as VX-765, an orally available pro-drug [96]. Moreover, a compound named MCC950 was developed to directly target NLRP3 inflammasome to block IL-1β release, and animal experiments show promising results which indicate its potent role in the treatment of auto-inflammatory syndromes [97].

Inflammasome	Members	Activators	Disorders
NLRP1	NLRP1, ASC (not required), caspase-1, caspase-5 (in human) / caspase-11 (in mouse)	Anthrax lethal toxin, Muramyldipeptide (MDP), beta amyloid, LPS	Alzheimer's disease, vitiligo, type I diabetes melitus, Addison disease, rheumatoid arhtritis, leprosy, glaucoma
NLRP2	NLRP2, ASC, caspase-1	LPS, IFNγ, IFNβ, ATP	Beckwith-Wiedemann syndrome
NLRP3	NLRP3, ASC, caspase-1	LPS, ATP, ROS Beta amyloid Crystals, alum adjuvant Prion proteins	Cryopyrinopathies Protein misfolding disease Particulate matter-induces disease
NLRP6	NLRP6, ASC, caspase-1	LPS, Viral RNA	Risk in IBDs
NLRP7	NLRP7, ASC, caspase-1	Microbial acylated lipopeptide, LPS	Hydatidiform mole
NLRP12	NLRP12, ASC, caspase-1	Yersinia Pestis, Salmonella typhimurium	Hereditary inflammatory disease, atopic dermatitis
NLRC4 (IPAF)	NLRC4, ASC (not required), caspase-1	Flagellated and non-flagellated pathogens	Macrophage activation syndrome (MAS), Alzheimer's disease

Table 2. Inflammasomes: intracellular regulators of infection and inflammation

2.3. NLRP3 inflammasome

NLRP3 was first described in 2002 as PYPAF1 (PYRIN-containing Apaf1-like protein1) or Cryopyrin that may regulate the activity of caspase-1 through its interacting partner, ASC [98]. Today, NLRP3 is the most studied member of the NLR family, and characterization of the NLRP3 inflammasome represents a considerable advance in the understanding of inflammatory molecular events.

Disorders		Triggers
Cryopyrinopathies	Familial cold autoinflammatory syndrome (FCAS)	Mutation in NLRP3 (Cold exposure)
	Muckle-Wells syndrome (MWS)	Mutation in NLRP3
	Neonatal-onset, multisystem inflammatory disorder (NOMID)	Mutation in NLRP3
Protein misfolding disease	Prion disease	Fibril PrP
	Alzheimer's disease	Beta amyloid
	Type 2 diabetes mellitus	Islet amyloid polypeptide (IAPP)
	Parkinson's disease	Alpha synuclein
Particulate matter-induces disease	Gout	Uric acid/urate crystals
	Silicosis	Silica crystals
	Asbestosis	Asbestos fiber
Others	Bleomycin-induced pulmonary neutrophilic disease	Bleomycin
	Allergy (allergic rhinitis, contact allergic dermatitis)	Several allergen

Table 3. Disorders associated with NLRP3-mediated IL-1β production.

Considering the key role of IL-1β in inflammatory processes, it is not surprising that uncontrolled NLRP3 inflammasome activity may cause serious inflammatory diseases. One group of disorders caused by the malfunction of NLRP3 inflammasome is Cryopyrinopathies. These auto-inflammatory syndromes are characterized by recurrent systemic inflammation that involves several tissues including joints and skin (Table 3). FCAS, MWS and NOMID are all caused by dominantly inherited or *de novo* mutation in NLRP3. Studies suggest that these mutations have a gain-of-function effect, probably through the loss of a regulatory step associated with NLRP3 inflammasome activation that results in the uncontrolled production of IL-1β [99, 100]. Studies have shown that NLRP3 inflammasome activation is also a key feature in the pathogenesis of protein misfolding diseases, including neuropathic diseases (such as Prion diseases, Alzheimer's disease and Parkinson's disease) characterized by protein aggregation in the central nervous system [101]. Protein aggregation contributing to

diseases can also appear in peripheral tissues as in the case of Type 2 diabetes (T2D). This non-neuropathic disease is a highly prevalent and chronic metabolic disorder, however recent evidence suggests that the formation of toxic aggregates of the islet amyloid polypeptide (IAPP) might contribute to β-cell dysfunction through activation of the NLRP3 inflammasome [102, 103]. The chronic histopathology of occupational diseases (gout, silicosis and asbestosis) is thought to result from several immunopathologic pathways, including the high concentration of IL-1β due to the NLRP3 inflammasome activation induced by urate or silica crystals, as well as asbestos [104, 105]. Other studies associated NLRP3 inflammasome`s function with a variety of allergic responses that is caused by several allergens, such as ragweed pollen or dust mite [106, 107].

Activation of the NLRP3 inflammasome is triggered by a broad spectrum of stimuli that belong either to PAMPs or DAMPs (Table 2 and 3). Due to the potent role of IL-1β in the regulation of various immune responses, and in the development of several diseases, the expression and effect of IL-1β are tightly controlled at both transcriptional and post-transcriptional level.

The NLRP3 inflammasome-mediated IL-1β secretion employs a two-step activation mechanism that is spatially and temporally separated. The first so called “priming” signal is typically induced by diverse pathogen-related molecules through specific receptors such as Toll-like receptors. This event results in the transcriptional upregulation of pro-IL-1β and the inflammasome components through diverse signal transduction pathways like those involving p38, ERK and NF-κB [108-110]. The second signal leads to the formation of inflammasome complexes, which initiates a proteolytic cleavage of caspase-1 and mediates the maturation of pro-IL-1β into active cytokine [111, 112]. This signal can be triggered by many microbial (PAMPs) or non-microbial (DAMPs) molecules.

Today it is generally accepted that NLRP3 inflammasome-mediated IL-1β secretion is not always associated with the presence of an infectious agent. This observation stems from the fact that IL-1β production was implicated in several ‘sterile’ inflammatory diseases that were induced by endogenous / self-derived (urate crystals, misfolded proteins) or exogenous / environmental (silica crystals, asbestos) compounds. In some cases of ‘sterile’ inflammation, these compounds directly trigger both signals required for inflammasome activation [113, 114]. On the other hand, diseases induces by ‘sterile’ compounds may lead to the production and accumulation of other inflammatory molecules; such as TNFα, which can prime the inflammasome, thereby mediates the sensation of self-derived or environmental compounds [115].

Accordingly, a wide variety of molecules are able to induce the activation of the NLRP3 inflammasome, upon either infections or in sterile inflammation, in the absence of pathogens (Table 2 and 3). Considering the broad spectrum of NLRP3 activators, it is unlikely that all of these different stimuli are sensed directly. Instead, it is generally believed that all of these signals converge to a common molecular event that eventually activates the complex. Several cellular mechanisms for NLRP3 activation have been proposed, such as sensing of K^+ ion efflux due to microbial toxins or ATP; mtROS or mtDNA due to mitochondrial destabilization or sensing of cathepsins due to lysosomal instability.

2.3.1. Potassium efflux as activator of NLRP3 inflammasome

One of the main signals for NLRP3 inflammasome activation is the intracellular potassium level which is crucial for the activation of caspase-1. Potassium efflux may be achieved either by microbial toxins such as nigericin acting as potassium ionophores, or by the opening of potassium channels as a response to increased extracellular ATP concentration. [116, 117].

ATP is the source of free energy in most of cells, but tissue damage and inflammation may lead to the release of high doses of ATP, that act as danger signal, leading to the activation of an immune response. Extracellular ATP may be recognized by purinergic P2 receptors, specifically, either by G-protein coupled P2Y or nucleotide-gated ion channel P2X receptors. Among these P2 receptors, P2X7 receptor sub-type has been associated with inflammasome assembly and IL-1β release. P2X7 receptor is expressed on both innate and adaptive immune cells; such as macrophages, lymphocytes, mast cells and monocytes [118-120], and appears to play a predominant role in inflammation and autoimmune diseases [121]. P2X7 receptor is a homotrimer, with each monomer containing two transmembrane domains and an ATP-binding extracellular loop. In response to ATP, P2X7 receptor mediates the influx of Ca^{2+} and Na^+ and the efflux of K^+.

However, compared to wild-type animals, LPS-activated peritoneal macrophages from P2X7R-/- animals fail to generate mature IL-1β when challenged with ATP. This defect is not because of an inability of the macrophages to express pro-IL-1β, but rather the inability of the inflammasome activation [122]. In contrast to P2X7 receptor, activation of the other ATP receptor P2Y2 has been recently implicated in the directed migration (chemotaxis) of immune phagocytes, such as macrophages and neutrophils. In macrophages, extracellular ATP

released from an extrinsic source; such as apoptotic cells, directly acts as a ’find-me’ signal, and this recruitment was significantly decreased in P2Y2-/- mice [123].

Activation of NLRP3 inflammasome by binding of ATP to P2X7 receptor was shown years ago to act through a mechanism dependent on potassium (K^+) efflux [124]. When potassium efflux is prevented experimentally by drugs or high concentration of K^+ in the cell culture media, activation of the NLRP3 inflammasome in response to almost all known activators is abolished [125, 126].

Extracellular concentration of ATP is controlled by ectonucleotidases in a coordinated process (Figure 1). First, ATP is hydrolyzed to AMP by member of the ectonucleoside triphosphate diphosphohydrolase CD39 (NTPDase1), in a Ca^{2+} and Mg^{2+} -dependent manner [127]. AMP is then rapidly converted to adenosine by surface ectoenzyme CD73 (ecto-5’-nucleotidase) [128, 129]. CD39 and CD73 expression on macrophages may change depending on the macrophage activation state.

Adenosine accumulates extracellularly in response to inflammation, cell stress and death by release from cytosolic stores or sequential dephosporylation from ATP. Adenosine, which regulates many physiological processes, may be recognized by four adenosine receptor subtypes: A1, A2a, A2b and A3. These G-protein-coupled receptors contain seven transmembrane domains, and belong to the P1 purinergic receptor family. A1 and A3 subtypes inhibit adenylate cyclase, whereas A2a and A2b activate adenylate cyclase enzyme, resulting in the elevation of cAMP and the activation of protein kinase A (PKA) [130, 131].

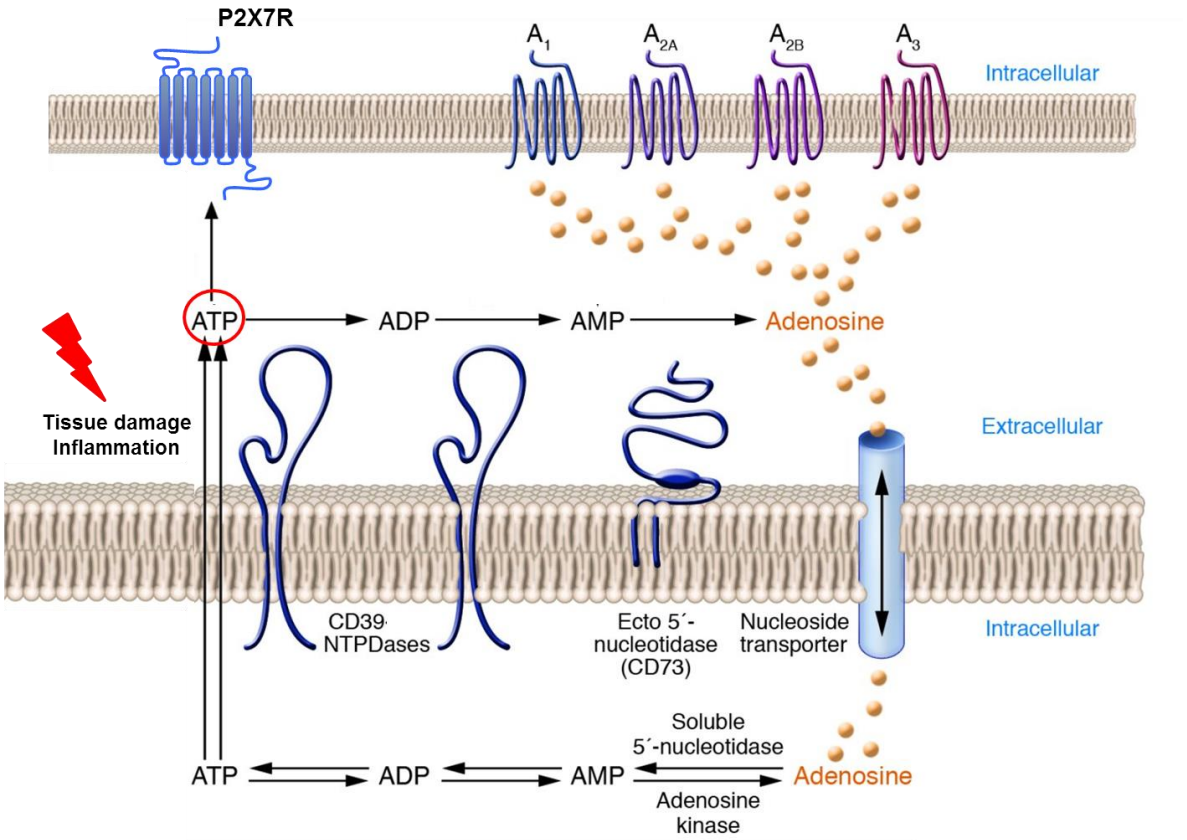


Figure 1. Regulation of extracellular ATP level (modified from Tilley SL and Boucher RC, Journal of Clin. Invest, 2005)

2.3.2. ROS as activators of NLRP3 inflammasome

As a second signal, Reactive Oxygen Species (ROS); toxic substances produced by immune cells in response to microbial invasion, have been widely implicated in NLRP3 inflammasome activation due to the many diverse stimuli (ATP, alum, uric acid, nigericin etc.) that induce ROS generation.

ROS is a term used to describe a number of reactive molecules and free radicals derived from molecular oxygen. These molecules are produced as byproducts during the mitochondrial electron transport of aerobic respiration, or by the activity of cellular enzymes. All aerobic life forms provide a balance between production and removal of ROS for survival. There are a number of antioxidants that play a role in ROS detoxification, and the general observation is that antioxidants are potent inhibitors of NLRP3-dependent IL-1β production.

It has been reported that antioxidants can inhibit IL-1β secretion after LPS stimulation [132], as they prevent NLRP3 expression, indicating that ROS may act on the level of the priming step [133]. However, Zhou et al. demonstrated that the oxidative stress mediator thioredoxin-interacting protein (TXNIP) associated with NLRP3, leading to inflammasome activation upon increase of cellular ROS [134]. These results show that ROS may influence both the priming signal, and the activating signal for NLRP3 inflammasome activation.

Mitochondria constitute the biggest source of cellular ROS, and NLRP3 has been reported to associate with the mitochondria upon activation, suggesting that NLRP3 might sense these molecules [135]. Following studies also supported the fact that scavenging of mtROS efficiently suppresses activation of the NLRP3 inflammasome [136, 137]. Furthermore, Wu et al. revealed that targeted inhibition of mtROS but not inhibition of NADPH oxidase-derived ROS, prevented IL- 1β production in alveolar macrophages. [138].

Theoretically, the membrane-bound NADPH oxidase (NOX) complex may be an alternative intracellular ROS source. Multiple lines of evidence suggest that induction of ROS production by NLRP3 activators may involve NOX, as NOX2 deficiency impairs ATP-mediated ROS generation by macrophages [139]. However, studies suggested that NLRP3 stimulation is NOX1-4 independent in macrophages [140], since macrophages derived from patients with chronic granulomatous disease due to mutations of this complex were still capable of secreting IL-1β in response to DAMPs [141, 142].

Despite of intensive studies, there is still much controversy in the field, and the precise mechanism of ROS-regulated NLRP3 activation is still unknown. There are other enzymatic systems that contribute to ROS production, including xanthine oxidoreductase (XOR), lypoxygenases (LOXs), cyclooxygenases (COXs), and cytochrome P450s, whose implication in NLRP3 inflammasome activation remains to be explored.

2.3.3. Mitochondrial dysfunction as activator of NLRP3 inflammasome

Recent evidence suggests that mitochondrial events appear to be closely related to NLRP3 inflammasome activation. In addition to mtROS, oxidized mitochondrial DNA (mtDNA) released into cytoplasm from the damaged mitochondria has also been reported to directly induce NLRP3 inflammasome activation [143, 144]. In healthy host cells, DNA is enclosed in the nucleus and mitochondria, precluding its ability to activate cytoplasmic DNA sensors. The mechanism of how danger signals derived from the damaged mitochondria affect the activation of NLRP3 inflammasome is still unexplored. There are also conflicting results

regarding the connection between mitochondrial damage and NLRP3 activation. Yu et al. suggest that mitochondrial damage might be the consequence of inflammasome activation, rather than the cause [145]. In contrast, recent studies show that accumulation of damaged mitochondria and excessive release of mtDNA in the cytoplasm due to impaired autophagy can lead to hyperactivation of the NLRP3 inflammasome [132, 146]. Recently, mitochondria-selective autophagy; termed mitophagy, has emerged as a central player for prevention of hyperinflammation triggered by NLRP3 inflammasome activation. Limitation of IL-1β production by autophagy is targeting ubiquitinated inflammasomes for destruction [147]. It was first published in 2012, that pharmacological inhibition of NLRP3 deubiquitination completely blocked NLRP3 activation in both mouse and human cells, indicating that deubiquitination of NLRP3 is required for its activation [148].

2.3.4. Lysosomal destabilization and increased icCa^{2+} level as activators of NLRP3 inflammasome

Sterile particulate and crystalline; including monosodium urate crystals (MSU), alum, silica, amyloid-β, calcium pyrophosphate dehydrate as well as infectious bacteria (i.e. Mycobacterium tuberculosis) are phagocytosed by antigen presenting cells. This mechanism results in lysosomal rupture and the cytoplasmic release of lysosomal proteases, including cathepsin B, which has been reported to be involved in NLRP3 activation [149]. However, the requirement for this protease in this process is controversial. The role for cathepsin B in NLRP3 activation was suggested by studies showing that a specific cathepsin B inhibitor (Ca074Me) suppresses IL-1β production induced by different stimuli that results in lysosomal degradation [149-151]. In contrast, subsequent studies demonstrated that knock-down of cathepsin B had no or moderate effect on NLRP3 inflammasome activation [125, 152]. Thus, the efficacy of Ca074Me in selectively inhibiting cathepsin B is unclear. Indeed, Orlowski et al. demonstrated that multiple cathepsins promote NLRP3-mediated IL-1β production, especially cathepsin X [153]. Furthermore, other studies suggest that the internalization of particulate matter via phagocytosis induces lysosomal membrane damage, which triggers K^+ efflux [125].

It is well known that potassium can regulate Ca^{2+} signaling through its effect on plasma membrane polarization, thus promoting Ca^{2+} influx. Notably, lysosomal destabilization or rupture is sufficient to trigger Ca^{2+} mobilization [154]. Based on the ability of the selective Ca^{2+} chelator BAPTA-AM in inhibiting IL-1β secretion, Ca^{2+} was suggested

as a key molecular regulator of the NLRP3 inflammasome [155]. This consequence was also proposed by observations showing that extracellular ATP; which activates NLRP3 inflammasome through P2X7R, also induces the elevation of intracellular Ca^{2+}. Furthermore, induction of calcium-sensing receptor (CASR) also causes NLRP3 inflammasome activation [156]. Collectively, these findings indicate that Ca^{2+} signaling may play a common role in NLRP3 inflammasome activation.

2.3.5. Intracellular localization of NLRP3 inflammasome

Under resting conditions, most NLRP3 proteins were shown to associate with the endoplasmic reticulum (ER) homogeneously in the cytosol. This localization changed significantly after inflammasome stimulation, where NLRP3 located into the perinuclear space and co-localized with ER and the mitochondria-associated ER membranes (MAMs) [135]. The research group of Oslowski recently demonstrated that NLRP3 inflammasome activation emerges dominantly from the ER stress-associated inflammation [157]. MAMs are part of the ER and regulate calcium homeostasis, mitochondrial function as well as autophagy, all of which have been shown to be implicated in activation of the NLRP3 inflammasome. Recent studies also indicate that impaired contact between MAMs and mitochondria might underlie the pathology of several human neurodegenerative diseases, including Alzheimer's disease, which is associated with NLRP3-mediated IL-1β overproduction [158].

Recently, mitochondria were shown to serve as molecular platforms for the NLRP3 inflammasome. It was reported that the mitochondrial antiviral signaling (MAVS) protein associates with NLRP3 and facilitates its oligomerization, leading to caspase-1 activation [159]. Subsequently, it was explained by that the activation of RIG-I/MAVS pathway converges into an intracellular potassium lowering step, which induces NLRP3 activation [160]. These observations confirm that the intracellular localization of NLRs is emerging as a critical determinant of its function.

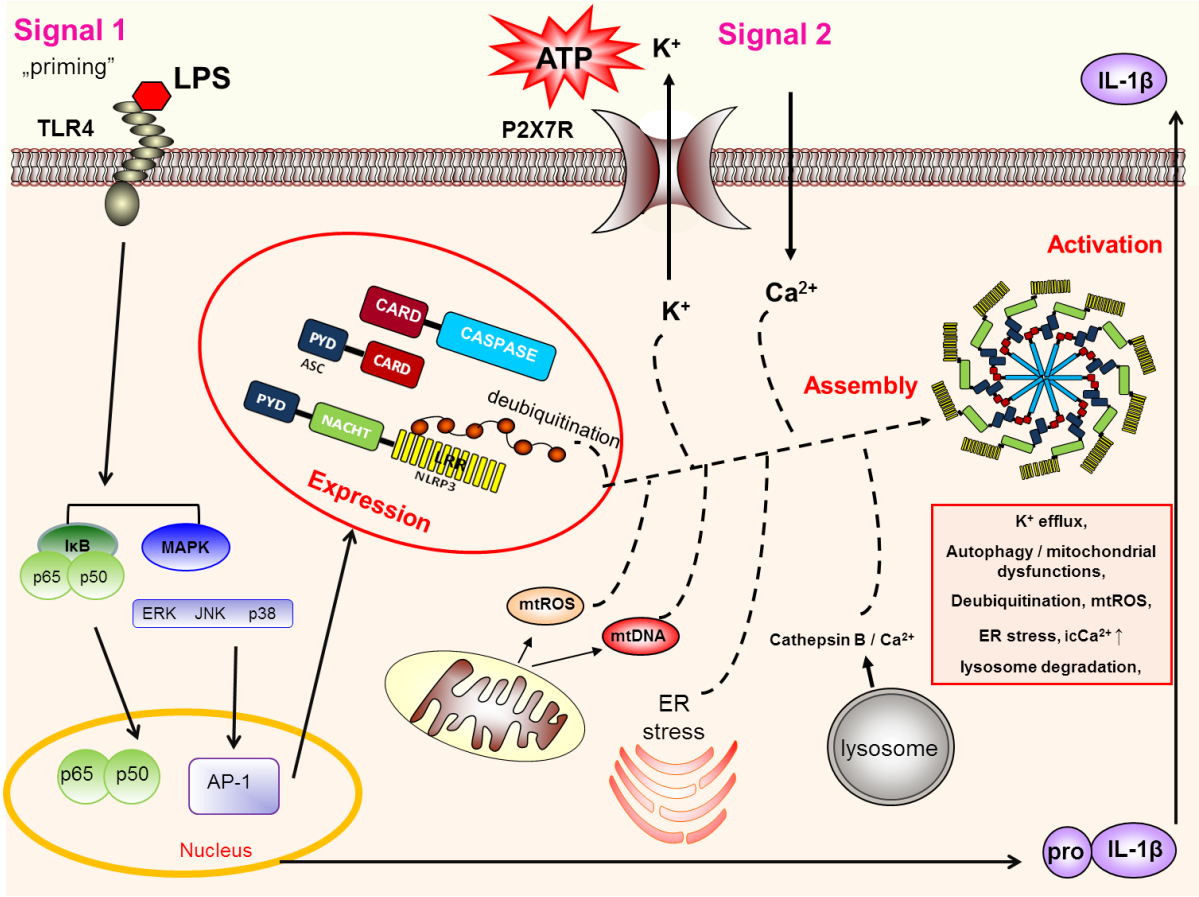


Figure 2. Mechanisms of NLRP3 inflammasome activation. Activation of the NLRP3 inflammasome occurs in two steps. The priming step involves an initiating signal in which PAMPs (e.g. LPS) recognized by TLRs (e.g. TLR4) lead to the activation of signal transduction pathways (e.g. NF-kB, MAPKs), which in turn up-regulates transcription of inflammasome - related components. The second step of inflammasome activation which involves the assembly of NLRP3, ASC and pro-caspase-1 into a complex may be triggered by many stimuli (e.g. K^+ efflux, autophagy dysfunctions, mitochondrial dysfunctions, mtROS, deubiquitination, lysosome degradation, ic. Ca^{2+} increase, ER stress).

