DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

RETINOIC ACID MODULATES NOD-LIKE RECEPTOR-MEDIATED RESPONSES IN HUMAN MACROPHAGES

by

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1. List of abbreviations

AIM2: Absent in Melanoma 2

ATRA: All-trans retinoic acid

Arg-1: Arginase-1

ASC or PYCARD: Apoptosis-associated speck-like protein containing a caspase-recruitment domain

PGN: Bacterial peptidoglycan

CLIC: Chloride intracellular channel protein

GM-CSF: Colony-stimulating factor

DAMPs: Danger- or damage-associated molecular patterns

DCs: Dendritic cells

DMSO: Dimethyl sulfoxide

ECAR: Extracellular acidification rate

ERSR: ER stress response

Erk: Extracellular signal–regulated protein kinase

FAO: Fatty acid oxidation

PFKP: phosphofructokinase P

GCC: Glucocorticoid

HAMPs: Homeostasis-altering molecular processes

HK: Hexokinase

iNOS: Inducible NO synthase

IFNs: Type I interferons

IFNβ: Interferon beta

IFN-γ: Interferon gamma

IRF3: Interferon regulatory factor 3

IRF7: Interferon regulatory factor 7

LPS: Lipopolysaccharide

mTOR: Mammalian target of rapamycin

MAMs: Mitochondria-associated membranes

MAPK: Mitogen-associated protein kinase

NTD: N-terminal domain

NAIPs: NLR family apoptosis inhibitory proteins

NO: Nitric oxide

NLR: NOD-like receptor

NF-KB: Nuclear factor-kB

OxPhos: Oxidative phosphorylation

OCR: Oxygen consumption rate

PAMPs: Pathogen-associated molecular patterns

PRRs: Pattern recognition receptors

PPP: Pentose phosphate pathway

PI3K: Phosphoinositide 3-kinase

PKB or Akt: protein kinase B

RA: Retinoic Acid

ROS: Reactive oxygen species

RIPK2: Receptor-interacting serine/threonine-protein kinase 2

RALDHs: Retinaldehyde dehydrogenases

Th1: T helper type 1

TRIF: TIR domain-containing AP inducing interferon-β

TNF-α: Tumor necrosis factor-α

TRAF6: TNF receptor-associated factor 6

TIR: Toll-IL-1 receptor

TNF-α: Tumor necrosis factor alpha

TFs: Transcription factors

2. Introduction

Macrophages (MΦs) are crucial innate immune effector cells involved in a wide range of biological processes, including host immune defense and tissue homeostasis. MΦs surveil the local environment by utilizing several groups of sensing receptors, such as cytokine receptors, nuclear hormone receptors and pattern recognition receptors (PRRs). Activation of these receptors in turn determine the MΦs fate and the outcome of immune responses. Among the PRRs, NOD-like receptors (NLRs) are a group of conserved cytosolic proteins, that recognize microbial and host danger signals and initiate innate immune responses. Besides, NLRs are implicated in regulation of gene expression, signaling pathways, reproduction; and some NLRs trigger inflammasome formation. However, dysfunction of NLRs is associated with various inflammatory diseases and autoimmune diseases, in line, targeting these receptors or modulate its functions may provide promising option for novel therapeutics targets.

Since MΦs functions can be also regulated by nuclear receptors that sense endogenous or exogenous lipid compounds, all-trans retinoic acid (ATRA) arises as tissue-derived signal and an endogenous ligand for nuclear receptors that may modulate the MΦs activity. ATRA is the most metabolically active form of vitamin A, and has been approved by Food and Drug Administration (FDA) for treating acute promyelocytic leukemia. ATRA is an essential component in certain tissue microenvironments where it is necessary for the proper functions of immune cells. However, there is less known about the role of ATRA in regulating specific NLR-mediated response in MΦs.

We hypothesized that ATRA modulates selected, NLR-mediated functions in human MΦs. In our study, we targeted NLRs characterized with different functions: (1) inflammasome forming NLR (NLRP3); and (2) regulatory NLRs of inflammatory signaling (NOD1 and NOD2). These proteins belong to those few NLRs that have well-characterized agonists and antagonists for molecular manipulation. Our data reveals that ATRA is capable to modulate the NLR-mediated responses via several potential mechanisms. Our findings raise the importance of ATRA in regulating NLR-mediated pathways and the outcome of related inflammatory responses, and highlights that targeting nuclear receptors is a promising strategy for the treatment of various infectious and chronic inflammatory conditions that are associated with NLR dysfunction.

3. Theoretical background

3.1. Macrophages (MΦs)

As a member of innate immune effector cells, macrophages (MΦs) play a crucial role in maintaining the immune homeostasis under normal physiological conditions, as well as regulating inflammatory responses. MΦs form various subpopulations and their phenotypic and functional properties are determined by their developmental origin and local tissue microenvironmental factors. They are implicated in a wide array of functions, including host defense, antigen presentation, phagocytosis, efferocytosis, metabolic regulation, tissue repair and remodeling, and secretion of several cytokines and growth factors (1,2). During embryonic development, MΦs derive from precursor cells of the yolk sac or fetal liver to form tissue-resident MΦs, such as brain microglia, liver Kupffer cells and alveolar MΦs. These MΦs are usually long-lasting, self-renewing cells that constantly monitor their local environment. However, monocyte-derived MΦs originate from hematopoietic stem cells, and infiltrate the target tissues from blood circulation in partially polarized states, and are further shaped by the surrounding tissue microenvironment under either homeostasis or disease (2,3).