2.4. The lipopolysaccharide receptor, TLR4

Toll-like receptors (TLRs) were the first identified PRRs [161, 162]. Discovery of the role of TLRs has greatly advanced the field of innate immunology, and was honored with the Nobel Prize to Jules Hoffmann and Bruce Beutler in 2011. Until now, 10 functional members (TLR1-TLR10) of TLR family were described in human, with each having a different PAMP or DAMP specificity. Some TLRs are expressed on the cell surface (e.g. human TLR1, 2, 4, 5, 6 and 10) and activated by microbial components including proteins, lipoproteins or lipids, while others are expressed in intracellular vesicles (e.g. human TLR3, 7, 8 and 9) to recognize nucleic acids of pathogens that have been taken into cells by phagocytosis or receptor-mediated endocytosis. Interestingly, although TLR11, TLR12 and TLR13 are expressed and functional in mice, human TLR11 is a nonfunctional pseudogene and humans completely lack TLR12 and TLR13.

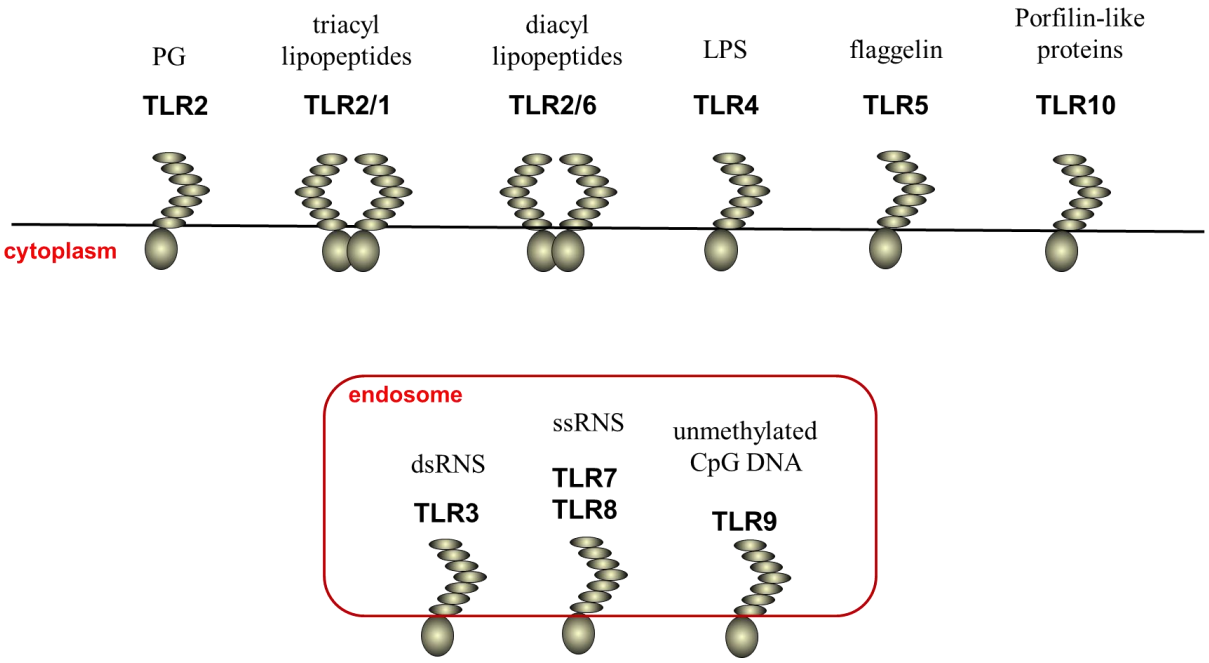


Figure 3. The cardinal ligands of Toll-like Receptors

TLRs are type I transmembrane proteins, characterized by an extracellular domain containing leucine-rich repeats (LRRs) that mediate the recognition of a wide range of molecular patterns, and are involved in co-receptor interactions. The cytoplasmic tail contains a conserved region called TIR (Toll–IL-1 receptor) domain that activates downstream signaling pathways. TLR signaling involves a family of five adaptor proteins. Different

adaptors engage different receptors, and the particular adaptor used determines which signaling pathway will be activated. TLRs trigger signaling cascades that promote gene transcription of cytokines, interferons, and other pro-inflammatory or antimicrobial proteins [163].

Beutler's group has revealed that TLR4 is activated by a potent PAMP, the lipopolysaccharide (LPS), which is the major component of the outer membrane of Gram-negative bacteria. LPS comprise a hydrophobic component known as lipid A, which is responsible for the major bioactivity of the endotoxin, and a hydrophilic polysaccharide termed the O antigen, which is attached to the core oligosaccharide. The surface membrane-bound TLR4 is expressed by many immune cells; such as monocytes, macrophages, neutrophils and dendritic cells, or the non-immune endothelial cells. The pro-inflammatory action of LPS is crucial to orchestrating an appropriate antimicrobial defense, but excessive host responses to LPS can lead to systemic inflammatory conditions such as severe sepsis, with a mortality rate of 30-50%.

Stimulation of TLR4 facilitates the activation of two pathways: the MyD88- (myeloid differentiation primary response protein 88) dependent and MyD88-independent pathways. Macrophages from MyD88-/- mice are defective in LPS-induced secretion of cytokines (e.g., IL-6, TNFα and IL-1β), highlighting the importance of MyD88 in the expression of TLR4-mediated pro-inflammatory cytokines [164]. Many studies have demonstrated that LPS stimulation of human monocytes or macrophages activates several intracellular signaling pathways that include the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway and three MAPK (mitogen-activated protein kinase) pathways: ERK 1/2 (extracellular signal-regulated kinases 1 and 2), JNK (c-Jun N-terminal kinase) and p38. These signaling pathways in turn activate a variety of transcription factors, which coordinate the induction of many genes encoding inflammatory mediators [165].

Upon LPS stimulation, MyD88 mediates the activation of the downstream molecules, including IRAKs (IL-1-receptor-associated kinases) and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6), leading to activation of the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex). Thereafter the IKK complex (IKKβ, IKKα, and NEMO) phosphorylates IκBα, which leads to its ubiquitination and proteasomal degradation. This allows NF-κB transcription factor complex (p65-p50) to translocate to the nucleus and induces the expression of its target genes [166]. Crucially, p65 also stimulates an auto-regulatory loop to resynthesize IκBα that eventually restores the pathway to an inactive state [167]. Exposure of mammalian cells to LPS has also been shown to activate MAPKs, leading

to the activation and nuclear translocation of AP-1 and its binding to DNA responsive elements, which initiates the release of pro-inflammatory cytokines [168]. To limit or terminate the inflammatory response, virtually all immune cells can also produce anti-inflammatory cytokines. IL-10 is a key component of this cytokine system, which regulates and suppresses the expression of pro-inflammatory cytokines during the recovery phases of infections, consequently protecting the host from tissue damage caused by inflammatory cytokines [169]. Regulation of IL-10 production in immune cells is complex and depends on cell type [170].

Activation of MyD88-independent pathway involves the recruitment of adaptor proteins TRIF (TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule). Activation of the downstream molecules TBK1 (tank-binding kinase 1) and IKK mediate the nuclear translocation of IRF3 (IFN regulatory factor 3) transcription factor, inducing transcription of Type I interferons [171].

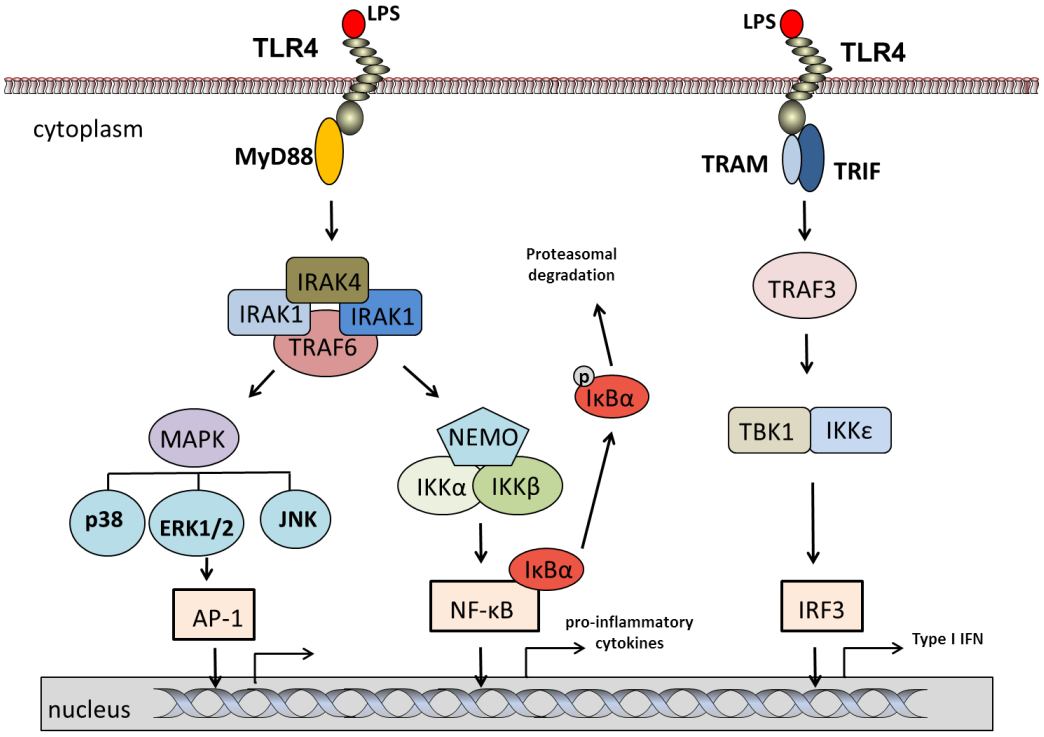


Figure 4. Overview of major TLR4 signaling pathways.

2.5. Macrophages

Macrophages belong to the mononuclear phagocytic system and are present in virtually all tissues. Macrophages were identified by the Russian zoologist, Élie Metchnikoff more than 100 years ago. His discovery of phagocytes and major defense mechanisms in innate immunity was awarded with the Nobel Prize for Medicine in 1908. Due to his crucial discoveries about phagocytosis, he had a huge impact on understanding this field, and we have learned a lot about the distribution of macrophages, their heterogenous phenotypes, and their complex functions in tissue homeostasis as well as in innate and adaptive immunity.

Macrophages originate from hematopoetic stem cells in bone marrow, which develop into blood circulation monocytes, followed by regulated migration into different tissues, either in steady state or upon inflammation. There, they finally differentiate into tissue-specific macrophages [172]. Macrophages are potent inducers and regulators of immune responses, additionally, they have a central role in the orchestration of many other physiological functions as well. In wound healing for instance, macrophages participate in inflammation, but also in new tissue formation and tissue remodeling through communication with keratinocytes [173], fibroblasts and myofibroblasts [174], as well as endothelial cells [175]. It is now widely accepted that macrophages are also major cellular mediators of chronic inflammation in almost all tissues and diseases [176].

Macrophages have a remarkable role in different phases of the inflammatory response to infections or damages. They produce cytotoxic compounds; pro-inflammatory cytokines, to fight invading microbes. They then contribute to the resolution of inflammation by producing various growth factors to promote the function of other cells or anti-inflammatory cytokines, to counteract the tissue damage. These factors are produced by different subpopulation of macrophages.

Macrophages are very plastic and may be characterized by different morphological and functional properties. The development and polarization of MFs is highly dependent on the specific microenvironment [177-179]. Homeostatic control of monocyte/macrophage development is mostly influenced by macrophage colony stimulating factor (M-CSF), and it supports the steady-state differentiation of macrophages. Granulocyte-macrophage colony stimulating factor (GM-CSF) is another factor involved in the development of mononuclear phagocytes, but only during the inflammatory state and not under homeostatic conditions [180]. Several protocols have been developed in order to model these systems and enable the detailed study of MFs in *in vitro* conditions. In murine, MFs are purified from the peritoneum

or the alveolar lavage, or differentiated from bone-marrow in the presence of M-CSF. While in human, MFs are differentiated from monocytes, and either M-CSF or GM-CSF provides the condition for their development. It is important to note that classification of macrophages described by the general terms of M1 or M2 phenotypes are based on the different stages of macrophage activation. This terminology originated from an observation that classically activated macrophages are induced by microbial products and cytokines, particularly IFN-γ produced by Th1 cells (M1). In contrast, alternatively activated macrophages are induced by IL-4 and IL-13 produced by Th2 cells (M2). M1 macrophages exhibit inflammatory functions, whereas M2 macrophages have more anti-inflammatory characteristics [181]. This classification of macrophages is now considered an oversimplified approach that does not adequately describe the spectrum of macrophage populations. For instance, the nomenclature has changed over time as nowadays M1 type has three subgroups, moreover, M2 type is broader and includes four or more subgroups [182-185]. The growing number of different subsets of macrophage population has recently put the heterogeneity of these cells into sharp focus. Today, most scientists agree that a more informative foundation for macrophage classification should be based on fundamental macrophage functions that are involved in maintaining homeostasis.

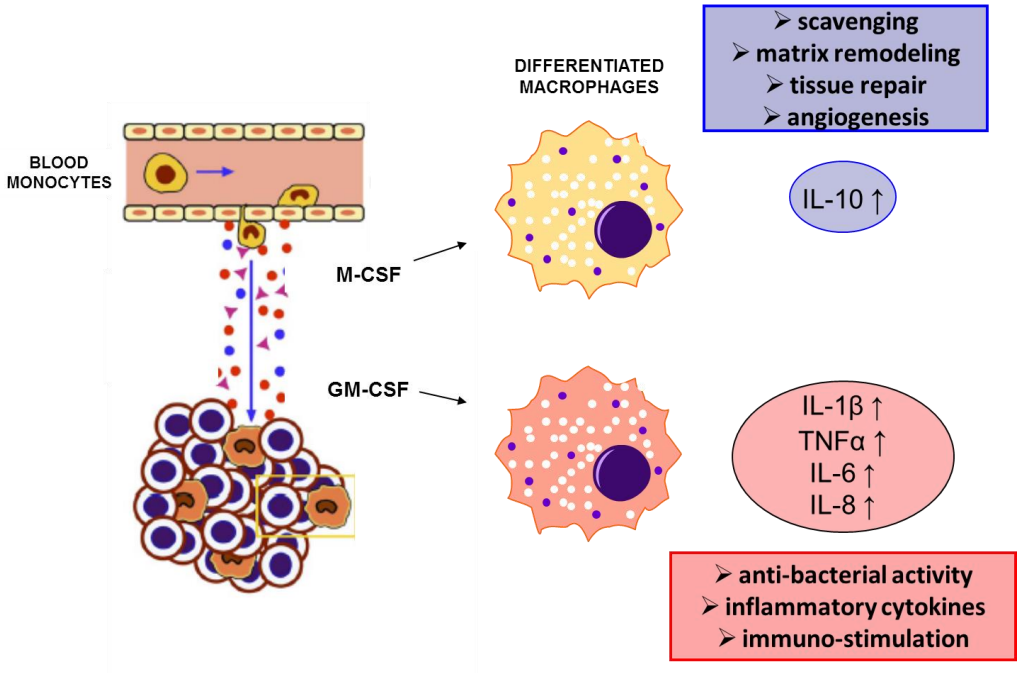


Figure 5. Effects of macrophage lineage-differentiation factors (M-CSF/GM-CSF) on the differentiation and main function of macrophages. (modified from Sica A. et al., Eur J Cancer. 2006)

2.5.1. Macrophages differentiated by M-CSF (M-MFs)

Macrophage colony stimulating factor (M-CSF) may be produced spontaneously or after stimulation by numerous cell types; such as endothelial cells, fibroblasts [186], monocytes [187] activated B cells [188] and T cells [189], or bone marrow-derived stromal cells [190]. M-CSF binds to a single high affinity; type III tyrosine kinase receptor, the M-CSFR, and induces its dimerization and activation. Consequently, numerous signal transduction pathways are initiated, including Stat1, Stat3 [191], phosphoinositol-3 (PI-3) kinase [192], protein kinase C [193], Mona [194] and MAPK/ERK pathways [195].

M-CSF can be detected in plasma at ~ 10 ng/ml concentration, and increased levels of circulating M-CSF have been observed in several autoimmune diseases, including arthritis, kidney inflammation, pulmonary fibrosis, obesity, inflammatory bowel disease, and cancer metastasis [196-199]. Furthermore, mutations in the M-CSF receptor in humans lead to myelodysplastic syndromes or acute myeloid leukemia [200], and the mutations have also been associated with hereditary diffuse leukoencephalopathy [201].

A continuous M-CSF stimulation is required to induce monocytes to differentiate. In the presence of M-CSF, monocyte differentiation is associated with early (day 3) substantial changes in cell-cycle genes. Further analysis of the effects of M-CSF also highlighted a concerted down-regulation of chemokine receptors, cystatins, defensins and pro-inflammatory cytokines; such as IL-1 and IL-8, but the opposite effect was detected for complement components. In contrast to pro-inflammatory cytokines, expression of the anti-inflammatory IL-10 increases dramatically in response to TLR activation in M-CSF-differentiated cells [202]. IL-10 is an inhibitor of activated macrophages and dendritic cells, and thus, involved in the control of innate immune reactions [203, 204]. IL-10 functions as a negative feedback regulator, because it is produced by macrophages, and simultaneously, it is able to inhibit their functions. In M-CSF- differentiated macrophages, relevant percentage of highly expressed genes are associated with cellular metabolic activities; such as active transport and oxidoreductase activities. In an effort to characterize M-CSF-differentiated macrophages, several lines of evidences suggest a major role in host defense, wound healing and immune regulation [205].

2.5.2. Macrophages differentiated by GM-CSF (GM-MFs)

Granulocyte macrophage colony stimulating factor (GM-CSF) is produced by a wide variety of cell types, including activated T cells, B-cells, macrophages, endothelial cells, fibroblasts and tumor cells [180, 206]. The GM-CSF receptor is a heterodimer comprising a cytokine-specific α-chain and a common β-chain which is shared by two others receptor family of cytokines, IL-3 and IL-5. The α-chain contains a binding site for cytokine and the β-chain is involved in signal transduction. Receptor activation, which occurs through receptor-associated Janus kinases (JAKs); predominantly JAK2, leads to activation of further downstream molecules, such as STAT, MAPK and PI3K pathways [207, 208].

Human macrophages generated by GM-CSF are associated with induction of inflammatory properties. GM-CSF promotes these characteristics via induction of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα, and chemokines, such as CCL22, CCL24, CCL5 and CCL1, which contribute to leukocyte recruitment [209, 210]. Furthermore, these macrophages strongly express the costimulatory receptors CD86 and CD80 or HLA-DR underlining their role as potent antigen presenting cells.

Nevertheless, elevated tissue levels of GM-CSF have been detected in multiply inflammatory conditions, including RA, multiple sclerosis, obesity, lung disease and cancer [202].

It is important to note that in mouse, GM-CSF treatment of bone marrow cells induces the development of dendritic cells, while M-CSF-treated bone marrow cells are commonly used to generate macrophages. In contrast, in human, immature DCs are differentiated from monocytes in the presence of GM-CSF plus IL-4. At the same time, M-CSF- or GM-CSF-treated human blood monocytes are a frequently used, which comprise a convenient population to study macrophage function and signaling. Though both differentiation methods results in the generation of macrophages, M-CSF shifts the phenotype of macrophages toward an anti-inflammatory direction, whereas GM-CSF promotes a pro-inflammatory phenotype. These opposing effects of M-CSF and GM-CSF on macrophages have been linked to “M2 and M1 macrophages”, respectively, although such terminology should be applied with caution, as M- and GM-MFs represent the differentiation state and not the activation state of these cells.

2.6. *Aloe vera*, an immunomodulatory natural compound

The development of novel therapies for immunologic diseases is based on the understanding of basic science, and on new promotions of experimental immunology. The mainstay of therapy for inflammatory diseases for many years has been synthetic, anti-inflammatory drugs; particularly non-steroidal drugs, corticosteroids, monoclonal antibodies that deplete lymphoid cells or target cytokines, as well as agents that inhibit cell-cell interactions and migration. Although these synthetic drugs have been beneficial in patients with inflammatory diseases, their long-term use increases the risk of several side effects. At present, the trend towards the use of natural remedies with fewer side effects has given rise to natural compound as an alternative therapy for diseases.

For the last 20 years, *Aloe vera* has been at the center of a global interest due to its therapeutic and nutritive substances extracted from leaves that are used in commercial preparations for pharmaceutical, cosmetic or alimentary purpose and as a fresh food. The more than 400 species of *Aloe* belong to the family *Liliaceae* that originates from dry subtropical and tropical climates, including South Africa and the Southern USA. Only a few species of *Aloe* have commercial importance, of which *Aloe barbadensis miller*, commonly referred to as *Aloe vera* is considered the most potent plant. *Aloe vera* has been used in traditional medicine in many cultures for over 2000 years, such as in China, India and Japan. Fresh leaves of *Aloe* contain various groups of chemical compounds such as: glycoproteins, polysaccharide, enzymes, vitamins, minerals, aminoacids, anthraquinones (emodin) and many others, which are under intense study. Over 75 active components have already been identified in leaf gels [211] and many of them have been implicated as immunomodulatory compounds.