Broadly, MΦs have been phenotypically classified into two main groups: classically activated MΦs (M1) with proinflammatory functions, including host defense, tumor suppression, secretion of high level of proinflammatory cytokines (TNF-α, IL-1β, IL-12), and antigen-presenting capacity through highly expressed MHC class II. The alternatively activated MΦs (M2) exhibit anti-inflammatory activity and are involved in tissue repair, resolution of inflammation, parasitic infections, and asthma. In addition, these MΦs can produce regulatory cytokines (such as IL-10 and TGF-β) and are characterized by poor antigen presentation (4). However, the M1/M2 terminology represents the most phenotypically distant poles of the polarization states of MΦs, and it does not sufficiently describe the heterogeneity of MΦ populations. Besides, importantly, MΦs are capable to change their functional phenotypes in response to different microenvironmental cues (5).

As the source and context of the activation signals determine the fate of MΦs, a further sub-classification has been extended to encompass various distinct MΦ phenotypes. M1 MΦs have been sub-grouped into classical (M1a)- and innate (M1b)-activated MΦs. M1a develops in response to T helper type 1 (Th1) cytokines, IFN-γ, TNF-α or GM-CSF; while M1b develops

following activation by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). Experimentally, concomitant treatment with IFNγ combined with LPS, TNF-α; or GM-CSF alone is generally used to obtain classically activated MΦs (2,6,7).

However, alternatively activated MΦs are categorized into four subgroups, each with characteristic functions: while M2a exhibits wound healing properties, M2b, M2c and M2d are involved in various regulatory roles. M2a is stimulated by IL-4 and/or IL-13, which are mainly secreted by Th2 cells, mast cells, and basophils. M2b is obtained by complex activation of TLR and/or IL-1 receptor or through activation of Fcγ receptors by immune complexes, and exhibits proinflammatory function accompanied with high IL-10 secretion. The M2c subgroup is induced by IL-10, TGF-β or glucocorticoids, and these MΦs have a high capability of phagocytosis of apoptotic cells. The M2d subgroup is induced by co-activation of TLRs and adenosine A2A receptor and exhibits pro-angiogenic capacity (6,8,9). Notably, M-CSF-differentiated monocytes are often considered as a model for tissue MΦs, and belong to M2-like alternatively activated MΦs (Figure 1) (4).

In addition, other non-classical MΦ subtypes have been characterized under pathogenic conditions such as MΦ-ox (oxidized phospholipids), MΦ-Hb (hemoglobin–haptoglobin complexes) and MΦ-hem (heam) that are associated with atherosclerosis (10); tumor-associated MΦs (TAMs), which have high influence on tumor pathogenicity; and adipose tissue-associated MΦs (ATMs), which are characterized by distinct transcriptomes, chromatin landscapes, metabolic markers and functions mainly in obesity (2).

In response to the molecular microenvironment, MΦs adopt distinct transcription program that is determined by a complex hierarchical and collaborative interaction of lineage-determining transcription factors (TFs), signal-dependent TFs and the accessibility of the regulatory elements (enhancers and promoters) (11,12). The lineage-determining TFs (such as Ps U.1, MYB, c-MAF and MAFB) mediate DNA accessibility through chromatin rearrangement to promote the binding of signal-dependent TFs (such as NF-κB and STATs), which in turn activate several enhancers by histone modification (e.g., H3K27me3 to H3K27ac transition) (12). For instance, activation of NF-κB signaling - as a dominant inflammatory pathway that regulates gene expression - mediates the recruitment of H3K4me3 to several proinflammatory promoters (e.g., NLRP3) to activate transcription (11). However, IL-4 signaling modulates chromatin accessibility and mediates

transcriptional repression of several proinflammatory genes (such as NLRP3 and IL-1β) through STAT6-repressed enhancers to promote an anti-inflammatory state (13).

The functional plasticity and heterogeneity of MΦs are also associated with cellular metabolism, and MΦs acquire distinct metabolic signatures for proper effector functions. These cells are highly sensitive and affected by the microenvironmental changes and the availability of resources, which accordingly drive their constant metabolic adaptation or metabolic rewiring to maintain the bioenergetic demands under a quiescent state or polarization/activation conditions. This metabolic activity, in turn, determines the phenotype and the functional properties of the MΦs. Several key metabolic pathways have been identified to regulate MΦ phenotypes and functions, including glycolysis, pentose phosphate pathway, oxidative phosphorylation (OxPhos), fatty acid metabolism (including oxidation and synthesis) and amino acid metabolism (mainly arginine, glutamine and tryptophan) (14,15). Notably, the metabolic index or the balance between of glycolysis and OxPhos highly represents the activation status of MΦ subpopulations (16).

Based on metabolism, the classically activated (M1) MΦs adopt glycolysis as a source of energy as well as exhibit increased glucose consumption and lactate release. The upregulation of glycolytic metabolism in M1 provides rapid ATP production (around 100 times faster than OxPhos) to support the high secretory and phagocytic functions, to supply the pentose phosphate pathway (PPP), and provides NADPH for reactive oxygen species (ROS) production through NADPH oxidase. Further, glycolysis and PPP support the generation of the building blocks for protein and nucleotide synthesis (15,17).

In addition, the mitochondria in M1 MΦs possess several breakpoints in TCA cycle; like modulation of isocitrate dehydrogenase (IDH) or succinate dehydrogenase (SDH) may lead to the accumulation of signal metabolites such as citrate, succinate and itaconate. While itaconate is considered as a microbicidal metabolite and has anti-inflammatory effects, succinate leads to stabilization of hypoxia-inducible factor 1a (HIF-1a), which is responsible for the expression of several inflammatory genes (e.g., *IL1B*). Accumulated citrate is exported to the cytosol, where it is utilized to produce itaconate for fatty acid biosynthesis (FAS) and NO production (14,18). The alternatively activated MΦs (M2) have intact mitochondrial TCA cycle, exhibit enhanced OxPhos, and mainly rely on fatty acid oxidation. However, glucose through glycolysis is required to fuel TCA cycle by acetyl-CoA in these MΦs (19). Of note, at basal level, the human M-MΦs exhibit higher respiration to glycolysis ratio compared to GM-MΦs. In addition, GM-MΦs display

higher glycolytic metabolism, which is associated with high accumulation of glycolytic catabolites (such as pyruvate and lactate), TCA metabolites (succinate, fumarate, malate), and high expression of several glycolytic genes (such as *hexokinase3* (*HK3)*, *fructose-1,6-bisphosphatase* 1 (*FBP1)* and *phosphofructokinase P* (*PFKP*)) compared to M-MΦ (20).

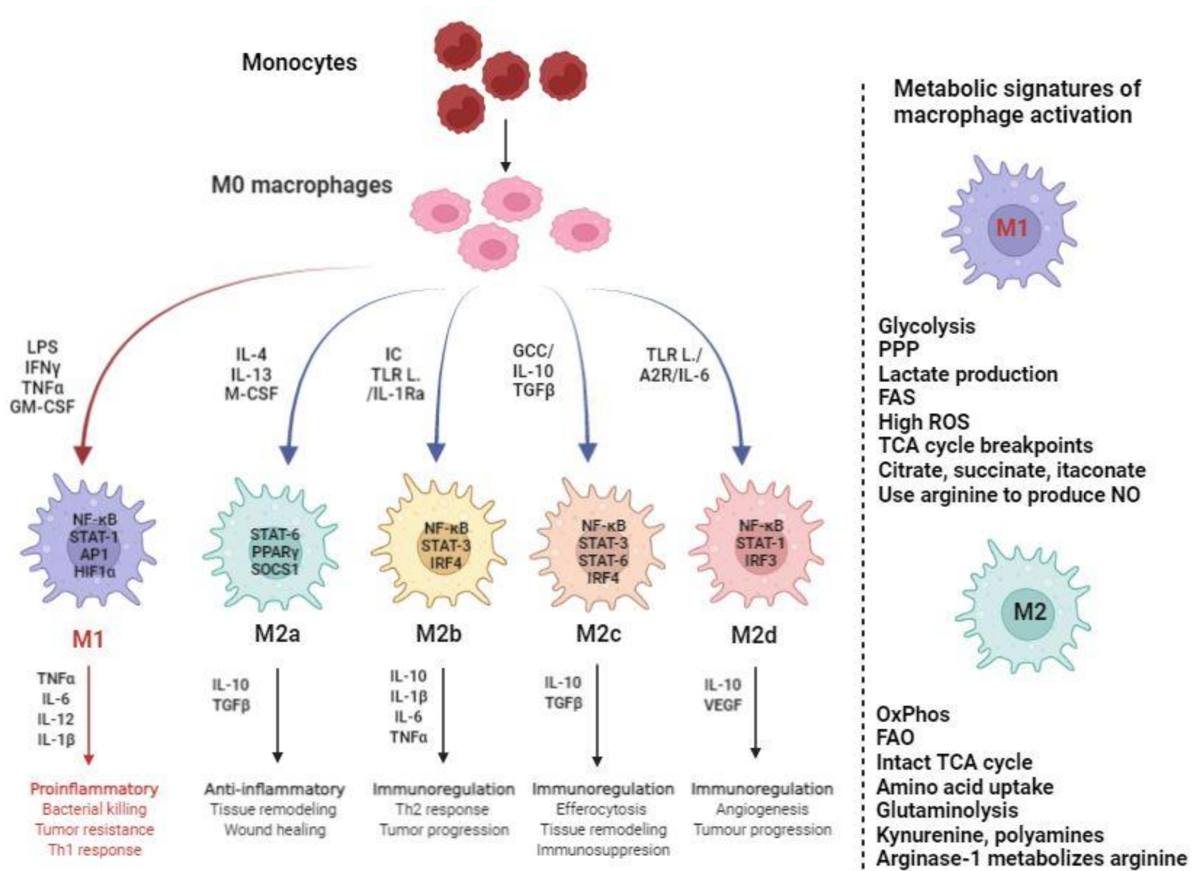


Figure 1. Schematic overview of the heterogeneity of MΦ subtypes. *Based on microenvironmental signals, MΦs adopt a wide spectrum of polarization states ranging from classically polarized (M1) to alternatively polarized (M2) cells, that determine their functional and metabolic characteristics. BioRender template with modifications. BioRender.com.*

Amino acids are essential for proper MΦ functions and have ability to regulate several signaling pathways (e.g. mTOR and NO production), particularly during immune responses. For example, arginine is converted to nitric oxide (NO) by inducible NO synthase (iNOS) in M1 MΦs, and inhibiting iNOS halts the phenotypic polarization of M1. Conversely, in alternatively activated MΦs, arginine is metabolized via arginase-1 (Arg-1) to urea and L-ornithine, which are needed for tissue repair and remodeling. Glutamine amino acid is required for the polarization of M2 MΦs

and is considered as one of the main sources of carbon that feeds TCA cycle. Furthermore, the absence of glutamine leads to decreased expression of M2-associated genes (such as *CD206, CCL22, IRF4, KLF4*). In addition, tryptophan is catabolized to kynurenine by indoleamine 2,3-dioxygenase (IDO), whose activity is a hallmark for M2 phenotype; and inhibition of IDO leads to a proinflammatory MΦs profile (14,15,21). These previous reports highlight the importance of the transcriptional program and the metabolic status as determinants of MΦs functions, particularly when the activation of pattern recognition receptors (PRRs) is needed.