Commonly, *Aloe vera* has been used externally to treat various skin conditions; such as cuts, burns and eczema. *Aloe* gel has a wound healing effect, and several mechanisms have been proposed as an explanation for its activity, including maintaining wound contraction, increase epithelial cell migration, more rapid synthesis of collagen and reduction in inflammation [212-214]. *Aloe* gel and its different bioactive molecules and formulation have been extensively studied to determine its anti-inflammatory and immunomodulatory properties. Studies, which examined anti-inflammatory activities of the *Aloe vera* gel, suggested that these effects occur *via* suppression of pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6) [215-217]. However, polysaccharides or the anthraquinone component, emodin in *Aloe vera* gel were demonstrated as activators of macrophages and NK cells [218-220].

Besides the anti-inflammatory effects, anti-microbial properties of *Aloe vera* has also been shown by studying bacterial infections, including Shigella flexneri, MRSA (Methicillin-Resistant Staphylococcus aureus), Enterobacter cloacae and Enterococcus bovis [217, 221].

Since *Aloe vera* has been traditionally used for skin-related injuries (e.g. burns, frostbite, cuts) or skin-care (e.g. hydration), there is high interest in the biological effects of this herbal on the dermis. *Aloe vera* was shown to increase water content of the stratum corneum, resulting in its moisturizing effects [222]. Furthermore, *Aloe vera* gel was identified as a skin permeation enhancer, which is explained by a probable pull effect of complexes formed between the drug and the aloe gel [223]. For this reason, *Aloe* is considered a potential penetration enhancement agent for delivery of drugs, but the proposed mechanism of action has to be further investigated and confirmed.

It has been reported by several studies that *Aloe* gel has anti-oxidant effects, probably through its components; such as glutathione peroxidase, superoxide dismutase enzymes and phenolic anti-oxidants [216, 224].

Other studies indicated the anti-cancer [225], anti-diabetic [226, 227], anti-rheumatoid effects [228] and also hematopoietic activity [229] for *Aloe vera* gel, confirming its benefits to the immune system. Moreover, *Aloe vera* is cheap, easily available and applicable with minimal equipment, properties which enable future development of multidirectional therapeutic action.

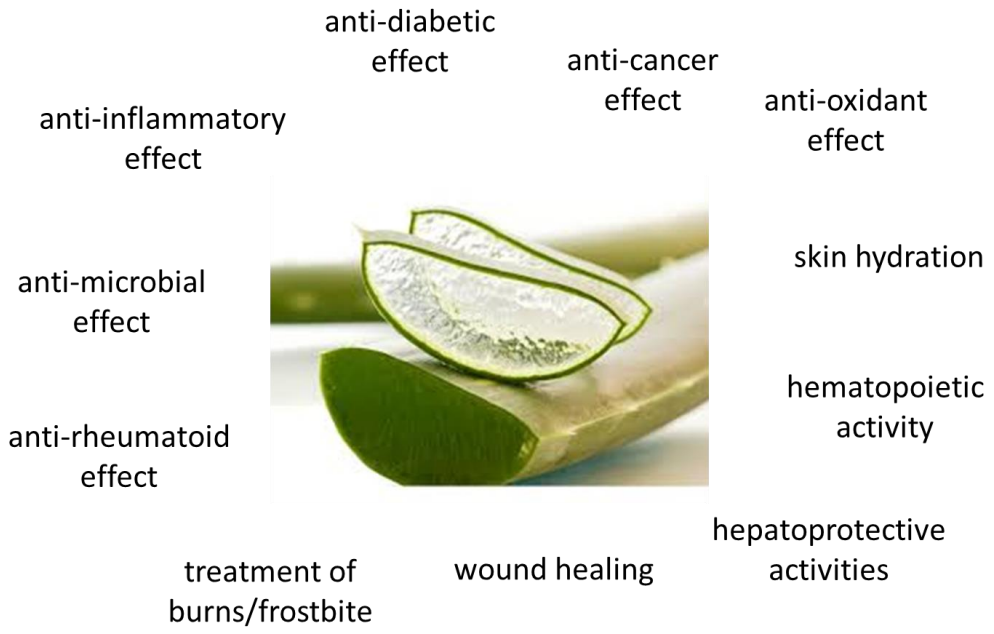


Figure 6. Effects of *Aloe vera*

2.7. Aims of the study

Aim 1. Study the different dynamics of NLRP3 inflammasome-mediated IL-1β production in human GM-MFs and M-MFs.

Though NLRP3 is one of the most studied inflammasome complexes, there are no comparative studies focusing on the LPS-primed IL-1β secretion *via* NLRP3 inflammasome activation in human MFs differentiated by M-CSF or GM-CSF. Despite plasticity and different morphological and functional characteristics of human macrophages, previous studies have focused mainly on general gene expression differences and some specific functions (such as phagocytosis, migration, etc.) of these cells. In order to gain a better understanding of the regulation of NLRP3 inflammasome by LPS-primed human macrophages our study aimed to:

- Monitor the time-dependent pattern of cytokine expression of LPS-primed M-MFs and GM-MFs.
- Identify the involvement of NLRP3 inflammasome-dependent caspase-1 activity in LPS-induced IL-1β production by both macrophage subtypes.
- Examine the signal transduction pathways and expression pattern of inflammasome components in the two macrophage types.
- Analyze the modulatory effect of released endogenous ATP and that of extracellular adenosine on IL-1β secretion.
- Investigate the effect of robust IL-10 secretion by M-MFs on IL-1β secretion.

Aim 2. Study the effect of immunomudolatory *Aloe vera* on LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages.

The anti-oxidant, anti-bacterial and anti-inflammatory benefits of *Aloe vera* have been examined and described in several studies, nevertheless, its role on the function of human monocyte-derived MFs; especially on the production of potent pro-inflammatory cytokine IL-1β, has not been investigated. Therefore, we aimed to study the effect of *Aloe vera* on

- Cytokine secretion, especially that of IL-1β by human macrophages.
- Expression of NLRP3 inflammasome members as well as the precursor of IL-1β.
- Possible modulatory effect of *Aloe* on the activation of the LPS-induced main signaling pathways.
- Expression of ATP-sensing P2X7 receptor in LPS-primed macrophages.

3. MATERIALS AND METHODS

Reagents

Ultrapure LPS from *Escherichia coli* was purchased from InvivoGen (San Diego, CA, USA). ATP, A740003 (selective P2X7 purinoceptor antagonist), ARL67156 (ecto-ATPase inhibitor), apyrase (high activity), ZM241385 (A2a-specific antagonist), PSB1115 (A2b-specific antagonist), DPCPX (A1-specific antagonist) and VUF5574 (A3-specific antagonist) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Z-YVAD-FMK (caspase-1 inhibitor) was purchased from BioVision Technologies (Milpitas, CA, USA). Anti-human IL-10 was obtained from Thermo Fisher Scientific (San Diego, CA, USA) and recombinant human IL-10 was from PeproTech (Rocky Hill, NJ, USA).

Commercially available *Aloe vera* gel (Forever Living Products Hungary Ltd) was centrifuged with 2000 rpm for 30 min to remove the insoluble components and obtain a clear solution. Solvent utilized for the preparation of the gel was used in the mock experiments. The supernatant of the centrifugation was used in the experiments.

THP-1 cell culture and differentiation into macrophage

The human monocytic THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 2mM L-glutamine and supplemented with 10% heat-inactivated-FCS, 500 U/ml penicillin-streptomycin (Thermo Fisher Scientific). Differentiation of THP-1 cells was induced by incubating cells with 0.5 μM phorbol myristate acetate (Invivogen) for 3 hours, as described previously (Grahames et al, 1999). Then cells were washed three times with PBS and plated at $1{,}3x10^6$ /ml. After 24 hours, cells were treated with 100 ng/ml LPS in the absence or presence of *Aloe vera*.

Monocyte isolation and macrophage differentiation

Leukocyte-enriched buffy coats were obtained from healthy blood donors in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen (Debrecen, Hungary). Informed consent of all participating subjects was obtained. Mononuclear cells were isolated by density gradient centrifugation using Ficoll Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) and $CD14^+$ monocytes were separated with anti-CD14–conjugated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). Finally, monocytes

were plated in 24-well tissue culture plates ($1,5x10^6$ cells/ml) in RPMI 1640 (Sigma-Aldrich) containing 2 mM L-glutamine and supplemented with 10% heat-inactivated-FCS, 500 U/ml penicillin-streptomycin (Thermo Fisher Scientific) and differentiated for 5 days in the presence of either 50 ng/ml M-CSF (PeproTech) or 80 ng/ml GM-CSF (Gentaur Molecular Products, Kampenhout, Belgium). On day 2, M-CSF or GM-CSF was replenished. On day 5, macrophages (M-MFs, GM-MFs) were stimulated with LPS (500 ng/ml) for the indicated time periods. When indicated, prior to sample collection, supernatants of macrophages were replaced with FCS-free RPMI media supplemented with ATP (5 mM) for 45 minutes. In some experiments, adherent $CD14^+$ monocytes following isolation were primed with LPS.

Cytotoxicity assay

Monocytes were plated and differentiated in 96 well plates at a concentration of $2x10^5$ cells / well. On day 5, macrophages (M-MFs, GM-MFs) were incubated with LPS for the indicated time at 37°C. At the end of treatment, the medium from control and LPS-treated cultures were discarded and 100 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] (0,5 mg/ml) containing PBS was added to each well. The cells were incubated for 2 to 4 hours at 37°C. Finally the MTT crystals were dissolved by adding 100 µl of solubilization solution (81 v/v% isopropanol, 9 v/v% 1M HCl, 10 v/v% TritonX-100) and the formazan dye was measured in a microplate reader (FlexStation 3 Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 550 nm. All experiments were performed in triplicates.

Measurement of ATP

Media collected from primary macrophages were centrifuged, and ATP content was determined from cell-free supernatants immediately after collection by ATPlite luminescence assay kit (PerkinElmer, Budapest, Hungary) according to the manufacturer's instructions. Luminescence was measured by Wallac 1420 Victor2 fluorimeter-luminometer (Wallac Oy, Turku, Finland).

ELISA

Supernatants collected from MFs were centrifuged and stored at -20 °C until further use. IL-6, TNFα, IL-10 and IL-8 were detected from the ATP-untreated samples, and IL-1β was measured from ATP-untreated and -treated samples by ELISA (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Analysis was performed on a

FlexStation 3 Microplate Reader. The minimum detectable doses are 0.8 pg/ml for IL-1β and IL-8, 2 pg/ml for IL-10 and TNFα and 2.2 pg/ml for IL-6.

RNA preparation and RT-PCR

After removal of supernatants by centrifugation, total RNA was isolated from cell pellets by TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Concentration and homogeneity of RNA was determined by a spectrophotometer (NanoDrop ND1000; Promega Biosciences, Madison, WI, USA). Standardized amounts of RNA were digested with DNase (Ambion , Austin, TX, USA) then reverse transcribed into cDNA using SuperScript II First-strand Reverse Transcriptase and oligo dT primers (Thermo Fisher Scientific).

Quantitative real-time polymerase chain reaction (qPCR)

For gene expression analyses, PCR reactions of cDNA samples were measured using an ABI Step One Plus machine (Thermo Fisher Scientific). For the amplification reactions, all TaqMan Gene Expression Assays were purchased from Thermo Fisher Scientific (assay IDs: IL-1β Hs00174097_m1, NLRP3 Hs00918082_m1, caspase-1 Hs00354836_m1, P2X7R Hs00175721_m1, CD39 Hs00969559_m1, CD73 Hs00159686_m1, A1 Hs00379752_m1, A2a Hs00169123_m1, A2b Hs00386497_m1, A3 Hs00252933_m1). TaqMan assays provide confidence in results, PCR primer and probe design maximizes amplification efficiency. Taq DNA Polymerase (Fermentas, Vilnius, Lithuania) was used for amplification, and Rox Reference Dye (Thermo Fisher Scientific) was used for normalization of fluorescent reporter signal. Three PCR replicates per cDNA sample were used to real-time PCR. The amplification conditions were 40 cycles for two-stage PCR (95 ^{o}C for 12 sec and 60 ^{o}C for 1 min). The appropriate internal control gene for our experiments was the human cyclophilin. Expression levels of target transcripts in each sample were calculated by the comparative Ct method after normalization to the internal control ($2^{-\Delta\Delta Ct}$).

Western Blot analysis

Cells were harvested and centrifuged. From the supernatants, proteins were precipitated by 20% trichloroacetic acid (TCA). Pellet was washed in acetone and then dried, followed by re-suspension in loading buffer (62,5 mM Tris-HCl, pH 8.8, containing 25 % glycerol, 2 % SDS,

1 % β-mercaptoethanol and 1 % BPB). Cells were lysed in loading buffer. Before loading, all samples were boiled for 10 minutes. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk, washed briefly, thereafter incubated with primary antibodies (Abs) at 4°C overnight. Pro-IL-1β (No sc7884), procaspase-1 (No sc515), Akt1 (No sc5298) and ASC (No sc-30153) Abs were from Santa Cruz Biotechnology (CA, USA), cleaved IL-1β (p17) (No 2021) and cleaved caspase-1 (p20) (No 4199) Abs were from Cell Signaling Technology (Danvers, MA, USA), NLRP3 (No AG-20B-0014) Ab was from AdipoGen (San Diego, CA, USA), P2X7R (No APR004) Ab was from Alomone labs (Jerusalem, Israel), p-Akt (S473) (No AF887) was from R&D systems (Minneapolis, MN, USA), p-ERK1/2 (No M8159)was from Sigma-Aldrich (St. Louis, MO, USA), p-IκBα (No 9246), IκBα (No 9242), ERK1/2 (No 9102), p-SAPK/JNK (No 9251), SAPK/JNK (No 9252), p-p38 (No 9211) and p38 (No 9212) MAP Kinase Abs were obtained from Cell Signaling Technology (Danvers, MA, USA). Primary Abs were used in 1:1000 dilution overnight, then incubated with corresponding HRP-conjugated secondary Abs in 1:2000 dilution (goat anti-rabbit IgG, No 170-6515; goat anti-mouse IgG, No 172-1011) from Bio-Rad Laboratories (Hercules, CA, USA) for 1 hour at room temperature. Proteins were visualized by Supersignal West-Pico peroxide/luminol enhancer solution from Pierce Biotechnology (Rockford, IL, USA). To verify the loading of equal amount of protein sample β-actin (No A1978) (Sigma-Aldrich) expression was detected.

Measurement of caspase-1 activity

Caspase-1 activity in cell lysates was determined using the acetylated and AMC-conjugated fluorometric peptide substrate Acetyl-Tyr-Glu-Val-Asp-7-amino-4-methyl-coumarin (AnaSpec, San Jose, CA, USA). Lysis of cells was performed on ice for 30 min in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM EDTA, 0.5 % Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1.25 μg/ml leupeptin and 1 mM dithiothreitol. After centrifugation (10 000 g, 10 min at 4 ^{o}C), 30 μg protein lysate supernatant was incubated in 100 μl lysis buffer with 40 μM substrate (final concentration) in microtiter plate wells at room temperature, and the increase of fluorescence due to the release of AMC was detected at 460 nm, using 355 nm excitation wavelength in a Wallac 1420 Victor2 fluorimeter-luminometer (Wallac Oy, Turku, Finland). Data were normalized to the background signal of AMC.

siRNA transfection

On day 3 of differentiation, supernatants were collected and cells were suspended in PBS then washed once with OptiMEM (Invitrogen, Carlsbad, CA, USA). Cells were transfected with NLRP3-specific siRNA (s38591, s38592, s38593) or with siRNA for negative control (Ambion) using an electroporator (Gene Pulser Xcell, Bio-Rad). Finally, cells were plated in 24-well tissue culture plates ($1x10^6$ cells / ml). On day 5, macrophages (M-MFs, GM-MFs) were stimulated with LPS (500 ng/ml).

Statistical analysis

Significant differences between mean values of two groups were evaluated using a Student's *t* test. Data presented as mean ±SD. One-way ANOVA, followed by Bonferroni post hoc test, was used for multiple comparisons.

4. Results

4.1. Different dynamics of NLRP3 inflammasome-mediated IL-1β production in GM-CSF- and M-CSF- differentiated human primary macrophages

4.1.1. LPS treatment of M-MFs and GM-MFs results in different pattern of cytokine expression

Although macrophages originate from hematopoietic stem cells in the bone marrow, they are derived from circulating monocytes, which enter the vessel wall and differentiate into morphologically and functionally distinct macrophage populations depending on the tissue`s environment. The mechanisms mediating terminal differentiation have fundamental significance because of the critical and diverse roles of these cells. The different functions of various macrophage subtypes are manifested in part by the production of characteristic cytokine profiles. To compare how activation affects cytokine production of primary macrophages differentiated by M-CSF or GM-CSF from human monocytes, M-MFs and GM-MFs were treated with LPS for various time intervals, and cytokine production was determined using ELISA methods.

As shown in Figure 7, both macrophage subtypes produced detectable amounts of TNFα and IL-8 cytokines, but with significant differences in kinetics of cumulative values. M-MF released at an early phase of LPS treatment, which reached the maximum at 6 h, In contrast, TNFα secretion by GM-MFs was increasing, which resulted in significantly higher concentrations at later time points (12-24 h). Production of IL-8 increased by both macrophages upon activation, however, it was more robust at later time-points in case of GM-MF. Nevertheless, IL-6 and IL-10 production differed substantially between the macrophages. IL-6 was hardly detectable in the supernatant of M-MFs meanwhile its production was more intense and prolonged during the 24 hour-treatment by GM-MFs. However, we observed the opposite in case of IL-10 anti-inflammatory cytokine, as LPS treatment induced a continuously increasing IL-10 secretion by M-MFs, while GM-MFs were unable to produce a detectable amount of IL-10.

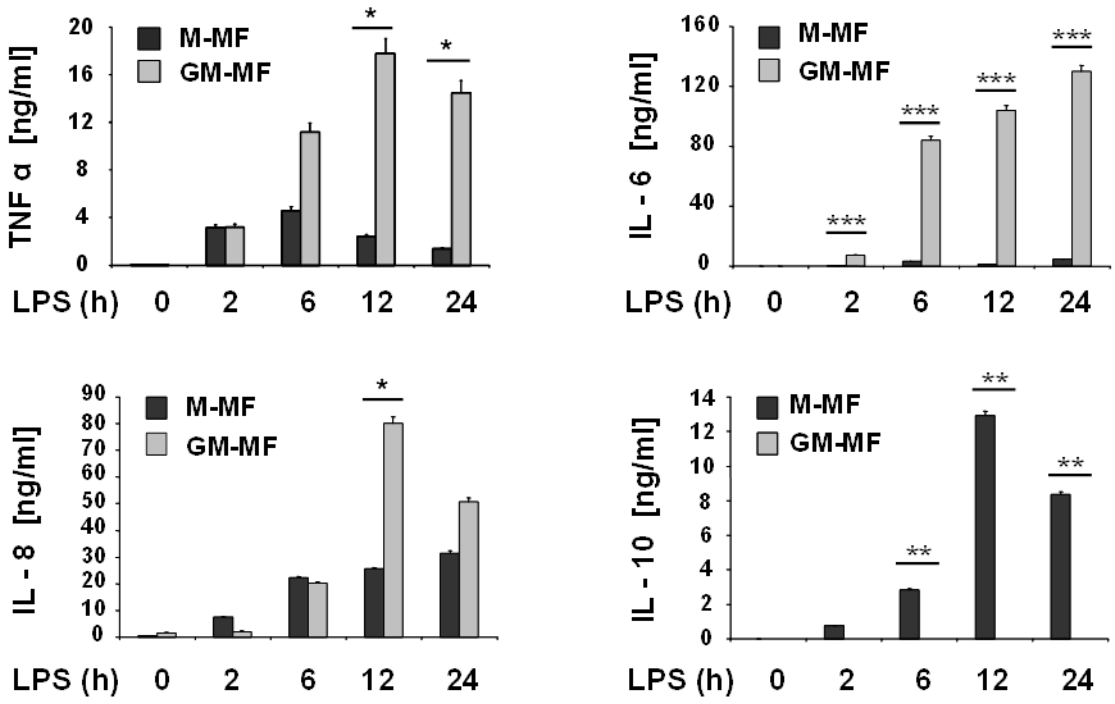


Figure 7. Cytokine secretion by human primary M-MFs and GM-MFs treated with LPS. M-MFs and GM-MFs were primed with LPS (500 ng/ml) for the indicated times and TNFα, IL-6, IL-8 and IL-10 cytokines were quantified from cell-free supernatants, n=3. (*p<0.05, **p<0.01, ***p<0.001).

Since several groups reported that IL-1β release by macrophages requires ATP supplementation, we collected media from both ATP-treated and non-treated cells. Surprisingly, following ATP exposure, the LPS-primed M-MFs released high amount of IL-1β at a very early time-point. IL-1β was detectable from the supernatant as early as 30 min after LPS treatment (data not shown), and it reached maximum production by 2 h (Figure 8A). Thereafter, it decreased successively, and by 24 h the amount of IL-1β was below the detection limit. In contrast, we found that IL-1β production by GM-MFs was barely detectable at 2 h following LPS treatment, however, it gradually increased by time, and its peak amount was comparable to that of the maximum of M-MFs (Figure 8A). Similar results were observed following the treatment with nigericin, the microbial toxin that acts as potassium ionophore. These results indicate that the dynamics of LPS-primed NLRP3 inflammasome-induced IL-1β secretion fundamentally differs in the two studied MF types, and is irrespective of the activating agent, since ATP activates purinergic receptors, while nigericin induces pore formation.

As expected, we did not detect secreted IL-1β cytokine from the supernatant of LPS-treated M-MFs without triggering by ATP (Figure 8B). However, to our surprise, LPS-primed

GM-MFs secreted moderate, but significant amount of IL-1β even without ATP supplementation, which was comparable to that produced by monocytes in similar conditions.