3.2. Pattern recognition receptors (PRRs)

During inflammatory response, innate immune system drives a protective defense against infectious stimuli and tissue damage, in order to maintain the host homeostasis, eliminate the damaged cells and initiate tissue repair (22,23). Innate immune functions depend on germ line-encoded PRRs that recognize exogenous or endogenous harmful factors and subsequently initiate downstream inflammatory cascades. PRRs sense highly conserved structures or molecular signatures of pathogens termed pathogen-associated molecular patterns (PAMPs), which are identified microbial components or its metabolic by-products (such as LPS, flagellin, bacterial toxins and viral ssRNA). Endogenous self-molecules that are released/produced or accumulated during tissue damage called danger- or damage-associated molecular patterns (DAMPs) can also trigger immune responses through PRRs. Several endogenous damage signals have been identified, such as high-mobility group box 1 (HMGB1), heat-shock proteins (HSPs), hyaluronan fragments from extracellular matrix, ATP, uric acid or DNA. Recently, the term metabolism- or homeostasis-associated molecular patterns (HAMPs) has emerged to describe the imbalance in cellular homeostasis as a trigger for the immune response (22,24–26). According to their localization, PRRs may be grouped into membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLR); or intracellular cytosolic receptors including nucleotide-binding domain leucine-rich repeat receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), absent in melanoma (AIM)-like receptors (ALRs) and proteins-containing tripartite motif (TRIM) (20–22).

3.3. Toll-like receptors (TLRs)

TLRs (TLR1-10 in human) are a family of transmembrane receptors, located in cell membrane and other membranes of intracellular compartments, such as in endosomal membranes.

These receptors play vital roles in recognizing pathogens or molecular signatures, including PAMPs and DAMPs, and subsequently drive the expression of antimicrobial genes, and direct the adaptive immunity. They have variable numbers of leucine-rich repeats (LRR) containing ectodomains that recognize various molecular patterns; and intracellular Toll-IL-1 receptor (TIR) domains that mediate signaling pathways to the cytosol. The ectodomain can form a homo- or heterodimer with a co-receptor or other molecule upon ligation with a ligand (27). LRR motifs provide multifaceted structure with the ability to interact with proteins, lipids, carbohydrates and nucleic acids (Figure 2) (28).

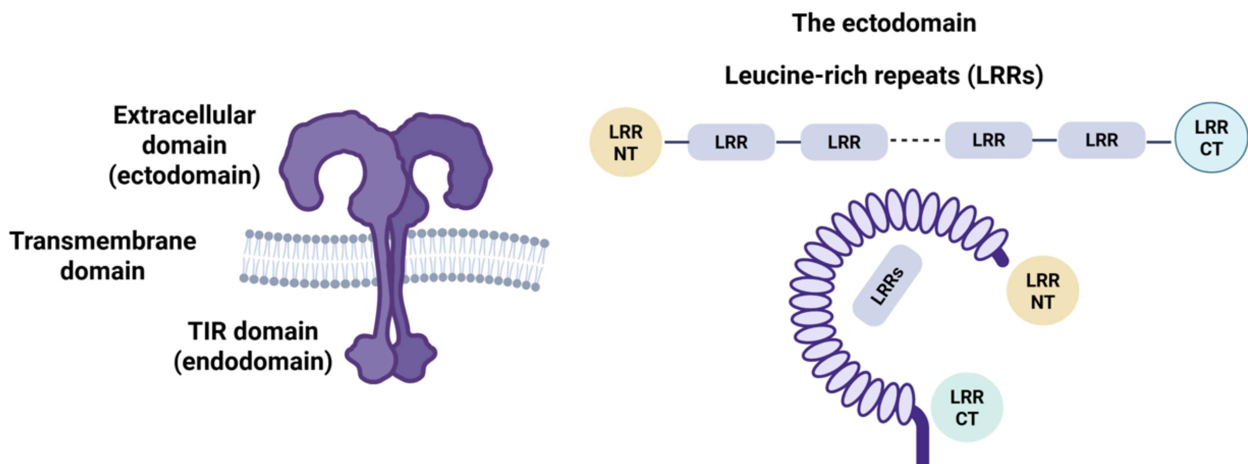


Figure 2. Schematic overview of the conserved TLR structure. *The ectodomain of LRRs is involved in ligand recognition, while the TIR domain mediates downstream signaling. LRRs: Leucine rich repeats; NT: N-terminal domain; C-terminal domain. Created in BioRender.com.*

Plasma membrane TLRs are specialized to recognize distinct microorganisms-derived components that present in extracellular space, for instance TLR4 detects bacterial lipopolysaccharide (LPS), while bacterial flagellin is recognized by TLR5. Other bacterial components such as lipoproteins, peptidoglycans and lipoteichoic acid are recognized by TLR1/TLR2 and TLR2/TLR6 heterodimers. Endosomal TLRs mainly recognize engulfed endogenous (self) and exogenous nucleic acids; for example, TLR3 recognizes double-stranded RNA derived from viruses or damaged cells, whereas single-stranded RNA is detected by TLR7 and TLR8 (27). TLR4 is the most extensively investigated receptor among the TLRs, that is expressed in a wide range of immune- and non-immune cells, including monocytes, MΦs, and epithelial- and endothelial cells. Besides being a potent sensor for bacterial LPS, TLR4 can also

recognize DAMPs derived from extracellular matrix molecules or intracellular factors, such as HMGB1 and cellular HSPs. TLR4-derived inflammatory response provides host defense mechanism against infections and tissue damage; however, dysregulated TLR4 functions can disrupt the immune homeostasis and lead to various inflammatory conditions such as sepsis and cancers (29,30).

Upon ligation of TLR4 ectodomain, the TIR domain recruits several downstream adapter molecules that trigger distinct signaling cascades to mediate gene transcription or activate other cellular functions involved in immune defense. Those adapters are key guiders for downstream signal transductions and determine the consequences of TLR4 activation (31). Several adapter proteins are identified to be involved in TLR4 signaling, like MyD88 (myeloid differentiation primary-response protein 88), TIRAP (TIR domain-containing AP), TRIF (TIR domain-containing AP inducing interferon-β), TRAM (translocating chain-associated membrane protein) and SARM (sterile-α and Armadillo motif-containing protein). Despite many differences, TLRs depend on MyD88-mediating signaling, except TLR3 that exclusively uses TRIF, and endosomal TLR4 which initiates TRIF-dependent/MyD88-independent signaling. Among these adapters, SARM is considered a negative regulator for TRIF, thus regulating TLR3 and TLR4 (Figure 3) (32).

In MyD88-dependent pathway, engagement of TLR4 recruits IRAK kinase family members to form what called Myddosome, that lead to recruitment and activation of TNF receptor-associated factor 6 (TRAF6). TRAF6 generates polyubiquitin chains leading to the recruitment and activation of TAK1 complex (33). This leads to activate the downstream IKK (IKKα, β and γ) complex- nuclear factor-kB (NF-κB) pathway through a phosphorylation process. The complex mediates the phosphorylation the NF-κB inhibitory protein IkBα, which induces its degradation by the proteasome, allowing NF-κB subunits (p65-p50) to translocate into the nucleus and activate the expression of the proinflammatory genes. Concurrently, the activated TAK1 triggers the mitogen-associated protein kinase MAPK family members Jun N-terminal kinases (JNKs), extracellular signal-regulated protein kinase (Erk) and p38, leading to the activation of activator protein 1 (AP-1) transcription factor complex (heterodimer of c-Fos and c-Jun) and cyclic AMP-responsive element-binding protein (CREB) to mediate inflammatory responses (27,32,34). In TRIF-dependent pathway, TRIF promotes TBK1 activation resulting in IRF3 dimerization by a phosphorylation process, and translocation into the nucleus, to induce the expression of Type I

interferons (IFNs) and IFN-stimulated genes (ISGs). In addition, TRIF interacts with TRAF6 via activation of RIP1 kinase, which in turn activates TAK1complex mediating NF-κB and MAPKs leading to induction of the inflammatory cytokines. Of note, LPS-activated TLR4 triggers both MYD88 and TRIF transduction pathways, in which these pathways are under tight regulation of balanced production of inflammatory cytokines, and type I IFNs act as a limiting factors to avoid excessive inflammatory responses (Figure 3) (27,32,33,35). In addition, phosphatidylinositol 3-kinase (PI3K) is activated through its upstream, TIR domain containing regulator B-cell adapter for PI3K (BCAP) leading to protein kinase B (PKB)/Akt activation; TBK1 also triggers the kinase Akt activation. Akt signaling is involved in the regulation of cellular metabolism and glucose utilization, limiting proinflammatory cytokine secretion and mediating anti-inflammatory cytokine production (IL-10 and IFNβ) (31,36).

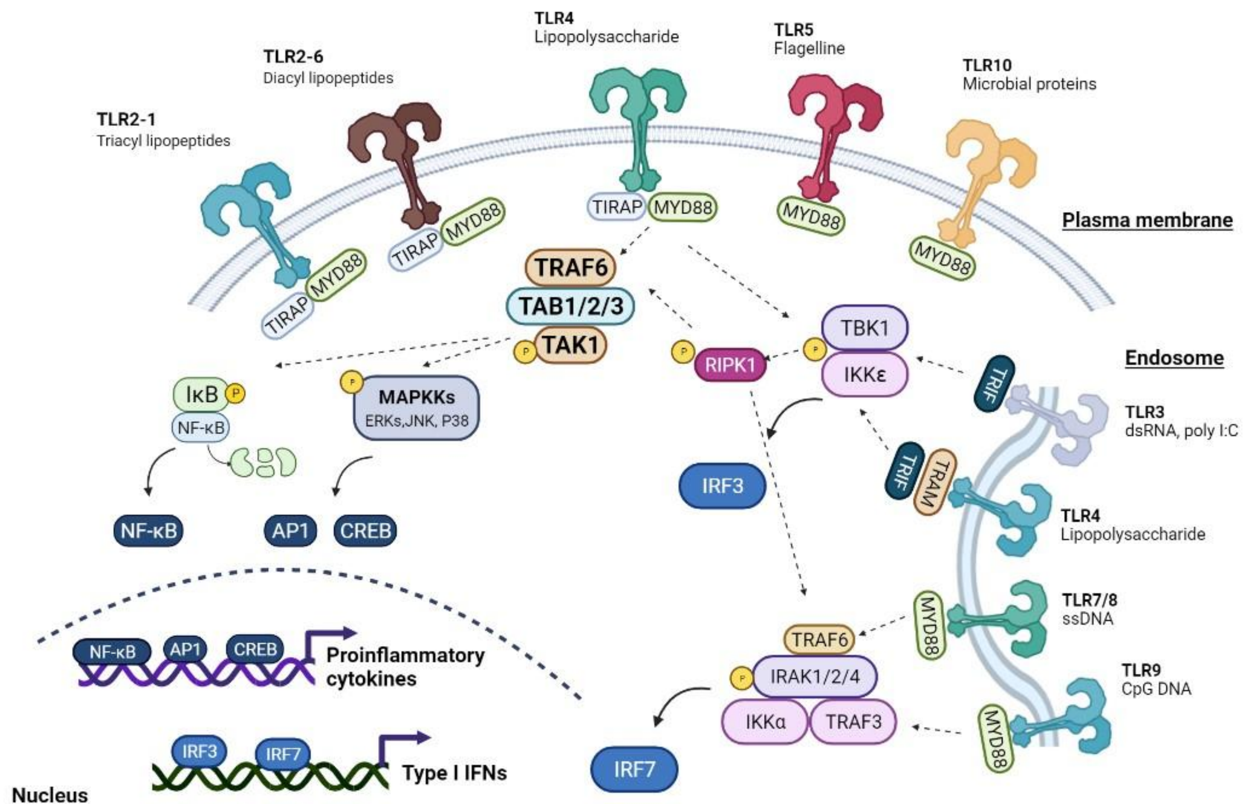


Figure 3. Schematic overview of basic TLR signaling pathways. *The functions of TLRs are mediated by several downstream adapter molecules, that drive gene expression and activate other cellular mechanisms involved in immune defense. Created in BioRender.com.*

3.4. NOD-Like Receptors

Besides TLRs, cells also express NLRs as another molecular defense line in the cytosol. NLRs are a family of evolutionary conserved cytosolic proteins, and are expressed in a variety of tissue types, including immune cells. They are specialized sensors that are activated in response to a wide range of intracellular microbial and danger-derived components, and initiate innate immune responses. Furthermore, their functions extend to regulate several other biological processes, such as transcription, differentiation, cell survival and metabolism. Moreover, certain NLRs may also be involved in reproduction and embryonic development (37).