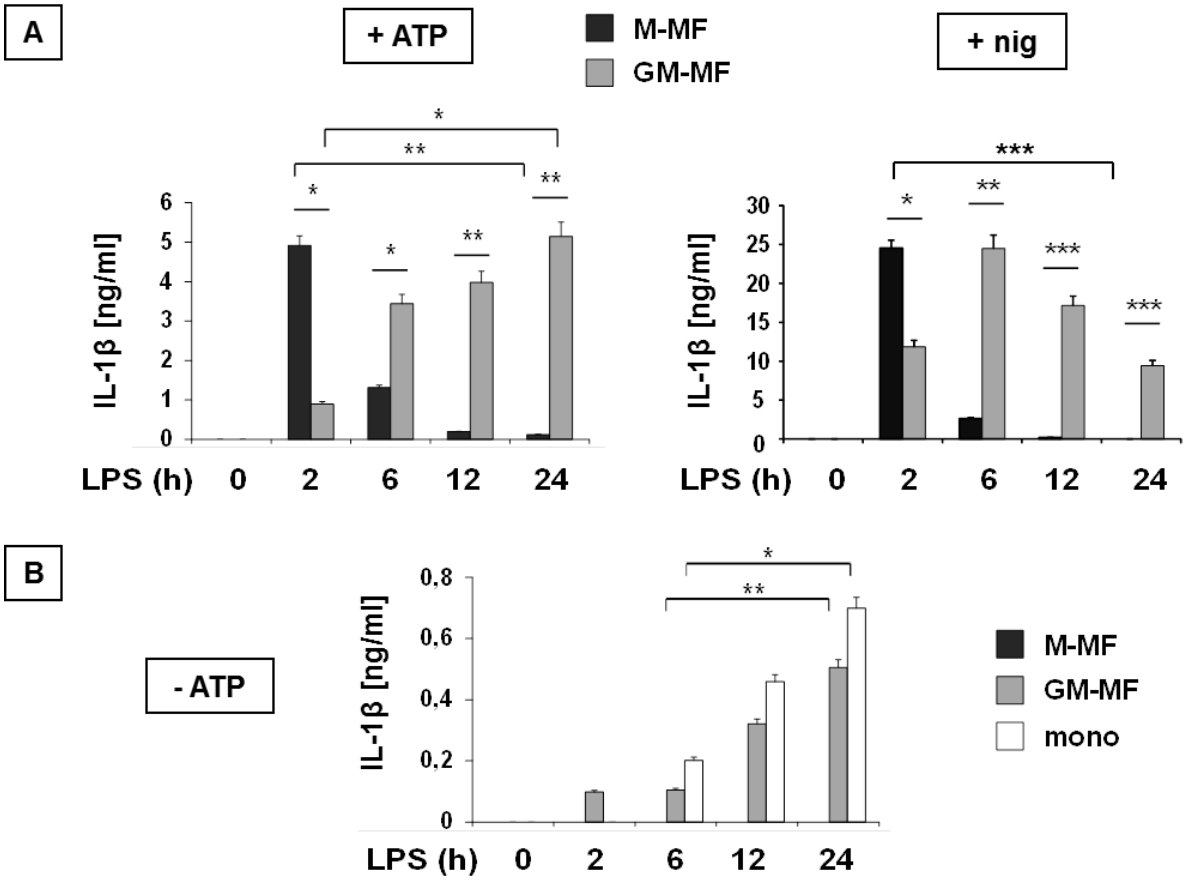


Figure 8. Triggers induce diverse ways of IL-1β secretion by human primary macrophages. (A) M-MFs and GM-MFs were primed with LPS (500 ng/ml) for the indicated times followed by treatment with ATP (left panel), n=6 or with nigericin (nig) (right panel), n=3. IL-1β was then measured from the supernatants (*p<0.05, **p<0.01, ***p<0,001). (B) Macrophages (n=6) and monocytes (n=3) were treated with LPS in the absence of ATP supplementation and IL-1β level was detected (*p<0.05, **p<0.01).

Altogether these results show that the same activating agent on the two different human macrophage types results in significant differences in the type and quantity of cytokine production. Also, the results of cytokine production support the general notion that GM-MFs possess more pro-inflammatory properties compared to M-MFs.

4.1.2. LPS-induced IL-1β production is mediated through caspase-1–dependent NLRP3 inflammasome pathway in both macrophage subtypes

The bioactivity of IL-1β is strictly controlled by inflammasomes. This cytokine is synthesized as an inactive precursor, and upon canonical activation, it is cleaved into active

IL-1β by caspase-1 enzyme. To study the role of caspase-1 on IL-1β production, we used a specific inhibitor of the enzyme, and monitored IL-1β secretion by MFs following LPS treatment (Figure 9). We found that the caspase-1 inhibitor Z-YVAD-FMK significantly reduced IL-1β production by both ATP-triggered macrophage subtypes. Moreover, ELISA results also demonstrated that caspase-1 enzyme is required for IL-1β release by LPS-primed GM-MFs in the absence of ATP treatment.

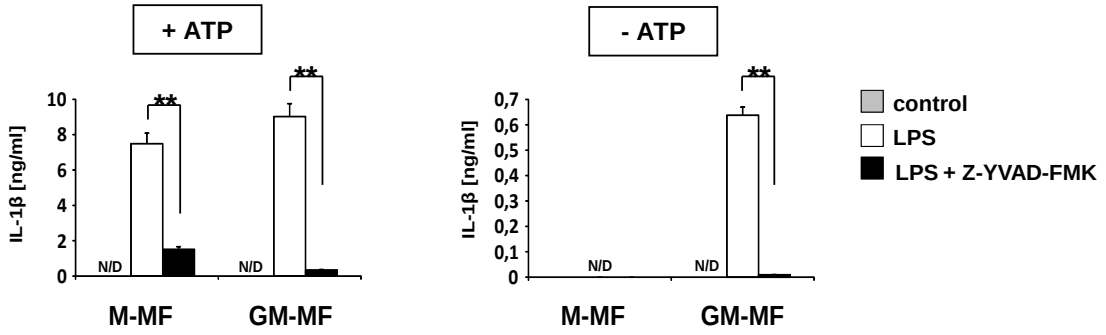


Figure 9. Caspase-1-dependent IL-1β secretion by human primary MFs. Macrophages were pre-treated with caspase-1 inhibitor (Z-YVAD-FMK, 20μM) for 1h, followed by priming with LPS for 2h (M-MFs) or for 12h (GM-MFs). IL-1β secretion was detected in the presence or absence of ATP treatment. N/D: not detectable. (**p<0.001).

To further clarify the type of inflammasome involved in LPS-primed IL-1β production, macrophages were exposed to LPS in the presence of high KCl concentration (30mM), which inhibits K^+ efflux. Previous studies have shown that reduction of intracellular K^+ concentration is sufficient to activate NLRP3 inflammasome. As Figure 10 A shows, we observed a significantly suppressed IL-1β production by both macrophage types. Of note, cell viability was not compromised by working concentration of KCl, although, we observed toxic effects of higher concentrations (60mM, 120mM) (Figure 10B).

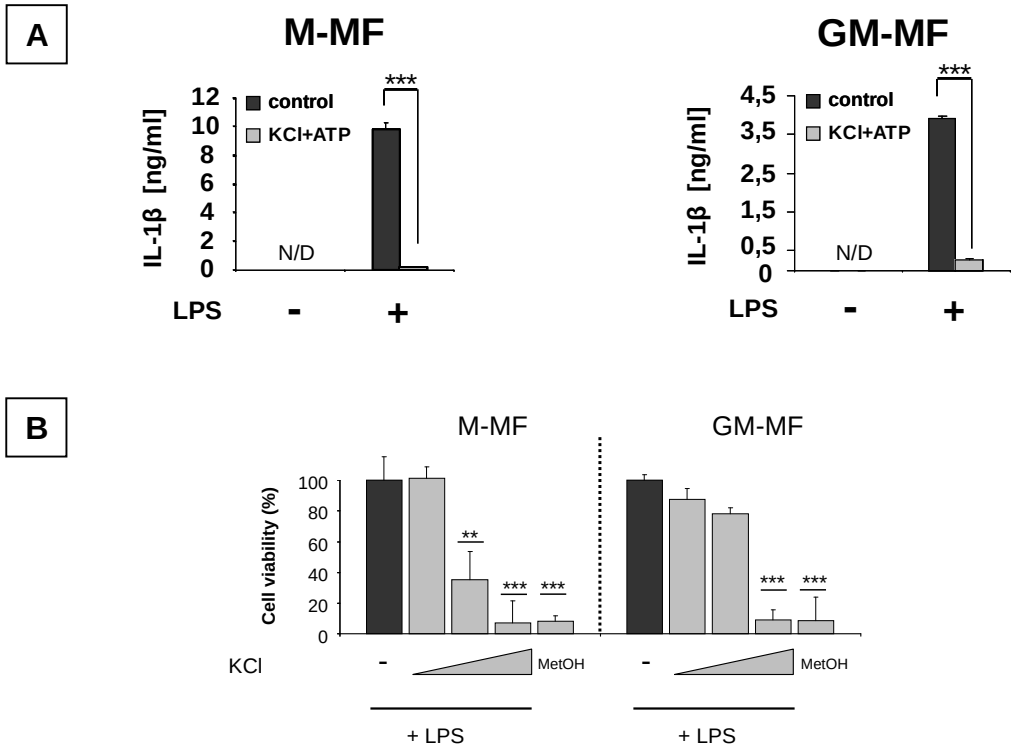


Figure 10. IL-1β secretion by M-MFs and GM-MFs is dependent on potassium efflux. (A) Macrophages were pre-incubated in KCl solution (30 mM) for 1h, and then cells were activated with LPS for 2h (M-MFs) or for 24h (GM-MFs). After incubation KCl were renewed in FCS free medium supplemented with ATP. For controls untreated or KCl treated cells were used. IL-1β secretion was detected in the presence of ATP, (***p<0.0001). (B) Macrophages were primed with LPS for 24h in the presence or absence of increasing concentration of KCl solution (30mM, 60mM, 120mM). For positive control cells were treated with methanol (70% v/v). **p<0.01, ***p<0.001 to correlate with LPS treatment (black column).

To identify the involvement of NLRP3 inflammasome in LPS-primed IL-1β production specific NLRP3 siRNAs were used. M-MFs were treated with LPS for 2 h and GM-MFs for 12 and 24 h when IL-1β secretion reaching the maximum after ATP stimulation. We detected IL-1β from the supernatant by ELISA and Western blotting. We found that the knockdown of NLRP3 protein was associated with a significant decrease in IL-1β release by M-MFs and GM-MFs, respectively (Figure 11). However, silencing had no effect on IL-6 secretion, signifying NLRP3 specificity.

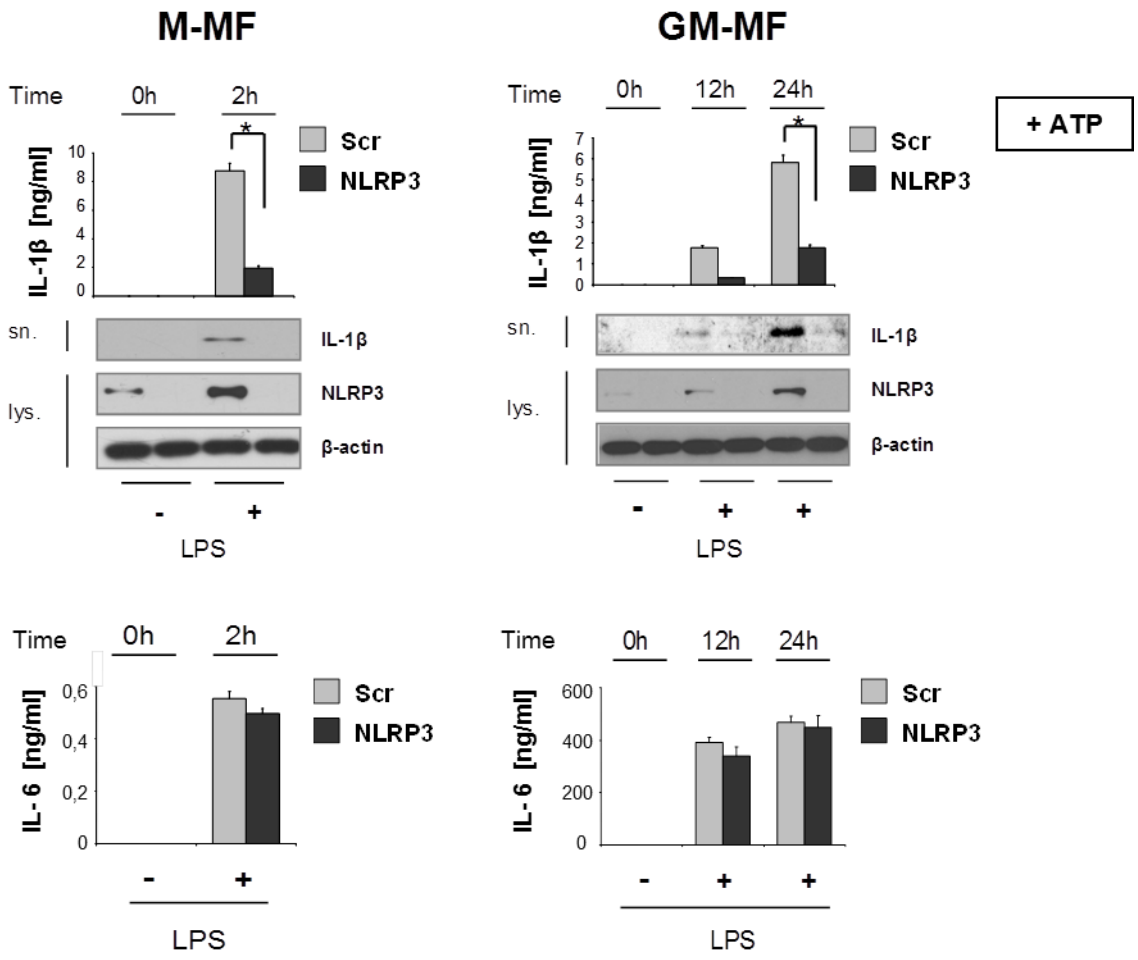


Figure 11. IL-1β secretion by M-MFs and GM-MFs is mediated by NLRP3 inflammasome. Macrophages transfected with NLRP3 siRNA or non-targeting siRNA (Scr) were primed with LPS for the indicated times, followed by ATP treatment. To verify silencing, NLRP3 protein was detected from cell lysates (lys) by Western blot. Secreted IL-1β was quantified from the supernatant (sn) by ELISA and verified by Western blot methods. IL-6 cytokine was detected from supernatant before ATP treatment. (*$p<0.05$).

Altogether, these data reveal that LPS-primed IL-1β production requires caspase-1 enzyme and K^+ efflux, and is mediated through the NLRP3 inflammasome complex in both M- and GM-MF subtypes.

4.1.3. Extracellular ATP released by GM-MFs does not affect NLRP3 inflammasome activation directly

Previous studies reported that monocytes secrete IL-1β upon LPS challenge as a result of paracrine or autocrine effects of released endogenous ATP that triggers NLRP3 inflammasome activation. In contrast, macrophages are unable to process IL-1β upon LPS treatment due to the lack of ATP release.

When we measured extracellular ATP content of the supernatant, we found that untreated GM-MFs release ATP in a significantly higher concentration compared to that of M-MFs. Interestingly, LPS treatment did not enhance secretion of ATP by the cells moreover we observed a decline in ATP concentration in the medium of GM-MFs 24 h following LPS activation (Figure 12). The levels of ATP released by GM-MFs increased at least up to 4-fold compared to the previously described level in LPS-treated monocytes.

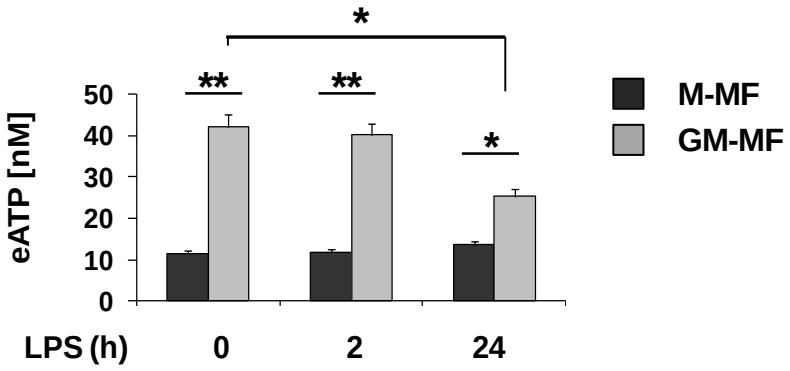


Figure 12. GM-MFs release significantly more endogenous ATP. Macrophages were activated with LPS for 2h and 24h and ATP levels were determined from cell-free supernatants. Data are expressed as mean ±SD of three independent experiments. *p<0.1, **p<0.01

We verified with a viability assay that the changes in ATP release were not caused by cell death (Figure 13).

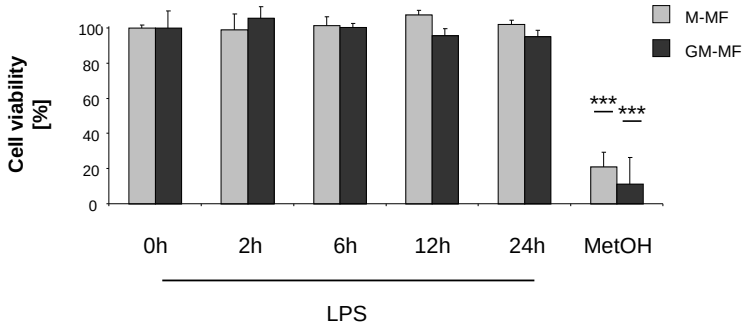


Figure 13. Determination of cell viability during time-dependent LPS treatment
Macrophages were treated with LPS for the indicated times. Control cells (0h) remained untreated. For positive control, cells were treated with methanol (70% v/v). Results were obtained from three independent experiments with three replicates as mean ±SD. ***p<0.001 between positive (0h) and negative controls (MetOH).

Increased extracellular ATP level related to inflammation triggers NLRP3 inflammasome through the purinergic receptor P2X7. To clarify the role of released ATP by

LPS-primed GM-MFs in IL-1β production, first we measured mRNA and protein expression of P2X7R in both macrophage types. As our results show, LPS treatment significantly induced the expression of the receptor in both macrophage types. However, we observed a faster decline in the expression in case of M-MFs, as compared to GM-MFs (Figure 14).

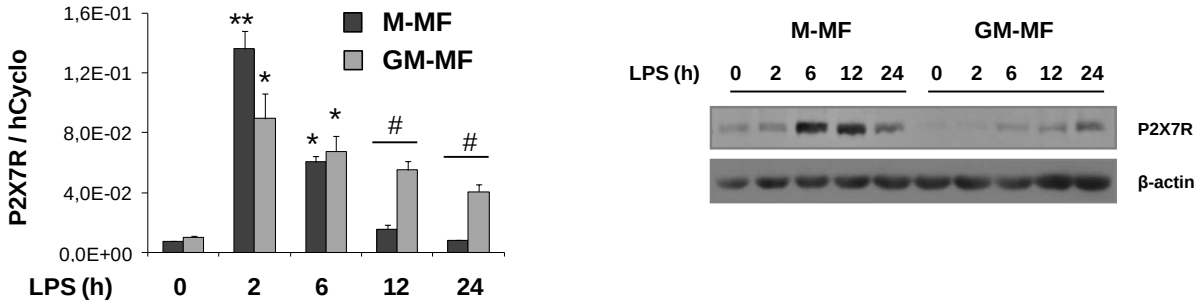


Figure 14. Expression of P2X7 receptor in LPS-primed macrophages. Cells were collected at the indicated time points after LPS stimulation, and P2X7 receptor expression was analyzed by qPCR (left panel) and Western blotting(right panel). *p<0.05, **p<0.01 between control (0h) and LPS treatment (2h, 6h, 12h) or #p<0.05 as indicated between LPS-treated M- and GM-MF (12h, 24h).

To test the functionality of P2X7R in macrophages, and their contribution to IL-1β secretion, we used P2X7R specific inhibitor A740003. We found that the inhibitor abolished ATP-triggered IL-1β secretion by both LPS-primed macrophage types (Figure 15A). However, the pharmacological inhibition of P2X7 receptor failed to reduce IL-1β secretion by LPS- primed GM-MFs without triggering with ATP. Similar results were obtained using apyrase enzyme that hydrolyzes extracellular ATP. We observed that although in the presence of apyrase the amount of extracellular ATP was significantly reduced, the concentration of secreted IL-1β did not change significantly (Figure 15B).

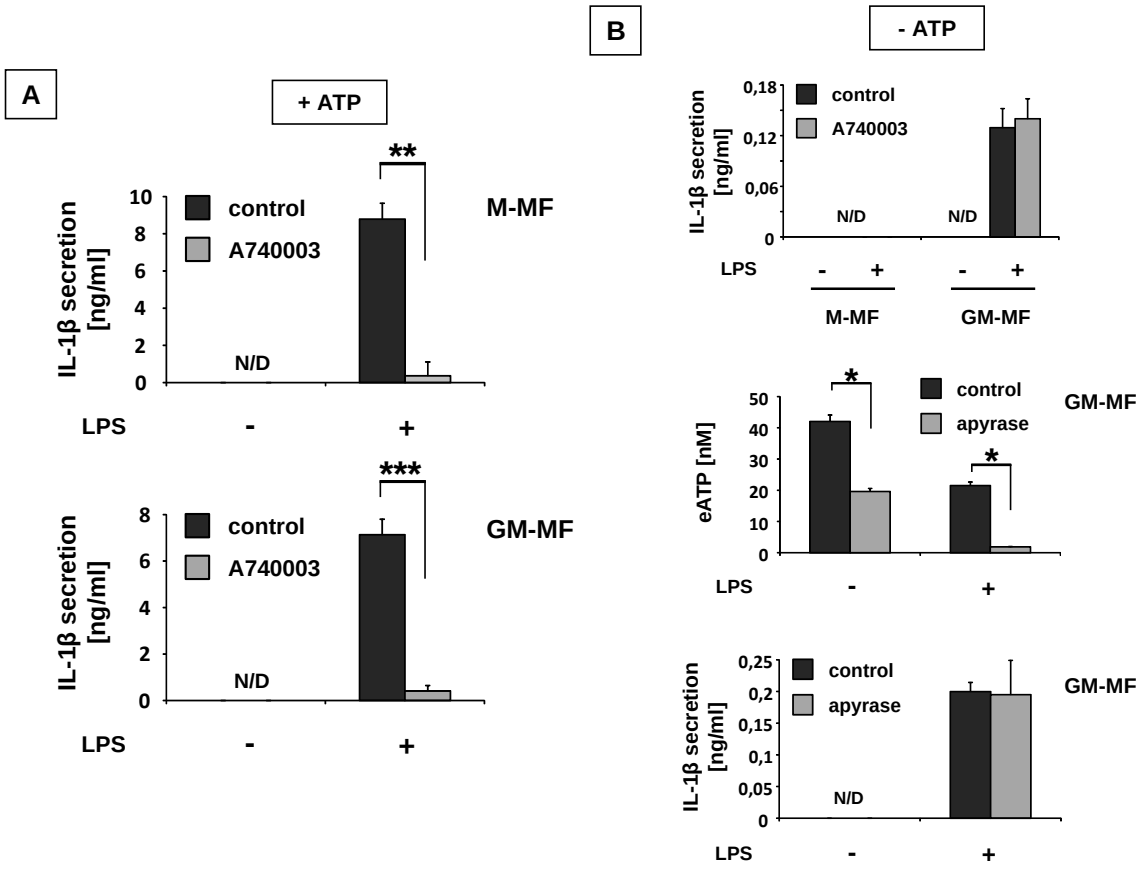


Figure 15. Released endogenous ATP is not involved in IL-1β secretion by GM-MFs. (A) Macrophages were pre-incubated with A740003 (100μM) for 1h, followed by priming with LPS for 2h (M-MFs) or 24h (GM-MFs). Supernatants were then replaced with A740003 containing FCS free media supplemented with ATP. The amount of secreted IL-1β was determined by ELISA. (**p<0,01, ***p<0.001). (B) In the absence of ATP, IL-1β secretion was quantified from supernatants of LPS-primed M-MFs (2h) or GM-MFs (24h) pre-incubated with A740003 (100μM) or apyrase (2,5U/ml) in the case of GM-MFs using ELISA method. Released ATP was detected from cell free supernatant of GM-MFs. (*p<0.05) N/D: not detectable.