In human, the NLR family consists of twenty-two members that share well-organized multidomain structures. With one exception (NLRP10), the C-terminal of each NLR contains a leucine-rich repeats domain (LRRs) with a variable number of repeats, which is responsible for the recognition or the binding of agonists. All NLRs share a common central nucleotide-binding/oligomerization domain (NACHT), which is required for self-association and oligomerization (38,39). The N-terminal domain exhibits variations among the NLRs, like it can be caspase recruitment domain (CARD), pyrin domain (PYD) or the baculoviral inhibitor of apoptosis protein repeat (BIR) domain. These domains are the effector domains that mediate hemophilic protein interactions and modulate downstream signaling pathways (39). Based on the N-terminal domains, NLRs are subcategorized into five groups (NLRA, NLRB, NLRC, NLRP, and NLRX) (Figure 4). Of note, some NLRs has unique N-terminal domains, like NLRC3 and NLRC5 carry CARD-like domain, while NLRX1 has an unconventional N-terminal domain without known function, that's why it is called 'X'. The only defined function of the N-terminus of NLRX1 is characterized as a mitochondrial localization signal (MLS). In steady state, NLRs are under autoinhibition, which is mediated by an interaction between LRR and NACHT domains, which represses NACHT-mediated oligomerization or NLRs auto-activation (40). Ligand sensing by NLRs induces a conformation change and exposes specific surface for interaction with their adaptor molecules or the effector enzymes. Based on their functions, NLRs can be categorized into subgroups. Some members of NLR family can positively regulate signaling cascades, such as NOD1 and NOD2. Following activation, NOD1 and NOD2 have been described to recruit receptor-interacting protein kinase 2 (RIPK2) through CARD domains to mediate downstream signaling cascades, including NF-κB signaling, MAPK signaling and the IRF3-/IRF7-dependent expression of IFNs (41). Other NLRs, like NLRC3, NLRP4, NLRP10 and NLRP12, act as negative

regulators for the inflammatory responses and inflammasome assembly via competitively binding to adaptor molecules and inhibiting complex assembly or downstream signaling pathways (39). NLRX1 is recognized as another negative regulator of immune response, and is uniquely implicated in the regulation of mitochondrial-associated immune responses (42,43). Another subgroup of NLRs, including NLRC5 (CITA) and CIITA, has been reported to shuttle from cytosol to the nucleus, to regulate the transcription of MHCI and MHCII, respectively (44,45). Furthermore, there is a subgroup of NLRs that plays essential role in the early mammalian embryogenesis and reproduction, such as NLRP2, NLRP7 and NLRP14 (46). And importantly, number of NLRs have been described as core proteins that trigger the inflammasome formation and related subsequent events, such as NLRP1, NLRP2, NLRP3, NLRP6 NLRC4 (47).

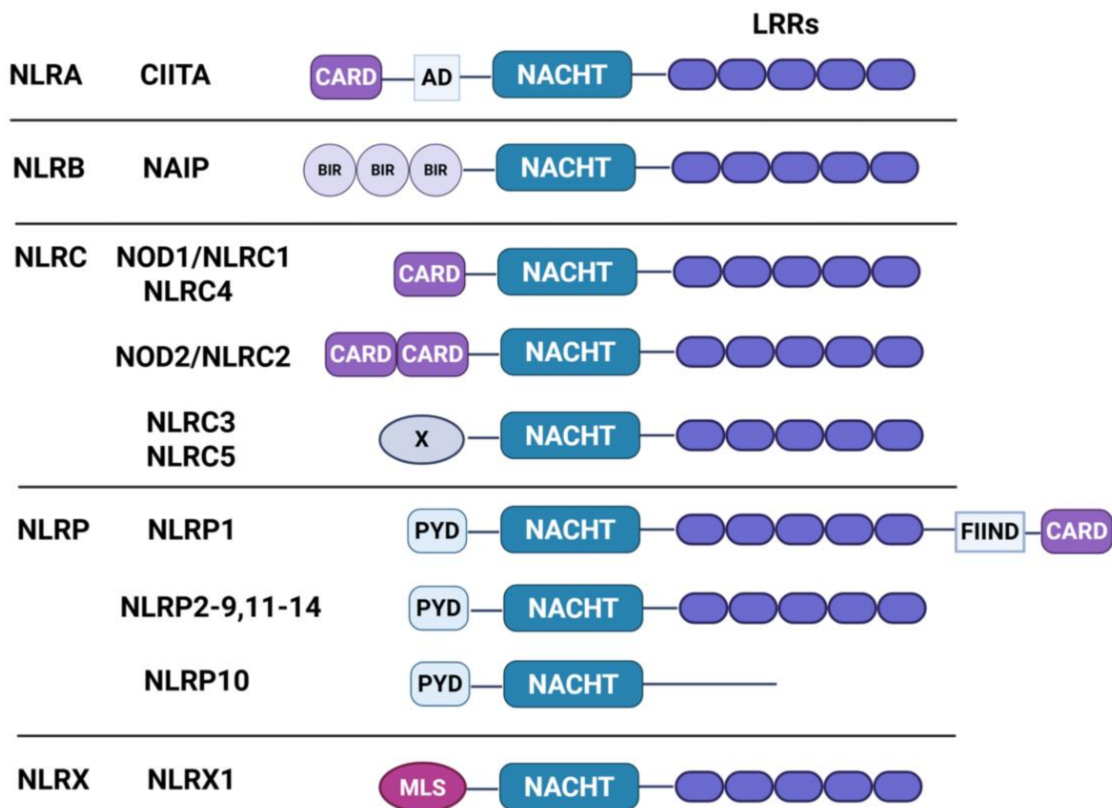


Figure 4. Schematic overview of human NLRs structures. *NLRs share common LRRs domain and central NACHT domain, and are categorized into five subgroups based on their N-terminal effector domains. The schematic does not reflect the actual number of the Leucine-rich repeats (LRRs). CARD: Caspase recruitment domains; AD: Acidic transactivation domain; BIR: Baculoviral inhibition of apoptosis protein repeat domain; X: Unknown domain; FIIND: Function to find domain, PYD: Pyrin domain; MLS: Mitochondrial localization signal domain. Created in BioRender.com.*

However, it is important to mention that due to the intensive studies of the NLR field, emerging researches show that some NLRs are characterized by multiple functions. For example, beside the regulation of MHC I expression, NLRC5 also affects signal transduction pathways (48); or NLRP2, which was originally described as an important regulator of reproduction, can also affect the cell migration of lung epithelial cells (49).

3.5. Inflammasomes

In response to the perturbation of cellular homeostasis (by PAMP or DAMP), group of cytosolic innate immune sensors initiate the formation of multiprotein scaffolds called inflammasomes that lead to cleavage and activation of their downstream substrates through inflammatory caspase enzymes (50). Inflammasome formation requires activation of a sensor protein that recruits an adaptor apoptosis-associated speck-like protein (ASC) containing PYD- and CARD domain (Figure 5). This adaptor ASC functions as a bridge between the inflammasome components, which recruits the pro-form of the cysteine protease caspase-1. The canonical inflammasome assembly triggers autocatalytic cleavage of caspase-1. The activated caspase-1 subsequently cleaves gasdermin D which drives the formation of membrane pores through its N-terminal fragment, leading to inflammatory-lytic cell death called pyroptosis (50). In addition, caspase-1 also mediates the cleavage of pro-IL-1β and pro-IL-18 to their biologically active forms, since their precursors lack the typical signal peptides and they do not go through the ER/Golgi secretory pathways. The matured IL-1β and IL-18 proinflammatory cytokines are subsequently released from the cells through the gasdermin D pores (51). Non-canonical activation of the inflammasome in response to cytosolic LPS, bacterial mRNA or endogenous oxidized lipids has also been described. In this pathway, activation of caspases-4 and caspase-5 in human (caspase-11 in mice) serves as upstream trigger for inflammasome assembly and mediates gasdermin D cleavage in a caspase-1-dependent manner (52).

Mechanistically, NLRs undergo a conformational change upon ligation, and several NLRs assemble through their NACHT domains. This leads to the recruitment of the adaptor ASC and initiates either PYD-PYD or CARD-CARD interactions. This initial construct facilitates the recruitment of pro-caspase-1 through CARD-CARD interaction between the ASC and pro-caspase-1. The autoproteolytic cleavage of caspase-1 results in two active subunits p20 and p10.

Notably, the active caspase-1 enzyme is a heterodimer of one p20 subunit and two molecules of p10 (Figure 5) (53).

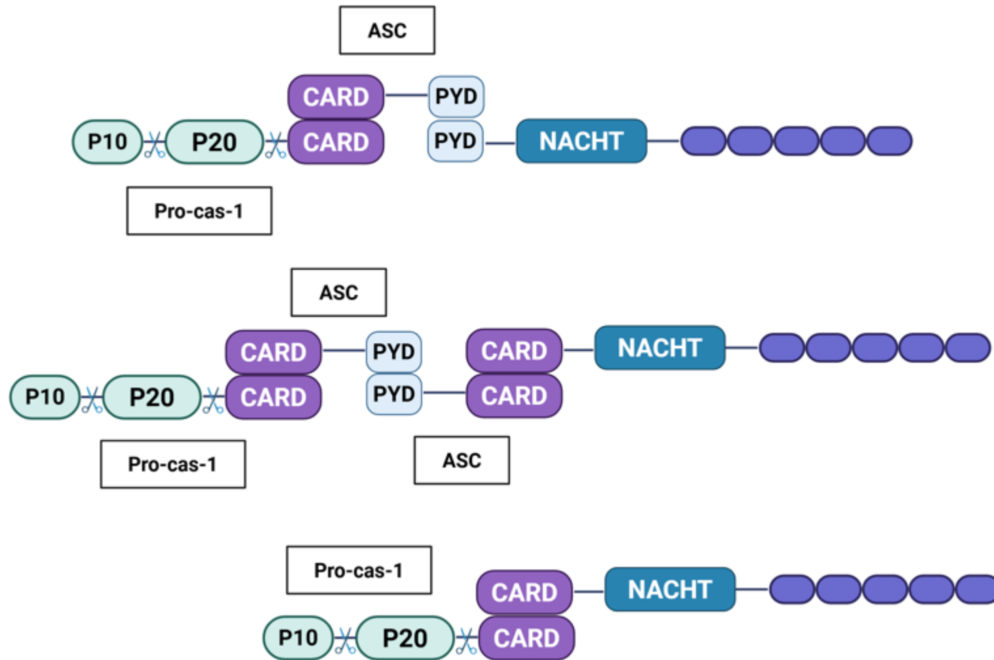


Figure 5. Schematic overview of possible mechanisms of the inflammasomes assembly. *NLR can recruit ASC through either PYD or CARD binding. NLR-containing CARD can directly recruit pro-caspase-1. Created in BioRender.com.*

Furthermore, ASC is a prerequisite for inflammasome formation for PYD-containing NLRs, however NLRs with CARD domains (such as NLRP1 and NLRC4) can initiate direct interaction with caspase-1 in an ASC-independent manner. Nevertheless, ASC-containing aggregates provide important scaffold for efficient inflammasome function (54).