These results altogether show that both macrophages could express functional P2X7R, required for IL-1β secretion in the case of ATP supplementation. However, these data also show that LPS-mediated IL-1β secretion in GM-MF is independent of released ATP, which is consistent with the lack of requirement for P2X7 receptor.

4.1.4. CD39 and CD73 ectonucleotidases contribute to IL-1β secretion in GM-MFs

The net amount of extracellular ATP reflects the balance between ATP released by cells and ATP hydrolyzed by surface ectonucleotidases. This purine nucleotide is hydrolyzed to AMP by CD39, which is rapidly converted to adenosine by CD73. These two enzymes can

determine the concentration of ATP in the extracellular milieu immediately surrounding the cells, therefore, we next studied expression of CD39 and CD73 by the two macrophage subtypes. We found that although prolonged treatment of macrophages with LPS resulted in induction of CD39 transcription, it was still significantly lower in GM-MFs compared to that of M-MFs (Figure 16). In contrast, while the transcription of CD73 was temporarily increased in M-MFs, it was markedly elevated in GM-MFs 24 h following LPS activation.

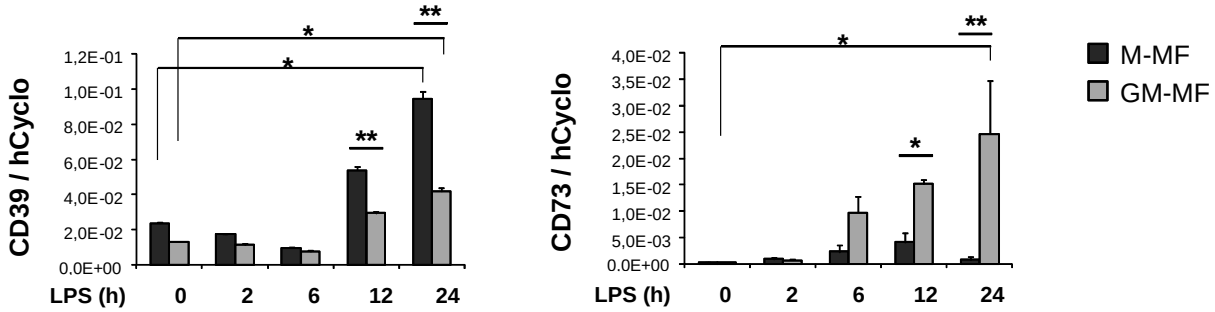


Figure 16. GM-MFs and M-MFs express different amount of ectonucleotidases. Cells were collected at the indicated time points after LPS stimulation, and the expression of CD39 and CD73 was analyzed by qPCR. Data are representative of three independent experiments. Gene expression is shown as the ratio of the studied transcripts relative to human cyclophilin expression (±SD) measured in triplicates. *p<0.1, **p<0.05.

To see the effect of ectonucleotidases on endogenous ATP catabolism and IL-1β release of GM-MFs, we treated cells with the ectonucleotidase inhibitor ARL67156. We found that in the presence of ARL67156, ATP content was significantly elevated in the supernatant of both LPS-treated and non-treated cells (Figure 17). Interestingly, when we measured IL-1β secretion of the GM-MFs, we detected a significantly decreased cytokine concentration in the ARL67156-treated cells, implying the possibility that in GM-MFs, not the endogenously released ATP, but the accumulating side- or end-product of ATP hydrolysis is required for IL-1β secretion. We still could not measure detectable IL-1β from media of M-MFs (data not shown).

These results show that the studied MFs express ectonucleotidases at different levels. Furthermore, inhibition of the enzymes resulted in an increased amount of extracellular ATP, which indicates their functional role in ATP metabolism. However, ELISA data also verified our previous findings, that IL-1β secretion by LPS-primed GM-MFs is independent of released ATP-mediated P2X7 receptor signaling.

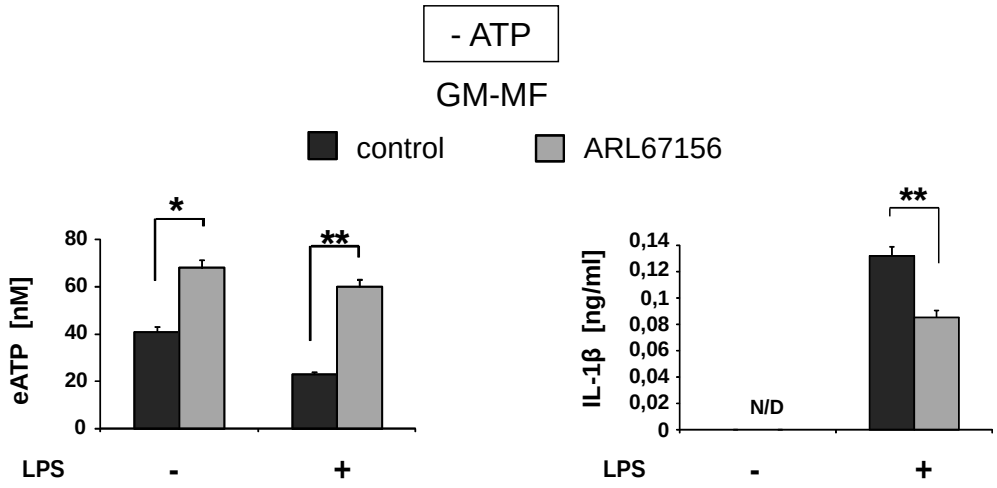


Figure 17. Ectonucleotidases expressed by GM-MFs influence their IL-1β secretion. GM-MFs were pre-incubated with ARL67156 (100μM) for 1h, cells were then primed with LPS for 24h in the absence of ATP supplementation. ATP was measured from the supernatant. IL-1β was analyzed from the same supernatants by ELISA. Mean ±SD values of three independent experiments are shown. *p<0.1, **p<0.05. N/D: not detectable.

4.1.5. Adenosine enhances IL-1β secretion in LPS-activated GM-MFs

Increased CD73 transcription in LPS-primed GM-MFs led us to assume that significant amount of adenosine is produced as a by-product or an end-product. To clarify the effect of adenosine on IL-1β production by M- and GM-MFs, first we measured the four adenosine receptor expression level upon LPS activation (Figure 18). As our quantitative PCR results show, expression of A2b and A3 were temporarily increased, whereas prolonged cultivation of LPS-primed macrophages significantly downregulates it. Meanwhile, transcription of A2a was significantly induced, and only moderately decreased in GM-MFs in contrast to M-MFs, where the A2a receptor was dramatically decreased. It is worth noting that in most time points, A2a and A2b receptors are expressed at a higher level in GM-MFs compared to M-MFs. Interestingly, A1 receptor was hardly detectable in both macrophages (data not shown).

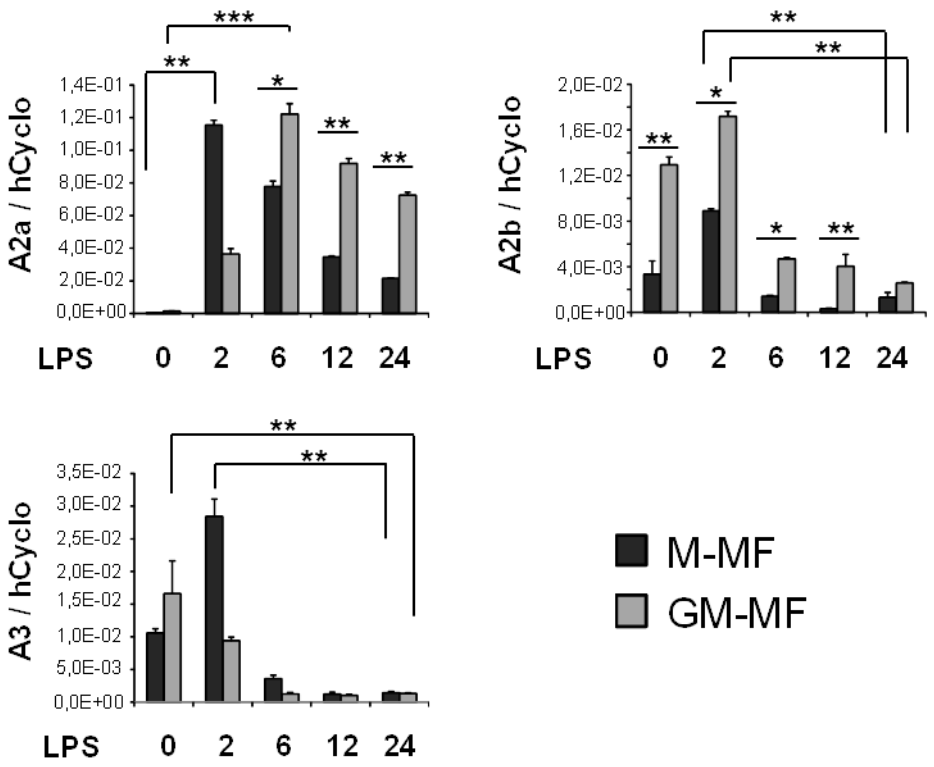


Figure 18. Expression of adenosine receptors in macrophages. Cells were treated with LPS and collected at the indicated times (h). A2a, A2b, A3 and A1 (data not shown) were analyzed by quantitative PCR. *p<0.1, **p<0.05, ***p<0.01.

To test whether released endogenous adenosine contributes to LPS-mediated IL-1β secretion in GM-MFs, we used adenosine receptor specific antagonists. As figure 19A indicates, A2a and A2b specific antagonists significantly decrease LPS-mediated IL-1β secretion, while A1 and A3 specific antagonists did not cause significant changes. Furthermore, when we treated GM-MFs with an increasing amount of adenosine, we detected enhanced LPS-mediated IL-1β secretion. Similar results were obtained for GM-MFs in the presence of ATP supplementation (Figure 19B). Interestingly, adenosine had no effect on IL-1β secretion by LPS-primed M-MFs. These results indicate that adenosine has an important regulatory effect solely on IL-1β secretion by GM-MFs, independently of ATP supplementation, while it does not affect IL-1β secretion by M-MFs.

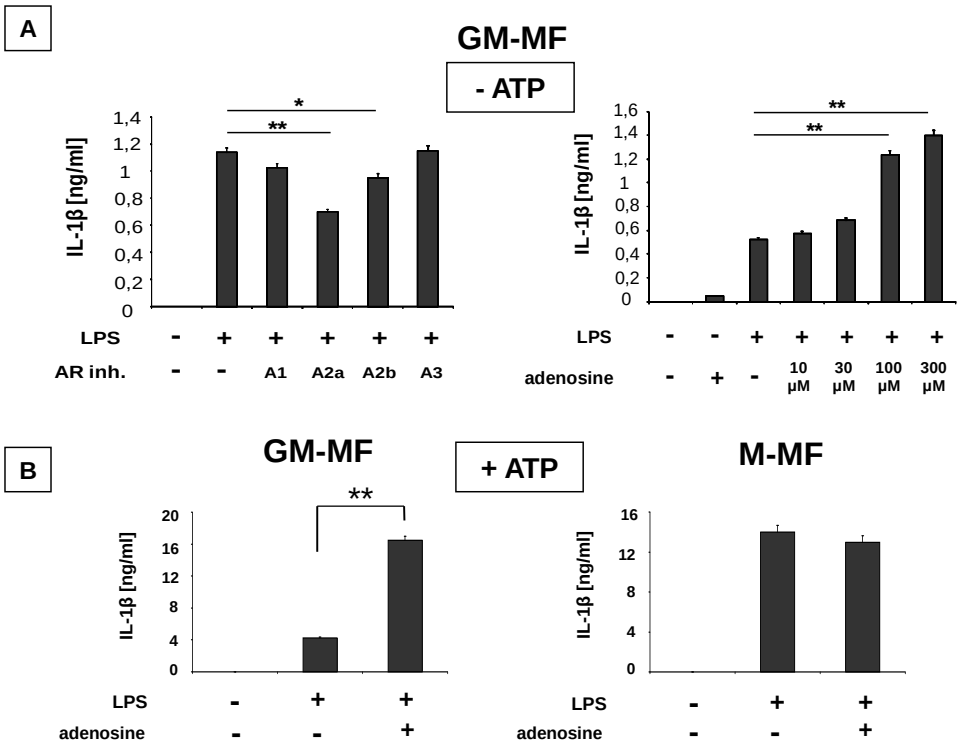


Figure 19. Adenosine affects IL-1β production by GM-MFs but does not affect M-MFs. (A) GM-MFs were pre-incubated with adenosine receptor (AR) specific inhibitors in 1 μM concentration for 1h (A1-DPCPX, A2a-ZM241385, A2b-PSB1115, A3-VUF5574) or with an increasing amount of adenosine (10 μM, 30 μM, 100 μM, 300 μM) followed by priming with LPS (24h). IL-1β levels were determined from supernatants of ATP non-treated cells. (B) Macrophages were pre-incubated with adenosine (100 μM) for 1h, thereafter cells were activated with LPS for 2h (M-MFs) or for 24h (GM-MFs). Before harvest, macrophages were treated with ATP and IL-1β secretion was analyzed from cell-free supernatant. (*p<0.1, **p<0.01).

4.1.6. Differences in expression of NLRP3 and pro-IL-1β as well as in caspase-1 enzyme activity mediate diversity in IL-1β processing by GM- and M-MFs

To further study the molecular basis of the observed differences in IL-1β production by M-MFs and GM-MFs, we stimulated cells with LPS, and assessed NF-κB and MAPK signal transduction pathways important for priming upstream of TLR4 activation (Figure 20).

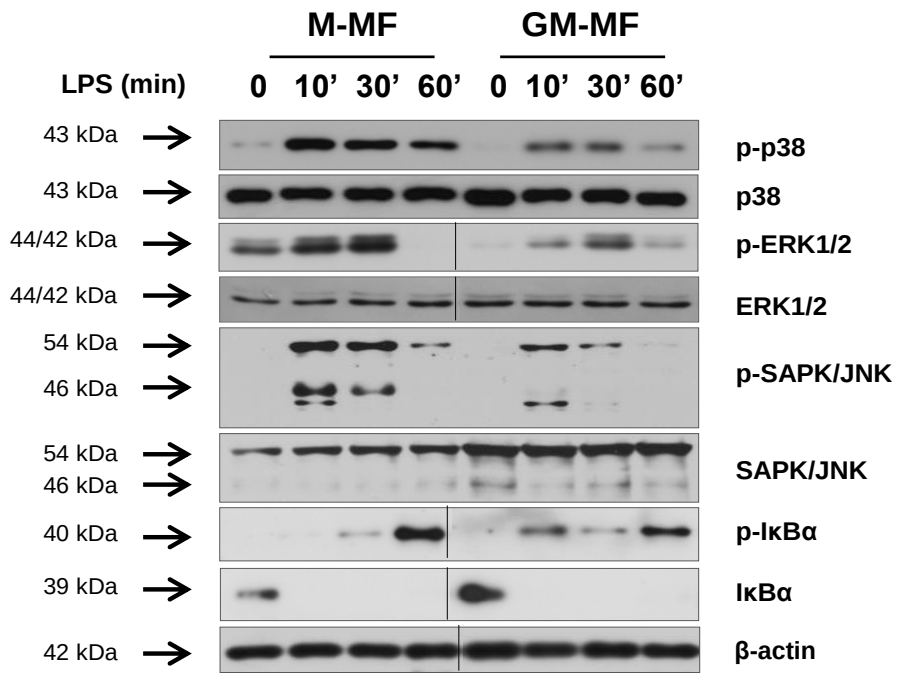


Figure 20. Analysis of signaling pathways in LPS-activated M-MFs and GM-MFs. Macrophages were primed with LPS and the time-dependent phosphorylation of p-p38, p-ERK1/2, p-SAPK/JNK and pIκBα were detected from whole cell lysates at the indicated time points by Western blot method. Total p38, ERK1/2, SAPK/JNK, IκBα were also detected using specific antibodies while β-actin amounts were probed to verify the same amount of proteins loaded. Western blots represented the part of M-MF and GM-MF come from the same experimental blot.

We found that ERK1/2 protein was already phosphorylated in the non-treated M-MFs, and phosphorylation was further induced for up to 30 min, after which it declined by 60 min. In GM-MFs, activation of ERK1/2 was observable by 30min after LPS treatment which was reduced at later time-points. Furthermore, in both macrophages, p38 and SAPK/JNK phosphorylation was detected at early time points (10min) after LPS treatment. However, while the intensity of phosophorylation during the studies time interval was only moderate in GM-MFs, in the case of M-MFs, we detected strong phosphorylation for up to 60 min (p38) or 30 min (SAPK/JNK) after LPS activation. Interestingly, while LPS treatment resulted in a strong phosphorylation of IκBα at an early time point (10 min) in GM-MFs, in the case of M-MFs, activation of this pathway showed a shift to later time-points, with a robust phosphorylation only at 60 min. We also observed the decrease of the non-phosphorylated IκBα form in parallel as the phosphorylated IκBα appeared.

Next, we aimed to compare the priming effect of LPS on the expression of NLRP3 inflammasome components in the two macrophage types (Figure 21). As our Western blot results show, both untreated macrophage types expressed the protein of ASC adaptor and pro-caspase-1, and we did not detect notable changes in expression levels upon LPS treatment. Nevertheless, we detected significant differences in the expression of the NLRP3 sensor molecule and pro-IL-1β. NLRP3 was clearly detectable in non-activated M-MFs, and enhanced by LPS treatment peaked by 2 h, and decreased gradually at later time points. In contrast, in untreated GM-MFs, expression of NLRP3 was hardly detectable, but LPS treatment resulted in a significant and continuously increasing NLRP3 protein expression. We found that untreated macrophages did not produce pro-IL-1β; the inactive form of the cytokine, though LPS priming strongly and rapidly induced its expression in both cell types, albeit with different dynamic. Pro-IL-1β was strongly detected by 2 h following LPS treatment in M-MFs, declined rapidly, and disappeared completely by 24 h, on the other hand, GM-MFs expressed this protein continuously during the studied interval. Similar changes were detected in NLRP3 and pro-IL-1β expression at mRNA level by quantitative real-time PCR method.

To detect the activation of NLRP3 inflammasome, active forms of IL-1β and caspase-1 were determined (Figure 21). The presence of IL-1β in the cell lysate and in the medium of macrophages was similar to that observed with ELISA. We detected intensive caspase-1 band following brief LPS treatment (2 h) in M-MFs from both cell lysates and the medium, which was abolished by 12 h. In the case of GM-MFs, the active form of caspase-1was weakly detectable, however, it showed an increasing tendency with time.

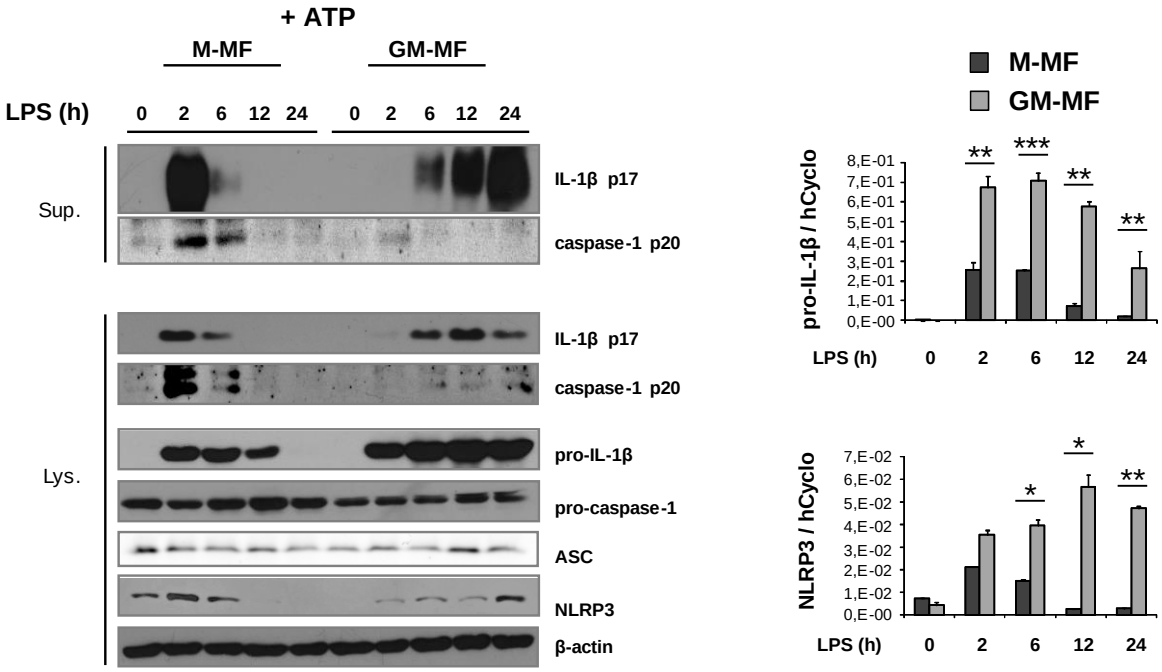


Figure 21. Comparative analysis of NLRP3 inflammasome members and caspase-1 activation between LPS-activated M-MFs and GM-MFs. Macrophages were primed with LPS for the indicated times, followed by treatment with ATP. Protein levels were determined from the whole cell lysates (Lys) by Western blot (left panel). The cleaved IL-1β (p17) and cleaved caspase-1 (p20) were quantified from precipitated supernatant (Sup) as well as whole cell lysates (Lys). Transcription of pro-IL-1β and NLRP3 was determined by qPCR (right panel). pro-IL-1β **p<0.005, ***p<0.001; NLRP3 *p<0.05, **p<0.005.

A clear mechanism of caspase maturation is still not well established, but data suggest that caspase-1 specificity is maintained within minutes of activation (~9 min), after which the protease is rapidly inactivated, thereby restricting the activity of this enzyme towards highly preferred substrates; such as IL-1β. To get a better understanding of caspase-1 function in IL-1β production by the two different macrophage types, we aimed to determine caspase-1 activity using a fluorogenic peptide substrate (Figure 22). We found that caspase-1 activity of M-MFs was hardly detectable in the absence of ATP, and LPS treatment did not have a notable effect on it. As expected, in LPS-primed M-MFs, the enzyme activity was significantly induced by triggering with ATP. It was increased in the first hour, thereafter it decreased gradually during the 24 hour-treatment. Furthermore, we detected significant caspase-1 activity even in the non-primed cells. Surprisingly, enzyme activity was clearly detectable in LPS-primed GM-MFs without triggering with ATP, even in the absence of LPS. Additionally, ATP enhanced caspase-1 activity at most time points in the GM-MFs.

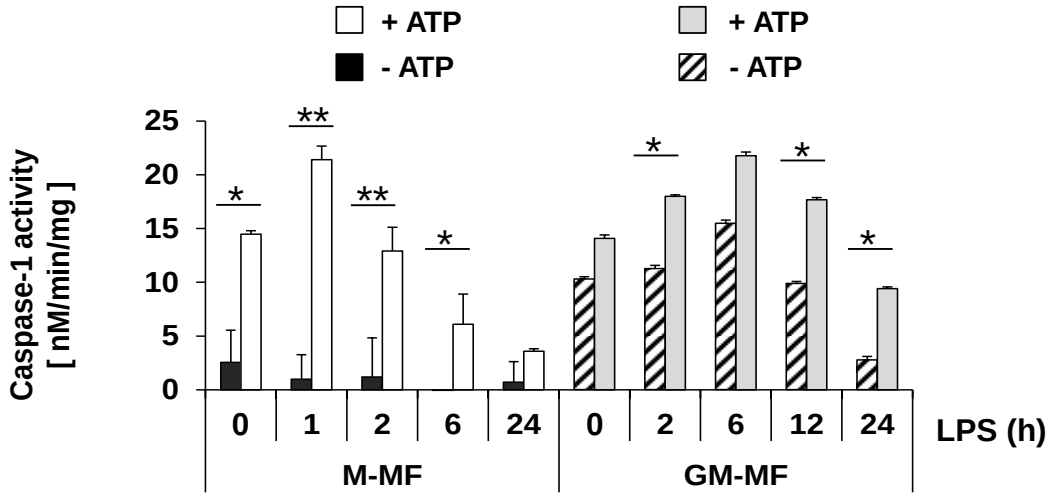


Figure 22. Analysis of caspase-1 activation in M-MFs and GM-MFs. Cell lysates were collected at the indicated time points after LPS or LPS plus ATP stimulation, and analyzed for caspase-1 activation using a fluorometric peptide substrate in triplicates. Values correspond to three representative experiments ±SD (*$p<0.1$, **$p<0.01$).

These results together show, that the substantially different expression and activity of the proteins in M-MFs and GM-MFs profoundly determine the outcome of LPS-induced IL-1β secretion.

4.1.7. Rapid attenuation of IL-1β secretion by M-MFs is the result of the robust secretion of IL-10

IL-10 cytokine has been reported as a negative regulator of many inflammatory responses. Our ELISA results showed a strongly increasing IL-10 expression by M-MFs in parallel with the decrease of IL-1β. Based on this observation, we aimed to investigate the role of released IL-10 in rapid down-regulation of IL-1β secretion detected in M-MFs. To test this correlation, we used recombinant human IL-10 (rhIL-10) or IL-10 neutralizing antibody (IL-10NE) in our experiments (Figure 23). We found that secretion of IL-1β was significantly reduced by the administration of rhIL-10 cytokine, in good agreement with the suppression of its transcription. Furthermore, by blocking IL-10 binding to their receptor, we demonstrated significantly elevated IL-1β secretion as well as transcription by M-MFs.

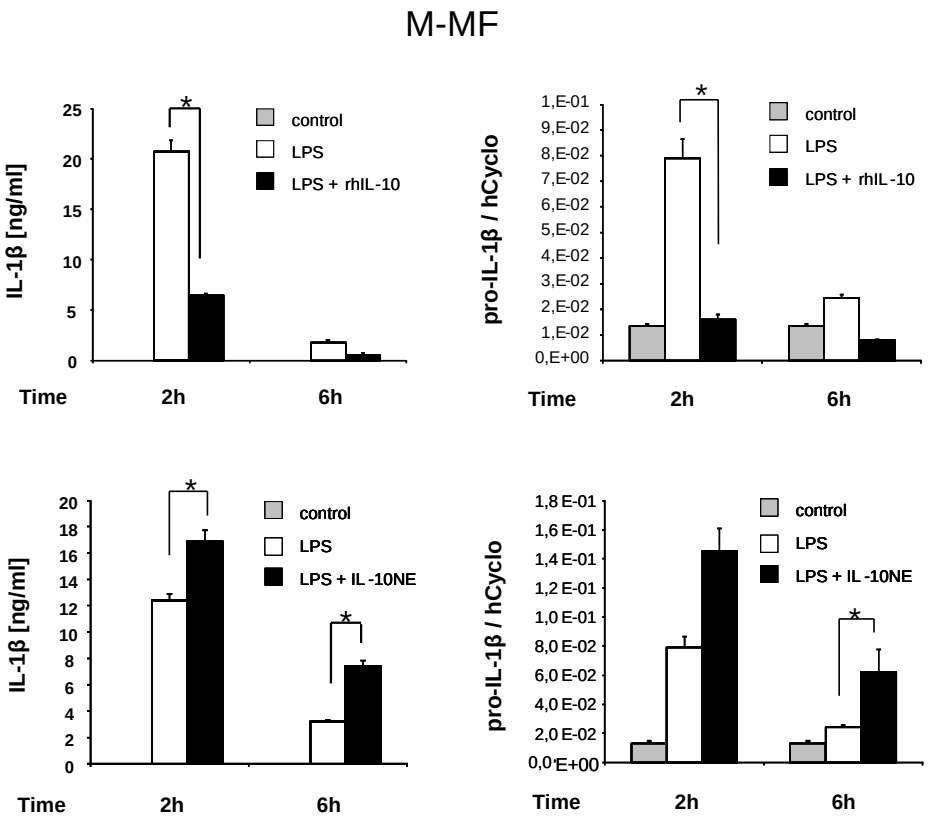


Figure 23. Released IL-10 initiates an auto-regulatory mechanism that partly limits IL-1β secretion by M-MF. M-MFs were pre-incubated with rhIL-10 (100 ng/ml) or with IL-10NE (10 μg/ml) for 1h, followed by priming with LPS. ATP treatment (45min) was used in all experiments in fresh FCS free medium prior to sample collection. Secreted IL-1β (left panel) and pro-IL-1β mRNA expression (right panel) are shown (*$p<0.05$).

One of the most important signaling pathways affected by IL-10 is the Akt signal transduction cascades, which is inhibited by IL-10 receptor ligation. To study this mechanism, we compared Akt phosphorylation in the two MF subtypes following LPS stimulation using a Western blotting. Our results show (Figure 24A) that Akt phosphorylation was significantly weaker in M-MFs at each time point following LPS stimulation compared to GM-MFs. When M-MFs were co-treated with LPS and rhIL-10, we observed a significantly reduced Akt phosphorylation compared to that induced by LPS alone, indicating that IL-10 indeed affects this pathway in M-MFs (Figure 24B).

These results indicate that the observed rapid down-regulation of IL-1β production by M-MFs, at least in part, may be explained as the indirect inhibitory effect of significantly induced IL-10 that resulted in down-regulation of Akt signaling pathway.

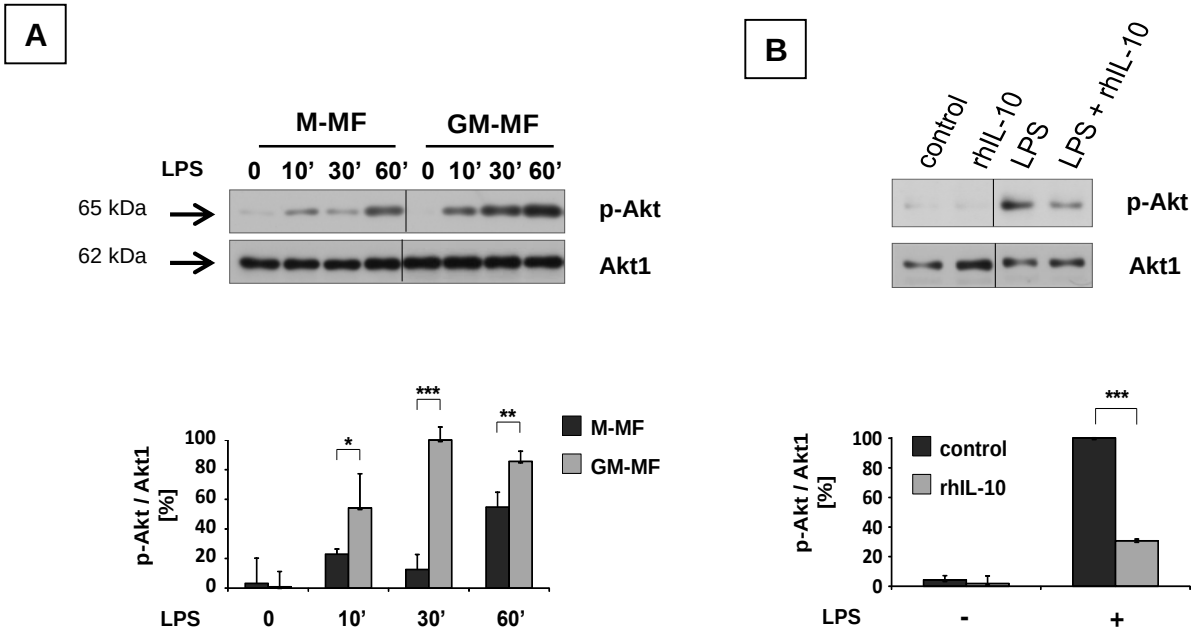


Figure 24. Akt phosphorylation in LPS-activated macrophages. (A) Cells were collected at the indicated time points after LPS stimulation and phospho-Akt (p-Akt) was detected from whole cell lysates by Western blot. The total level of Akt1 protein is shown to normalize protein loading (upper panel). The part of M-MF and GM-MF come from the same experimental blot. Western blots were quantified by densitometry (lower panel). (B) M-MFs were pre-incubated with rhIL-10 (100ng/ml) for 1h followed by LPS treatment. Western blot results show p-Akt and Akt1 protein expression level at 60 minutes after LPS treatment (upper panel). Western blots were quantified by densitometry (lower panel). *p<0,05, **p<0,01, ***p<0.001.

4.2. *Aloe vera* downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages

The anti-inflammatory activity of *Aloe vera* has been evaluated in a number of inflammation models partly via down-regulation of pro-inflammatory cytokine production. *Aloe vera* was shown to inhibit elevation of TNFα, IL-6 and IL-1β in mouse sepsis model and in human colorectal mucosa model as well [215, 216]. However, regarding human models, the effect of *Aloe vera* is usually studied in disorders involving the skin and epidermis [230, 231]. Only few studies examined the anti-inflammatory properties of this herbal in human immune cells, including peripheral blood leukocytes (PBLs) and undifferentiated THP-1 cells [217, 232]. Indeed, the molecular mechanisms behind the inhibitory effect of *Aloe vera* on IL-1β production by these cells remain unclear.

As we have shown, production of mature IL-1β is a strictly regulated process. In our primary macrophage models, M-MFs and GM-MFs, we demonstrated that IL-1β secretion is strongly induced by exogenous factors; such as LPS and ATP, but also several alterations during the activation may influence its production.

Here, we demonstrate the mechanism that likely contributes to the reduced IL-1β production by activated human macrophages treated with *Aloe vera*. For our experiments, we used macrophages differentiated from human monocytic THP-1 cells, in addition to primary macrophages. Our previous results revealed that macrophages generated by GM-CSF are associated with an induced and sustained inflammatory response. Therefore, we used GM-MFs as primary models to study the anti-inflammatory effects of *Aloe vera*. To induce strong inflammatory responses, we continued to use the TLR4 specific agonist, LPS.

4.2.1. GM-MFs are substantially more sensitive to Aloe treatment than THP-1 macrophages

First, we determined the cytotoxic effect of *Aloe vera* on human macrophages. THP-1 cells and GM-MFs were treated with an increasing amount of *Aloe vera* (1-10 v/v %) for 24 h, and cell viability was measured using an MTT assay**.** We found that using *Aloe vera* for up to 10 v/v % did not significantly affect the viability of THP-1 cells (Figure 25A). Interestingly, 5 v/v % of *Aloe vera* already resulted in more than 50% cell death of GM-MFs, and 10 v/v % of *Aloe vera* led to more than 70% death of those cells (Figure 25B). These results show that human primary macrophages are substantially more sensitive to *Aloe vera* treatment than THP-1 cell line, therefore we used different concentrations of *Aloe* for these cells.

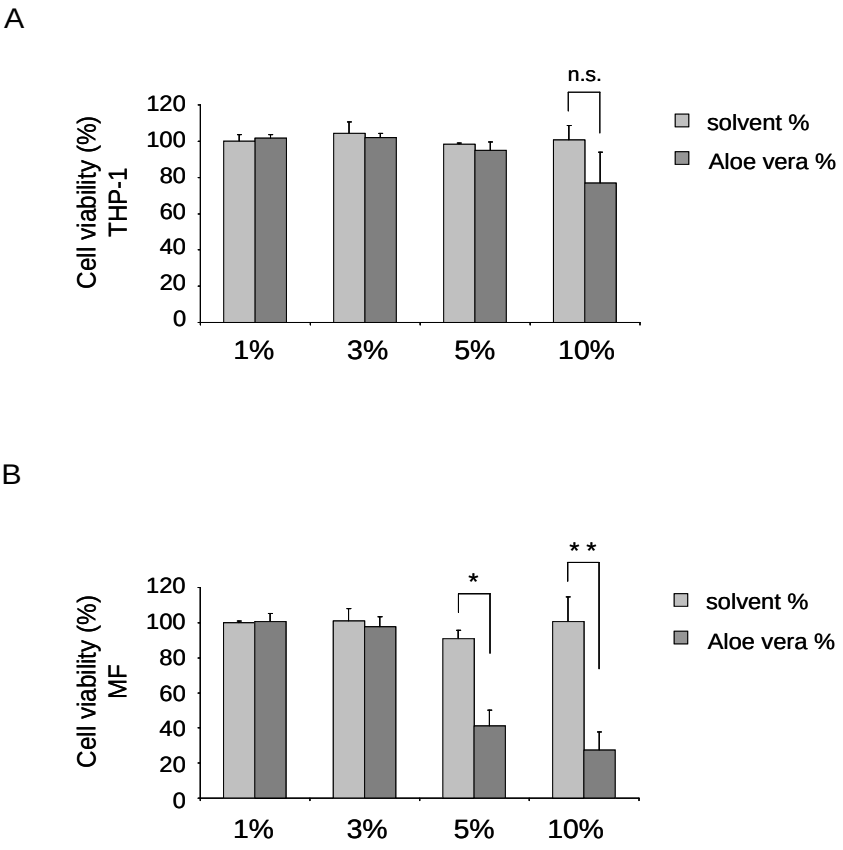


Figure 25. Determination of cell viability. PMA-differentiated THP-1 macrophages (A) and GM-MFs (B) were treated with increasing concentrations of solvent or *Aloe vera*. Results were obtained from three independent experiments with three replicates. Mean +/- SD values are provided as * $p < 0.01$, ** $p < 0.001$.

4.2.2. *Aloe vera* significantly decreases cytokine production of LPS-activated macrophages

To assess the impact of *Aloe vera* gel on cytokine secretion by THP-1 cells and primary GM-MFs, cells were treated with an increasing amount of *Aloe vera* in either the presence or absence of LPS. As in previous experiments, in this case, ATP was used for stimulating IL-1β release by LPS-primed GM-MFs. We differentiated THP-1 cells to macrophages using PMA; which is a ROS inducer in itself, and reactive oxygen species were generated to activate inflammasome. This way, THP-1 cells would not require a further activator such as ATP for IL-1β secretion.

Our ELISA results show that *Aloe vera* itself exerted anon-significant effect on the production of all measured pro-inflammatory cytokines in THP-1 cells, and had no effect on primary macrophages.

As our previous results indicated, GM-MFs release high levels of pro-inflammatory cytokines, such as IL-8, TNFα, IL-6 and IL-1β upon 24 hours of LPS priming. THP-1 cells were shown to respond to LPS treatment, similar to primary macrophages. *Aloe vera* significantly reduced LPS-induced secretion of these cytokines in both cell types in a concentration-dependent manner (Figure 26). Furthermore, our results also demonstrated that while in the case of GM-MFs the highest non-toxic concentration of *Aloe vera* (3 v/v %) resulted in more than 70 % reduction in cytokine production, attenuation of cytokine production was less pronounced in THP-1 cells, even with the 10 v/v % of *Aloe vera* treatment. Therefore, to explore the molecular basis of the previous observations, we used GM-MFs in the following experiments.

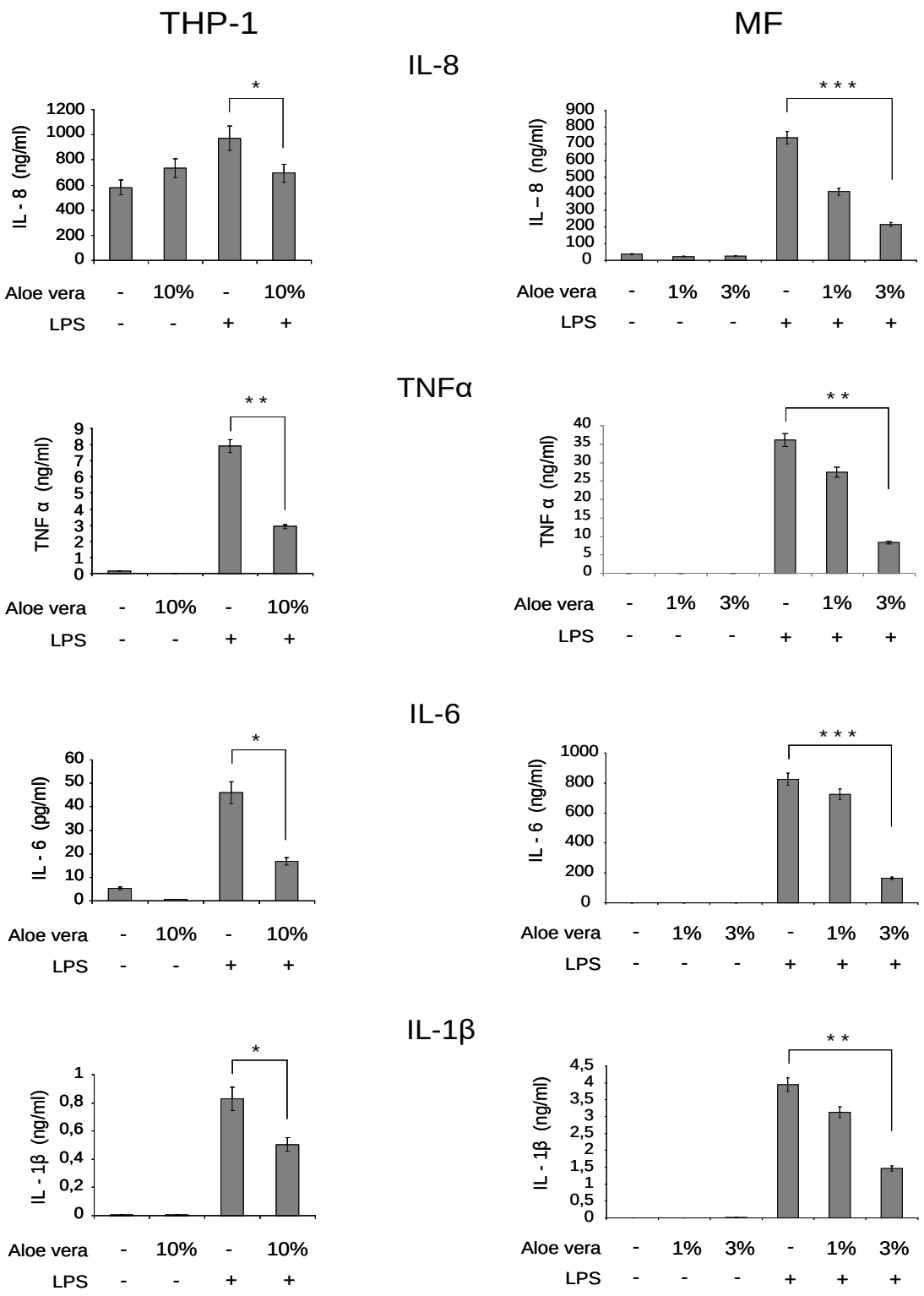


Figure 26. Effect of *Aloe vera* on LPS-induced cytokine production in THP-1 and GM-MF. PMA-differentiated THP-1 cells were pretreated with 10 v/v % *Aloe vera* for 1 hour, they were then subsequently treated with LPS for 24 hours (left panels). GM-MFs were pretreated with 1 v/v % or 3 v/v % *Aloe vera* for 1 hour and the cells were incubated with LPS for 24 hours (right panels). IL-8, TNFα, IL-6 and IL-1β were measured using an ELISA method. Experiments were performed three times and a representative result set is shown. Mean +/- SD values are provided as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

4.2.3. *Aloe vera* attenuates LPS-induced expression of IL-1β

We have previously shown how complex production of the master regulatory cytokine IL-1β can be. Production of IL-1β can be controlled at several steps. The first level of regulation is the priming step, which is necessary for pro-IL-1β expression. To determine whether *Aloe vera* influences IL-1β secretion, we studied changes in mRNA and protein levels of the precursor.

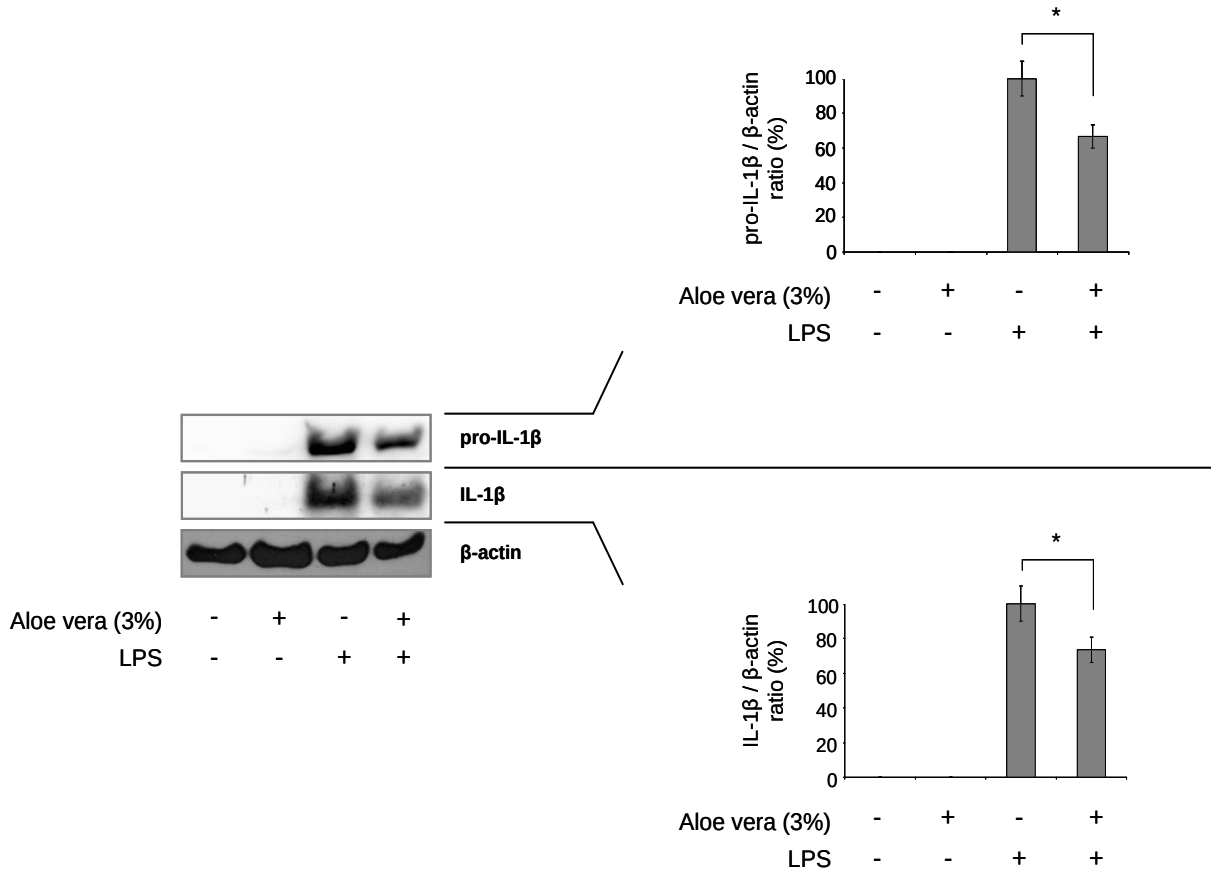


Figure 27. *Aloe vera* down-regulates LPS-induced protein expression of IL-1β. GM-MFs were pretreated with indicated amounts of *Aloe vera* for 1 h, cells were then stimulated with LPS for 24 hours, followed by ATP treatment (5 mM) for 45 min. Expression of pro-IL-1β and cleaved IL-1β was analyzed from the cell lysates by immunoblotting. Results obtained from three independent experiments and one representative Western blot is shown. Western blots were quantified by densitometry. * $p < 0.1$.

GM-MFs were treated with 3 v/v % of *Aloe vera* in the presence or absence of LPS. While *Aloe vera* alone did not have an effect on pro-IL-1β expression, LPS strongly induced it, resulting in the intracellular appearance of both the pro-IL-1β as well as cleaved IL-1β

proteins. In good agreement with the ELISA results, *Aloe vera* treatment significantly decreased the amount of both pro-IL-1β and cleaved IL-1β forms induced by LPS in the cell lysates (Figure 27).

According to these result, the substantial increase of IL-1β expression following LPS treatment can be explained at least partly by the enhanced transcription of the pro-IL-1β gene, as demonstrated by qPCR analysis. We also observed that *Aloe vera* treatment reduced LPS-triggered gene transcription in a dose-dependent manner (Figure 28). The dramatically induced transcription of pro-IL-1β in LPS-treated THP-1 cells was also significantly reduced in the presence of 10 v/v % *Aloe vera* (data not shown).

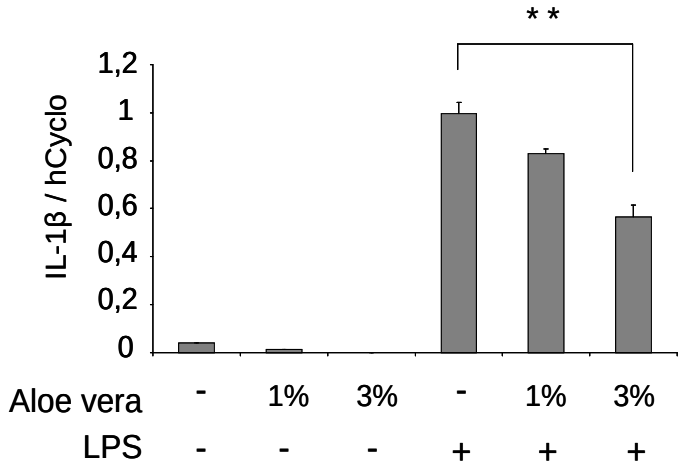


Figure 28. *Aloe vera* down-regulates LPS-induced mRNA expression of IL-1β. GM-MFs were pretreated with the indicated amounts of *Aloe vera* for 1 h, cells were then stimulated with LPS for 24 hours, followed by ATP treatment (5 mM) for 45 min. Pro-IL-1β mRNA expression was determined using qPCR. ** $p < 0.01$.

4.2.4. *Aloe vera* attenuates LPS-induced expression of NLRP3 and caspase-1 in GM-MFs

We have demonstrated previously; using specific siRNA or enzyme inhibitor, that LPS-primed and ATP triggered secretion of IL-1β by GM-MFs requires the caspase-1 dependent- NLRP3 inflammasome function. To see whether expression of NLRP3 inflammasome components are affected by *Aloe vera,* GM-MFs were activated with LPS in the presence or absence of *Aloe vera,* and the transcription as well as protein expression of NLRP3 and procaspase-1 was studied using quantitative RT-PCR and Western blot methods.

According to our previous results, we found that LPS significantly induced the expression of NLRP3 sensor at mRNA level resulting in induction of the protein. Caspase-1 was clearly detectable in untreated GM-MFs, and LPS stimulus elevated both the mRNA and protein levels by 24 hours. Except for the moderate decrease of caspase-1 protein, *Aloe vera* treatment alone did not affect the transcription and expression of the studied inflammasome components. However, it substantially down-regulated their LPS-induced transcription, and this effect was also detectable at the protein level (Figure 29).

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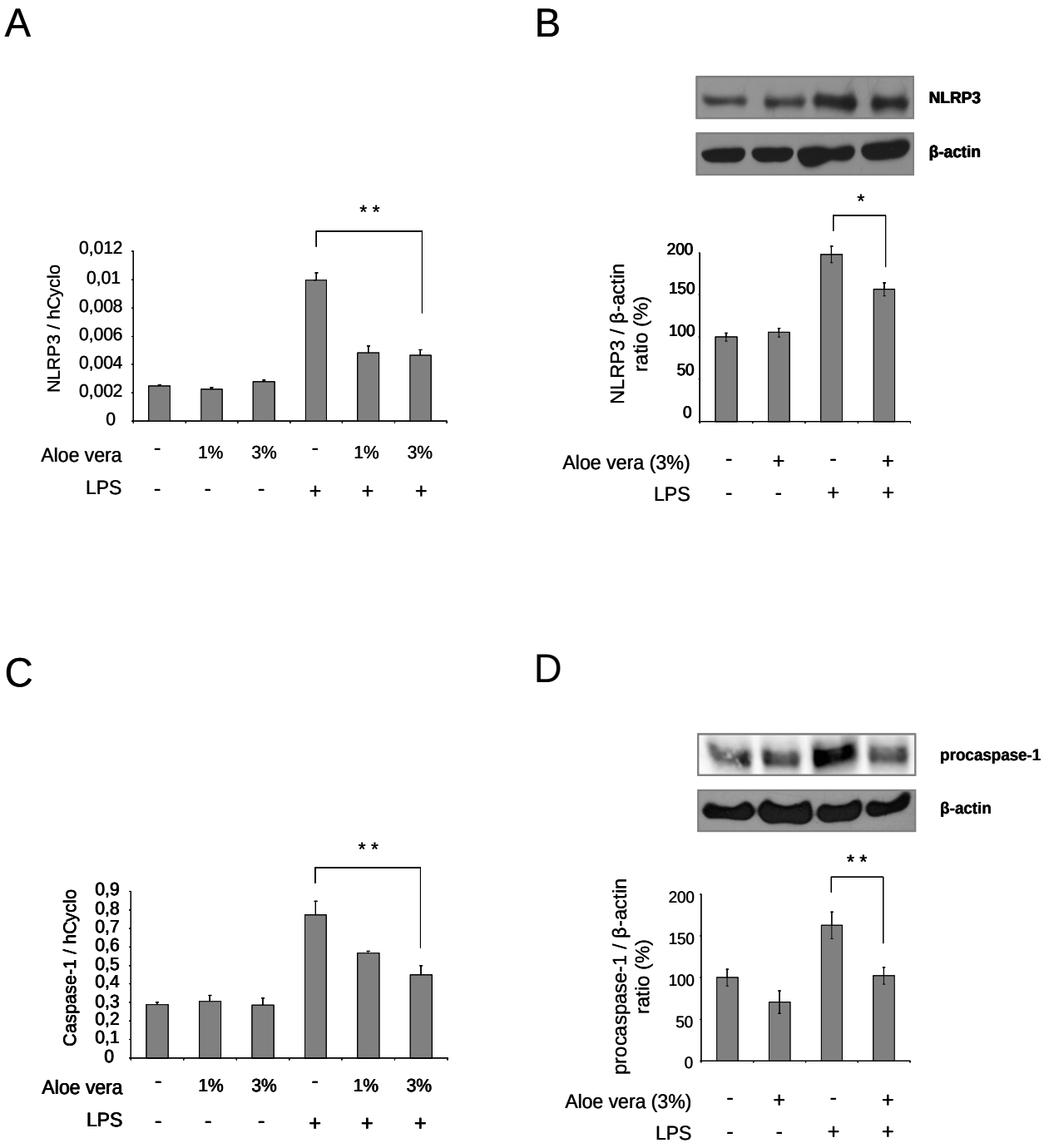

Figure 29. Effect of *Aloe vera* on LPS-induced expression of NLRP3 and procaspase-1. GM-MFs were pretreated with the indicated amounts of *Aloe vera* for 1 hour then cells were activated with LPS. After 24 hours, transcription of NLRP3 (A) and procaspase-1 (C) was determined by qPCR (* $p < 0.1$, ** $p < 0.01$). The protein levels of NLRP3 (B) and procaspase-1 (D) were determined from whole cell lysate using Western blot method. Western blots were quantified by densitometry. Results obtained from three independent experiments and one representative Western blot is shown. Mean +/- SD values of three independent experiments are shown.* $p < 0.1$, ** $p < 0.01$.

4.2.5. *Aloe vera* down-regulates the LPS-induced expression of P2X7 receptor

We have shown previously using specific receptor inhibitor, that IL-1β secretion triggered by ATP requires P2X7 receptor in LPS-primed GM-MFs.

Next, we aimed to study whether *Aloe vera* would decrease IL-1β secretion *via* influencing the expression of P2X7 receptor. GM-MFs were treated with LPS in the presence or absence of *Aloe vera*. As indicated, P2X7 receptor was strongly induced by LPS at 24 hour, an effect that was abolished by *Aloe vera* (Figure 30).

These results indicate the involvement of depleted P2X7 receptor store in reduced IL-1β secretion, while revealing the impact of *Aloe vera* on upstream mechanisms.

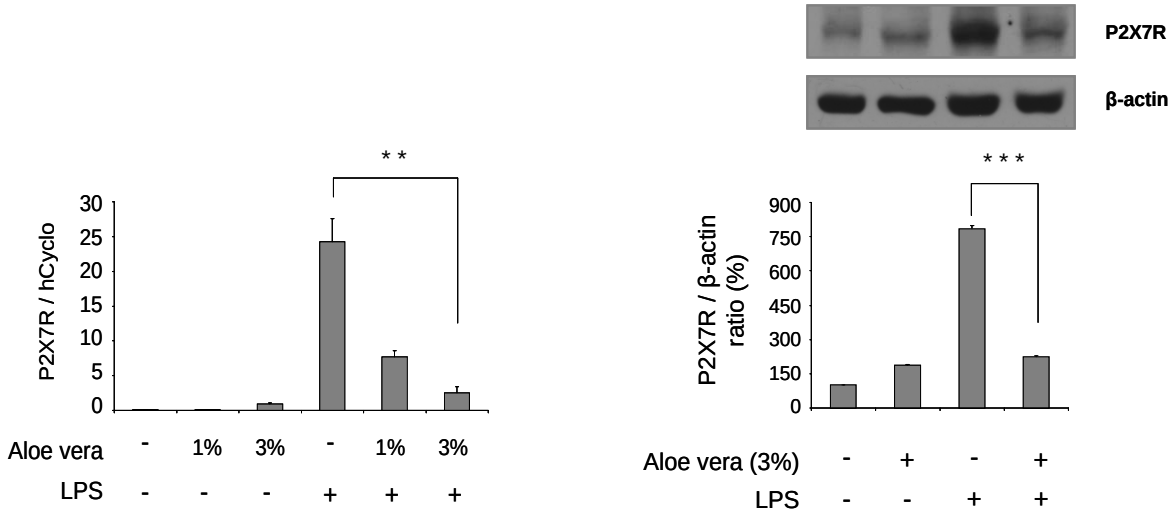

Figure 30. *Aloe vera* abolishes LPS-mediated enhancement of P2X7R expression.

GM-MFs were pretreated with the indicated amounts of *Aloe vera* for 1 hour. Cells were then activated with LPS for 24 hours. The mRNA level of P2X7R was determined by qPCR. The protein expression of P2X7R was detected by Western blot. Western blots were quantified by densitometry. Results obtained from three independent experiments and one representative Western blot is shown. ** $p < 0.005$, *** $p < 0.0005$

4.2.6. *Aloe vera* inhibits LPS-induced activation of NF-κB, p38, JNK and ERK signal transduction pathways

LPS induces a network of signaling pathways that, as a consequence, influence the expression of several genes, including pro-inflammatory cytokines. To explore whether *Aloe vera* has an effect on LPS-induced activation of signal transductions, macrophages were treated with LPS in the presence or absence of *Aloe vera*, and the time-dependent phosphorylation of key signaling molecules of the NF-κB, p38, JNK, and ERK signaling pathways were followed by Western blot (Figure 31).

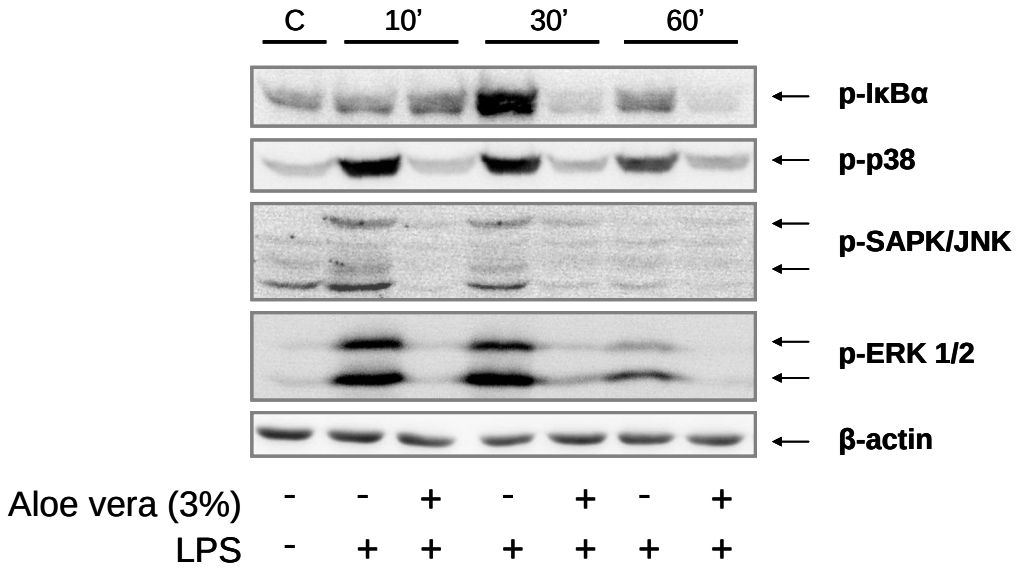

Figure 31. Effect of *Aloe vera* on LPS-induced signal transductions.

GM-MFs were pretreated with *Aloe vera* for 1 hour, cells were then activated with LPS for the indicated times. The p-IκBα,, p-SAPK/JNK,p- ERK and p-p38 MAPK were analyzed by Western blot.. Results obtained from three independent experiments and one representative Western blot is shown.

We described previously, that LPS strongly induces phosphorylation of IkBα in GM-MFs. Interestingly, this was completely abolished by *Aloe vera* treatment. Unlike NF-kB pathway, MAPK pathways were also strongly activated upon LPS stimulation. p38, ERK 1/2 and SAPK/JNK were already strongly phosphorylated 10 minutes after LPS treatment, which appeared to be slowly decreasing in the studied 60 min interval, however, this phosphorylation was inhibited by *Aloe vera* treatment.

Altogether, these results show that *Aloe vera* treatment results in a broad spectrum of inhibition of signal transduction pathways.

5. Discussion

IL-1β is a pivotal cytokine that regulates many immunologic and physiologic functions, including the activation and synchronization of various cells that lead to either acute or chronic inflammatory responses, but it also participates in processes such as wound healing and tissue regeneration. Due to the wide variety of its beneficial and harmful effects, secretion of IL-1β cytokine is tightly regulated by the activation of caspase-1, which occurs *via* recruitment to the multi-protein complex termed NLRP3 inflammasome. IL-1β is produced and secreted by a variety of cell types, although the vast majority of studies have focused on its production within cells of the innate immune system, such as monocytes and macrophages. Plasticity and diversity are the hallmarks of macrophage lineages and a huge spectrum of protocols were developed to enable the study of different types of MF in *in vitro* conditions. We reported for the first time, a comparative study about the dynamics and molecular mechanisms of NLRP3 inflammasome activation, in the generally accepted two distinct macrophage subtypes differentiated by M-CSF (M-MFs) and GM-CSF (GM-MFs), respectively. We focused on the LPS-induced IL-1β secretion by these human macrophages, using ATP supplementation, which is a triggering DAMP for NLRP3 inflammasome activation.

First, we compared the cytokine profile of these macrophages upon LPS stimulation, and found that GM-MFs display enhanced production of the pro-inflammatory cytokine, more markedly than that of M-MFs. Furthermore, only M-MFs were capable of secreting IL-10 anti-inflammatory cytokine. These results are in good agreement with the general notion that human macrophages generated from monocytes in the presence of GM-CSF predominantly have a common pro-inflammatory phenotype that leads to perpetuation of inflammation instead of its resolution. Contrarily, M-CSF treatment induces human monocytes to adopt a potent anti-inflammatory macrophage phenotype that dampens pro-inflammatory cytokines and may be essential for tissue repair and wound healing [205, 210, 233].

Surprisingly, we found that in the presence of ATP or even the ionophore nigericin, both macrophage subtypes produced comparable amount of IL-1β, although at very different time kinetics. While secretion by M-MFs was a very rapid process and lasted only for a few hours after LPS treatment, in case of GM-MFs, it was a slower process that led to a gradually increasing IL-1β secretion. Purinergic mediators, such as ATP are released from cells into the extracellular space in response to metabolic disturbances or other types of insults, including

inflammation and tissue injury in *in vivo* conditions. The purinergic system is an evolutionarily selected system enabled to fine-tune immune cell functions, such as cell-to-cell interaction, cytokine and chemokine secretion, and intracellular pathogen removal [234]. Most recent reports have highlighted the importance of purinergic signaling in tumor therapy due to the pro-inflammatory role of ATP and that P2X7 levels are significantly lower in various types of cancer cells compared with normal cells [235]. In the early stages of tumors, tumor-associated macrophages (TAMs) adopt the pro-inflammatory phenotype for the inhibition of tumor immunity. In contrast, TAMs shift to anti-inflammatory state in late-stage tumors, which promotes tumor progression and metastasis. [236]. ATP addition may inhibit cancer growth [237], and as our results show, high extracellular ATP level is able to change inflammatory state of MFs by inducing IL-1β production, which may contribute to tumor regression.

Production of IL-1β and activation of the NLRP3 inflammasome are tightly regulated, and in macrophages, require two signals. The priming signal ensures the expression of the required components, while the second signal; such as ATP, triggers the assembly and activation of the complex. As we expected, in the absence of a second stimulus, we did not detect IL-1β from the supernatant of the LPS-primed M-MFs, however, surprisingly, under similar conditions, we detected IL-1β production of GM-MFs, in an amount comparable to that produced by monocytes. Previous studies reported that human monocytes stimulated with microbial components release ATP in an autocrine / paracrine manner, which is responsible for the purinergic P2X7 receptor-dependent activation of NLRP3 inflammasome, and leads to IL-1β secretion [238]. Our results demonstrated that both macrophage subtypes released nanomolar ATP, however, it was significantly higher in the culturing medium of GM-MFs compared to that of M-MFs. Nevertheless, unlike in the case of TLR ligation in microglia and monocytes [239], the concentration of released ATP did not increase upon LPS stimulation. We also observed that both MFs expressed comparable amounts of P2X7 receptor, and we showed that these receptors were functional, since the receptor specific inhibitor efficiently blocked LPS-induced IL-1β production in the presence of ATP supplementation. However, we demonstrated that LPS-induced IL-1β secretion by GM-MFs was independent of the released endogenous ATP. In line with our results, murine bone marrow-derived DC (BMDC) was found to secrete substantial amounts of mature IL-1β upon TLR activation, which was dependent on the NLRP3 inflammasome, but independent of the P2X7 receptor [240]. However, a definite molecular explanation was not provided for the observed result. Based on our observations and published reports from the field, it seems that

the low sensitivity of P2X7 receptors to ATP in both M-MFs and GM-MFs [241] limits their activation to conditions in which immune cells or dying cells release millimolar concentration of ATP into the extracellular space. This threshold enables P2X7 receptor to function as a danger sensor associated with inflammation or cell injury [242, 243]. Indeed, ATP in a nanomolar range may induce signaling through high-affinity P2Y receptors, and influence other cell functions like chemotaxis or rapid remodeling of cytoskeleton for phagocytosis or migration[244, 245].

Following the release of ATP into the extracellular space, CD39 converts ATP into AMP, and then CD73 dephosphorylates AMP into adenosine. The function of these major nucleotide metabolizing enzymes determines the actual amount of extracellular ATP that regulate immunity and inflammation. Our results showed significant differences in the expression of both CD39 and CD73 after LPS treatment. It has been well documented that MFs express different ectonucleotidases to reduce inflammation by removing ATP and producing anti-inflammatory adenosine, the subtypes of MFs as well as their polarization and activation determine the expression level of those enzymes. For example, expression of the ATP hydrolyzing CD39 was reported to be up-regulated in peritoneal macrophages and IL-4-treated BMDMs that have immunosuppressive characteristics [246, 247], and we demonstrated that the LPS-induced increase of CD39 expression was more significant in M-MFs than in GM-MFs. In contrast, expression of CD73 that induces extracellular accumulation of adenosine resulted in a much more robust expression in GM-MFs compared to M-MFs. The ability of these macrophage subtypes to express different ectonucleotidases led us to speculate that the level of by- and end-products of ATP hydrolysis; such as adenosine, may have an important indirect regulatory effect on IL-1β production by GM-MFs. This notion is supported by our results showing a significant decrease in IL-1β secretion solely upon LPS activation in GM-MFs in the presence of ectonucleotidase inhibitor, while the extracellular ATP level was significantly increased.

Extracellular adenosine act as a signaling mediator, participating in tissue regeneration, wound healing [248], and immune responses, exerting mainly anti-inflammatory effects on dendritic cells, neutrophils, and MFs [249, 250]. It was reported that LPS treatment increases the extracellular level of adenosine in many cell types, including macrophages [251, 252]. Nevertheless, adenosine was also reported to be a key regulator of IL-1β secretion, increasing the duration of the inflammatory response *via* the A2a receptor, these studies however involved murine peritoneal macrophages, BMDMs [253, 254] or human THP-1 cell line [255]. Indeed, our results show that in the absence of ATP supplementation, adenosine

treatment increased the LPS-induced IL-1β secretion by GM-MFs in a dose dependent manner. We also found that IL-1β secretion by LPS primed GM-MFs was significantly reduced by A2a or A2b specific receptor inhibitors, which receptor's expression is more significant in the GM-MFs than in the M-MFs. A2a and A2b receptor activation stimulate cyclic AMP formation, which can bind to and modulate the function of nonselective cation channels that conduct calcium and potassium fluxes. Both are important triggers of NLRP3 inflammasome activation [256-258], strengthening the role of adenosine as an essential danger signal in IL-1β secretion by GM-MFs.

Finally, we tested whether adenosine can increase IL-1β secretion induced by the combination of LPS and ATP, both of which activate signals 1 and 2, respectively. This condition resulted in a high level of IL-1β production in both macrophage types, however, this was significantly increased by adenosine only in GM-MFs. Importantly, we did not notice a similar effect in case of M-MFs, the results of which seems to contradict observations published by the group of Ouyang, although they detected adenosine induced IL-1β secretion in murine macrophages differentiated by M-CSF [253]. Considering the versatile, opposing, yet sometimes complementary effect of ATP and adenosine, changes in the balance of the ATP-adenosine axis may have a fine-tuning effect on the regulation of IL-1β secretion, depending on the cell type and the activating conditions. Nonetheless, further studies are needed to provide a detailed explanation on the cell-specific effects of these purinergic compounds.

Next, we examined the role of NLRP3 inflammasome in the control of IL-1β secretion by both macrophage subtypes, for the reason of various inflammasome complexes beeing associated with IL-1β production. Using caspase-1 specific inhibitor or silencing of NLRP3 protein, have confirmed that IL-1β secretion by M-MFs and GM-MFs, respectively requires the canonical caspase-1–dependent NLRP3 inflammasome function. When we compared the expression profile of the inflammasome components between these cells, we observed vast differences in the expression dynamics of NLRP3 as well as pro-IL-1β, as in M-MFs, LPS treatment led to a rapid increase of these proteins, but only for a short time interval, while the same treatment induced a slow, gradually increasing expression in GM-MFs. Our results also demonstrated the requirement of the priming step for the pro-IL-1β expression in both types of macrophages, however, a striking difference was found in the expression of NLRP3 between these cells that was constitutively expressed in M-MFs. These are in accordance with the findings of Hornung and co-workers, who suggested that the first signal results in the *de novo* synthesis of proIL-1β, and the upregulation of NLRP3 [259]. We speculated different

molecular mechanisms underlying the observed differences in the expression of these proteins in the two cell types. Therefore, we analyzed various signaling pathways including p38, JNK and ERK MAP kinases or NF-kB, which were reported to induce pro-IL-1β and NLRP3 expression [108, 110, 260-262]. We found, however, that LPS stimulation induced each of those studied pathways in both MFs with different dynamics, and with the exception of NF-kB, the immediate activation was more pronounced in M-MFs. Furthermore, we assigned a substantial role to ERK pathway, which was described as the key transducer of M-CSF signaling, and we detected strong ERK phosphorylation in M-MFs even in non-treated cells [263, 264]. We conclude that the constitutive background activation of ERK in M-MFs induced by M-CSF provides a "half-primed" condition that result in continuous basal expression of NLRP3, which is normally provided by LPS in GM-MFs.

To investigate differences in the activation of NLRP3 inflammasome between these cell types, we analyzed the function of caspase-1 enzyme, which was shown above to play an essential role in IL-1β secretion by both macrophage subtypes. Our findings demonstrate that besides priming, that is required for both macrophage types for *de novo* synthesis of proIL-1β and upregulation of NLRP3, a second signal which can be provided by ATP is absolutely necessary for the activation of caspase-1 in M-MFs, but not in GM-MFs. We detected constitutive caspase-1 activity in GM-MFs in the absence of ATP supplementation, which was further induced when cells were treated with ATP. Previously, constitutive caspase-1 activation was reported in THP-1 monocyte cell line or in human monocytes purified from PBMC [265]. We assumed that the observed IL-1β release by GM-MFs in the absence of ATP supplementation is due to the constitutively active enzyme. Similar to our observations, it has been shown that, because of the constitutively active caspase-1, a single stimulation such as LPS leads to the release of IL-1β in monocytes, whereas MFs require two distinct stimuli for cytokine release. Notably, however, the studied MFs were generated in the presence of either 10% plasma or G-CSF [266]. There is an apparent contradiction between the detected caspase-1 activity and the absence of processed caspase-1 in the immunoblot. It has been demonstrated that caspase-1 is rapidly inactivated upon formation of the mature enzyme, thereby restricting the activity towards highly preferred substrates such as IL-1β and IL-18 [79]. Therefore, it is presumed that only a fraction of the immunologically detected caspase-1 is active. Conversely, active caspase-1 may not necessarily be detected by immunoblot because the fluorescent assay is substantially more sensitive. Furthermore, the inactive caspase-1 fragments may be further modified for rapid proteasomal degradation [267]. The different mechanisms and dynamics of activation and inactivation / degradation of

caspase-1 in the MF subtypes may also contribute to the observed results. Nevertheless, since caspase-1 is an essential component of NLRP3 inflammasome-mediated IL-1β production, further studies are required to provide the molecular explanation of the constitutive activity of caspase-1 enzyme.

We have found another interesting correlation between IL-1β and IL-10 secretion by M-MFs. Our results showed that LPS treatment strongly induces increasing IL-10 secretion by M-MFs, in parallel with the time-dependent decrease of IL-1β release. IL-10, as an anti-inflammatory cytokine, may inhibit expression of several pro-inflammatory cytokines [203, 268, 269]. By inhibiting IL-10 binding to IL-10R, we demonstrated that the observed decrease in IL-1β production by M-MFs is, at least in part, mediated by IL-10. This phenomenon may help to elucidate how tumor-associated MFs foster the development of a tolerant microenvironment of tumors, which may counteract effective immune responses and limits the efficacy of the chemotherapy [270].

Furthermore, we detected a significant weaker activation in Akt signaling pathway in M-MFs compared to GM-MFs following LPS treatment, which is one of the most important signaling pathways affected by IL-10 [248, 249]. Based on reports, IL-10 can abolish Akt phosphorylation that in turn leads to the decreased phosphorylation of downstream signaling pathways [271, 272]. These findings and our results may explain the decreased phosphorylation of JNK and ERK phosphorylation at 60 minutes following LPS stimuli in M-MFs. Considering that these pathways have an important regulatory role in the priming process of NLRP3 inflammasome, the observed down-regulation of NLRP3, pro-IL-1β expression and the following attenuation of IL-1β secretion in M-MFs, at least partly, may be explained by the indirect inhibitory effect of IL-10. However, it is worth noting that pro-inflammatory cytokines such as IL-6 are potent inducers of Akt phosphorylation and other cytokine production [273-275]. For this reason, the rapidly reduced expression of IL-1β in M-MFs could be a combined result of the significant IL-10 production and the lack of pro-inflammatory cytokines such as IL-6.

Our results may widen the general knowledge about macrophages, a heterogeneous population with diverse phenotypes that facilitate effective immune responses to the ever-changing environment. We have clarified the effect of several factors in the function of NLRP3 inflammasome between two macrophage types, GM-MF or M-MF, generated from human monocytes in the presence of the two classical hematopoietic growth factors GM-CSF or M-CSF. A high production of pro-inflammatory mediators have been closely related with a deficient activation of macrophages, as well as the pathogenesis of several chronic diseases

with inflammatory basis; such as rheumatoid arthritis, atherosclerosis, diabetes and cancer [276]. Therefore, further insight into IL-1β production of macrophages would be of great benefit to clinical immunology, providing an overview of potential targets that may promote therapeutic advantages for the treatment of different inflammatory diseases in the future.

Aloe vera is a high-quality wound care product that has been used traditionally for centuries by different cultures. In addition, the active components lead to broad health benefits; such as anti-diabetic, hypoglycaemic, anti-cancer, antimicrobial, antiviral and gastrointestinal activities [277]. The anti-inflammatory activity of *Aloe vera* has been intensively studied over the past few decades; however, detailed molecular mechanism of its effect is only being explored in recent years. In our work, we examined the effect of *Aloe vera* gel supernatant on IL-1β production of the earlier well defined inflammatory macrophage type GM-MF, and the human macrophage-like THP-1 cell line. Our results show that *Aloe vera* treatment significantly down-regulated LPS-induced IL-8, TNFα, IL-6 and IL-1β inflammatory cytokine production, in a concentration dependent manner in both THP-1 cells and GM-MFs. These results are in line with studies demonstrating anti-inflammatory effects of *Aloe vera* in animal models, without molecular explanation of the effect on IL-1β and TNFα production in non-differentiated THP-1 cells [217, 232, 278-280]. It should be noted that different amounts of *Aloe vera* solution was used for treatment of THP-1 cells and the monocyte-derived GM-MFs. While 10 v/v% of *Aloe vera* did not cause significant changes in the viability of THP-1, the highest non-toxic concentration used to GM-MFs was 3 v/v% indicating that primary cells are more sensitive than immortalized cell line. Furthermore, not only the cytotoxic sensitivity of primary GM-MFs was higher than that of THP-1 cells, but also the susceptibility towards *Aloe*-mediated down-regulation was more pronounced in these cells. THP-1 is the most common cell line utilized to study monocyte/macrophages differentiation and function [281]. While monocytic cell lines have obvious advantages in terms of ease of acquisition, as compared to primary macrophages, their differentiation state meant that inferences drawn from these experiments may not always accurately predict the behavior of differentiated tissue macrophages. For this reason, and to obtain results close to *in vivo* status, our further studies on the molecular characterization of the *Aloe*'s effects on IL-1β production focused on primary macrophages. Previously, we have shown the requirement of caspase-1 dependent NLRP3-inflammasome function in the IL-1β production of GM-MFs. Therefore, we analyzed the effect of *Aloe vera* on the expression of these essential inflammasome components. Our results demonstrate that the LPS-induced caspase-1 and NLRP3 expression was significantly decreased in the presence of *Aloe vera* solution, both at

mRNA and protein levels. Moreover, we also determined a down-regulatory effect of *Aloe vera* on the pro-IL-1β expression in LPS-activated GM-MFs. Since LPS-induced IL-1β production requires the presence and function of NLRP3 inflammasome, these results show that the decreased level of IL-1β after *Aloe vera* treatment may be the consequence of attenuated expression of not only the pro-IL-1β but the inflammasome components as well. As our previous results showed, the ATP-triggered NLRP3 inflammasome-mediated IL-1β production by LPS-primed GM-MFs was a P2X7 receptor dependent mechanism. Accordingly, we studied the effect of *Aloe vera* on the P2X7 receptor expression and we found that it completely abolished the effect of LPS induction on the expression of this ATP-sensor.

At the same time, expression of these components is regulated by signaling pathways such as NF-kB and MAPKs as discussed above. Our results showed that LPS induced phosphorylation of Ik-Bα, p38, JNK and ERK molecules, which was significantly inhibited by *Aloe vera* treatment. These results suggest that *Aloe vera* inhibits IL-1β production by GM-MFs at the level of the priming step, upstream of the NLRP3 inflammasome. However, the observed changes in the expression of the ATP-sensor P2X7 receptor may also contribute to the pronounced reduction of IL-1β secretion, underlying a versatile and efficient anti-inflammatory effect of *Aloe vera*. Thereby, *Aloe vera* could be a new therapeutic tool to target NLRP3 inflammasome-mediated cytokine production.

Our knowledge about the general mechanism of NLRP3 inflammasome function and its regulators is increasing rapidly. We have shown that the actual outcome of IL-1β activation and production is strongly determined by the molecular characteristics of the cell type harboring various intracellular or extracellular modulators. Our study sought to unravel the molecular mechanisms of LPS-primed NLRP3 inflammasome activation. These results may help clarify some controversial reports within the NLRP3 inflammasome field, the divergences of which are derived from methodological, cell type, and species differences in studies of NLRP3 inflammasome regulation.

6. Summary

Macrophage phenotype and function are dependent and driven by the complex and frequently changing cellular and molecular microenvironment *in vivo*. In human system, *in vitro* M-CSF and GM-CSF are the most frequently used to differentiate blood monocyte-derived M-MFs or GM-MFs, respectively. The general notion that GM-CSF treatment of monocytes results in the formation of macrophages with enhanced bactericidal capacity and secretion of pro-inflammatory mediators stands. In contrast, M-CSF treatment leads to the generation of macrophages with a decreased ability to produce inflammatory cytokines and participate in wound healing and tissue regeneration.

When we compared LPS-induced cytokine secretion by these cells, we found that pro-inflammatory cytokine (TNFα, IL-6 and IL-8) production was more pronounced in GM-MFs compared to that of M-MFs, while only M-MFs produced IL-10 anti-inflammatory cytokine. Surprisingly, our results showed that in the presence of the second signal, ATP, both LPS-primed MF subtypes produced comparable amount of IL-1β, albeit with very different dynamics. IL-1β secretion by LPS-primed M-MFs was a very rapid and short event, and is attenuated in part by the presence of IL-10 which reduces Akt signaling. However, IL-1β production by GM-MFs develops gradually, and these cells were capable of producing moderate amounts of IL-1β even in the absence of ATP supplementation, due to the constitutively active caspase-1 enzyme. The observed IL-1β release was independent of the P2X7 receptor signaling, and was not directly influenced by the released endogenous ATP by GM-MFs. The actual amount of extracellular ATP is determined by the balance of ATP released from cells and ATP hydrolyzed by the membrane-bound ectonucleotidases. We have shown that the ectonucleotidases CD39 and CD73 were differentially expressed in these macrophages, and they played an important regulatory role in the IL-1β secretion in GM-MFs. Furthermore, we provided evidences that adenosine; one of the end-products of ectonucleotidase function, enhanced LPS-primed IL-1β secretion by GM-MFs, this stimulatory effect however was not observed in M-MFs. Our results showed that due to the different activation status and expression levels of the NLRP3 inflammasome components, as well as the diverse signaling activity of the pathways, the two subtypes of macrophages responded very differently to the same stimuli.

To study the anti-inflammatory *Aloe Vera* on IL-1β secretion by human macrophages, we chose GM-MFs with prolonged pro-inflammatory properties. We showed that *Aloe vera*-mediated strong reduction of IL-1β appeared to be the consequence of the reduced expression

of both pro-IL-1β as well as NLRP3 inflammasome components, through suppression of specific signal transduction pathways. Furthermore, we demonstrated that expression of the ATP sensor P2X7 receptor was also downregulated by *Aloe vera,* which may have also contribute to the attenuated IL-1β cytokine secretion.

Intensive studies have advanced our knowledge about the general mechanisms of NLRP3 inflammasome function and its regulators. We believe that our results shed light on some controversial results in the field of the NLRP3 inflammasome, and also clarify that the actual outcome regarding the activation and production of IL-1β is strongly determined by the molecular characteristics of the cell type, involving various intracellular or extracellular modulators. Moreover, our results may provide a potential therapeutic approach, to better manage IL-1β- mediated systemic or chronic diseases.

Összefoglalás

A makrofágok fenotípusainak és funkcióinak *in vivo* kialakulása nagymértékben függ az összetett és folyamatosan változó mikrokörnyezetben található molekuláris és sejtes tényezőktől. Humán rendszerben, *in vitro* körülmények között, a perifériás vérből származó monocitákból leggyakrabban M-CSF (M-MF) és GM-CSF (GM-MF) jelenlétében történik a makrofágok differenciáltatása. A szakirodalomból ismert, hogy a GM-CSF kezelés során a monocitából nagy baktériumölő képességgel rendelkező makrofág keletkezik, mely nagy mennyiségben termel gyulladásos mediátorokat. Ezzel szemben az M-CSF kezelés hatására keletkező makrofágokra nem jellemző a gyulladásos citokinek termelése és leginkább a sebgyógyulásban és a szöveti regenerációban játszanak fontos szerepet.

Összehasonlítva a két LPS-aktivált MF típus citokin termelését azt tapasztaltuk, hogy a GM-MF sokkal intenzívebben termelte a TNFα, IL-6 és IL-8 citokineket, az M-MF-hoz képest. Ugyanakkor csak az M-MF esetében mértünk IL-10 anti-inflammatórikus citokin felszabadulást. Érdekes módon további eredményeink azt mutatták, hogy a második szignál, az ATP jelenlétében mindkét LPS-aktivált MF képes volt hasonló mennyiségben IL-1β citokint termelni, habár eltérő módon. Az M-MF rövid idejű LPS kezelés hatására szekretálta az IL-1β-t, aminek a szintje a hosszabb LPS kezelés során gyorsan lecsökkent. Ez a jelenség részben a felszabaduló IL-10 hatásának volt köszönhető, ami gátolta az Akt jelátviteli útvonalat. Ezzel szemben a GM-MF IL-1β termelése folyamatosan nőtt az LPS aktiválás során ATP jelenlétében. Érdekes módon ezek a sejtek ATP kezelés nélkül is képesek voltak IL-1β szekrécióra, melyben a folyamatosan aktív kaszpáz-1 enzim játszott fontos szerepet. Kimutattuk, hogy az LPS-indukált IL-1β termelést a GM-MF által felszabadított endogén ATP nem befolyásolta közvetlenül és P2X7 receptor független módon valósult meg. A pillanatnyi extracelluláris ATP koncentrációt a felszabaduló ATP mennyisége és a membránkötött ekto-nukleotidázok ATP bontása határozza meg. Eredményeink azt mutatták, hogy a két makrofág típus eltérő mértékben expresszálta a CD39 és a CD73 ekto-nukleotidázokat, melyek a GM-MF-ok IL-1β termelésében fontos regulátornak bizonyultak. Továbbá azt is kimutattuk, hogy az adenozin, az ekto-nukleotidázok működése során keletkező egyik végtermék, fokozza az LPS-indukált IL-1β szekréciót a GM-MF-ban, ezzel szemben az M-MF-nál ilyen jellegű hatást nem tapasztaltuk. Eredményeink azt mutatják, hogy az M- és a GM- makrofágok különböző stimulusokra adott eltérő válaszát az NLRP3 inflammaszóma expressziójában és aktivációs állapotában, valamint a jelátviteli útvonalak aktivációjában tapasztalt különbségek okozzák.

Az anti-inflammatórikus *Aloe Vera* humán makrofágok IL-1β termelésére gyakorolt hatásának vizsgálat a GM- makrofágokon végeztük, melyek gyulladásos tulajdonságait mi is bizonyítottuk. Kimutattuk, hogy az *Aloe vera* által erősen gátolt IL-1β szekréciót mind a pro-IL-1β mind pedig az NLRP3 inflammaszóma komponensek expressziójában bekövetkezett csökkenés eredményezte, mely specifikus útvonalak gátlásán keresztül valósult meg. Továbbá eredményeink szerint az *Aloe vera* az ATP szenzor P2X7 receptor kifejeződését is gátolta, mely nagymértékben hozzájárult az IL-1β szint csökkenéséhez.

Az intenzív kutatások eredményeként általános ismeretünk az NLRP3 inflammaszóma funkciójáról és szabályozásáról gyorsan bővül. Úgy véljük, hogy eredményeink segítenek az NLRP3 inflammaszómával kapcsolatos ellentmondások egy részét megérteni. Továbbá hozzájárulnak annak megértéséhez is, hogy az inflammaszóma aktiváció és az IL-1β termelődés nagymértékben a sejttípus tulajdonságaitól többek között különböző intracelluláris és extracelluláris szabályzó molekuláktól függ. Mindazonáltal, eredményeink egy új terápiás megközelítés lehetőségét kínálhatja, mely hatékonyabb IL-1β szabályozást nyújthat szisztémás vagy krónikus betegségekben.

7. References

7.1. References related to the dissertation

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7.2. Publication list prepared by the Kenézy Life Sciences Library

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List of publications related to the dissertation

1. **Budai, M. M.**, Tőzsér, J., Benkő, S.: Different dynamics of NLRP3 inflammasome-mediated IL-1[béta] production in GM-CSF- and M-CSF-differentiated human macrophages.
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2. **Budai, M. M.**, Varga, A., Milesz, S., Tőzsér, J., Benkő, S.: Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages.
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

List of other publications

3. Varga, A., **Budai, M. M.**, Milesz, S., Bácsi, A., Tőzsér, J., Benkő, S.: Ragweed pollen extract intensifies LPS-induced priming of NLRP3 inflammasome in human macrophages. *Immunology. 138* (4), 392-401, 2013.
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Tárgyszavak

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9. ABBREVIATION LIST

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	adenosine-5'-triphosphate
BMDC	bone-marrow-derived dendritic cells
DAMP	danger/damage-associated molecular patterns
cAMP	cyclic adenosine monophosphate
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IKK	inhibitor of NF-kB kinase
IkBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IRF	Interferon regulatory factor
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
M-CSF	macrophage colony stimulating factor
MF	macrophage
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary-response gene 88
NEMO	NF-kB essential modulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP3	NOD-like receptor family pyrin domain-containing 3
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PG	peptidoglycan
PRR	pattern recognition receptor
RIG-I	retinoic acid-induced gene I
RT-PCR	real-time polymerase chain reaction
ROS	reactive oxygen species
SAPK/JNK	stress-acivated kinase/c-Jun N-terminal kinase
TIR	toll-interleukin 1 receptor
TLR	toll-like receptor
TNFα	tumor necrosis factor-alpha

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