In human, several NLRs have been described to form inflammasomes. Like NLRP1, NLRP3, and NAIP/NLRC4 are already well-characterized, while others (like NLRP6, NLRP7 and NLRP9-12) have only been described recently and require further detailed studies. In addition, there are non-NLR proteins (such as AIM2, CARD8 and pyrin) that are also able to form inflammasomes (55). Each inflammasome formation/activation is regulated by distinct mechanisms that include different accessory proteins. For example, NLRP1 sensor is under auto-repression by a group of endogenous dipeptidyl peptidases (DPPs) that associate with FIIND domain of NLRP1 to restrain its activity. NLRP1 inflammasome may be activated by *Bacillus*

anthracis toxin (anthrax toxin), which is composed of a lethal factor and a protective antigen. The protective antigen forms membrane pores through which the lethal factor can enter the host cell to initiate inflammasome activation. The lethal factor may also interact with dipeptidyl peptidases (DPPs) to disrupt NLRP1 auto-repression. Targeting DPPs/NLRP1 interaction with a synthetic molecule, such as Val-boroPro (Talabostat), is considered a mechanism for NLRP1 inflammasome activation. In addition, a new mechanism has been suggested that NLRP1 activation is mediated by a "functional degradation" process of its N-terminal residue in a proteasome-dependent manner. This mechanism is initiated by an autoproteolytic cleavage of NLRP1-FIIND domain, leading to release its CARD-containing fragment to nucleate an inflammasome (56).

Traditionally, NLRC4 inflammasome activation requires NAIP proteins (NLRPB group) that act as upstream sensors for this inflammasome. NAIP may recognize flagellin (the typical protein of flagella in both Gram-negative- and Gram-positive bacteria) or type III secretion system (T3SS) (the needle and inner rod proteins components of Gram-negative bacteria) in the cytosol of the host cell. NAIP then binds to NLRC4 and induces its oligomerization. While murine has multiple NAIPs and each is dedicated to recognize certain bacterial components, human has only a single NAIP that is responsible for sensing all of the bacterial components (57).

In human, AIM2 belongs to the AIM2-like receptor family together with IFI16, MNDA and PYHIN1, and they sense cytosolic DNA. AIM2 protein consists of an N-terminal PYD domain that is required for ASC binding, and a highly conserved oligonucleotide-binding HIN200 domain at the C-terminal end that mediates dsDNA binding. AIM2 may form an inflammasome which is activated by various cytosolic double-strand DNA originating from exogenous (such as virus or bacteria) or endogenous sources (nuclear- or mitochondrial DNA) (58). The pyrin inflammasome is triggered by inactivation of Rho guanosine triphosphatase (Rho GTPase) via various bacterial effector proteins or toxins inside the cytoplasm. Pyrin is a uniquely evolved protein of the TRIM family that has N-terminal PYD domain, and its expression is restricted to innate immune cells (59). Pyrin protein is under an autoinhibition state through phosphorylation process that maintained by RhoA (GTPase)-dependent protein kinases PKN. The modulation of Rho GTPase activity leads to decrease pyrin phosphorylation, and in turn relieving its inactivation and subsequently initiation of pyrin inflammasome (60). CARD8 inflammasome shares common activation mechanisms and auto-processing of NLRP1 inflammasome, since both NLRP1 and CARD8 share similar domain organization. CARD8 protein is structurally organized in two

domains: N-terminal-FIIND and C-terminal-CARD domains, similar to those found in C-terminal region of NLRP1. NLRP1 is ubiquitously expressed in human tissues/cells and encoded in a single NLRP1 gene, while mice possess multiple NLRP1 paralogs. However, CARD8 is predominantly expressed in T-cells, and is missing in the murine system. In addition, viral protease is considered inducer for both NLRP1 and CARD8 inflammasomes through inducing direct cleavage of these proteins (61). Besides its role as inflammasome sensor, CARD8 has been reported to play anti-inflammatory roles, for example, it negatively regulates other inflammasomes through direct interaction with sensor proteins or caspase-1, or other signaling pathways such as NOD1, NF-κB and apoptosis (62). Importantly, NLRP3 inflammasome is the most deeply investigated inflammasome sensor, due to its wide range of activators, and its association with several inflammatory diseases (63).

3.6. NLRP3 inflammasome

NLRP3 inflammasome is a key signaling platform for innate immune responses against diverse threats to the host, that links innate to adaptive immunity. Since NLRP3 inflammasome activation initiates a strong inflammatory response and has potential detrimental consequences, it is tightly regulated by several mechanisms. In steady state, NLRP3 protein is under autoinhibition, through folding of its LRR domain onto the NACHT domain. This involves direct interaction of LRR domain with NACHT domain, that prevents the activation for the NLRP3 sensor. In addition, the NACHT domain has ATPase activity in its Walker A and Walker B motifs, that is required for ATP binding and hydrolysis, respectively. The ATPase activity of NLRP3 NACHT domain is essential for the NLRP3 conformational changes and oligomerization (64,65).

Importantly, the activation of the inflammasome complex is a process of two events: the priming signal (signal 1) includes the transcription and post-translational modification (licensing) of the inflammasome components; while the activation signal (signal 2) is required for the assembly of the complex, and evoked by a multitude of distinct stimuli or/and cellular perturbation. Furthermore, NLRP3 inflammasome is also regulated by a number of interacting proteins, such as NEK7, TXNIP, MAVS, HSP90, SGT1and DDX3X etc. (66). The NLRP3 protein itself undergoes various post-translational modifications (PTMs), including phosphorylation ubiquitination, and SUMOylation, that direct inflammasome activation either positively or negatively (67). Several epigenetic modifications are involved directly and indirectly in inflammasome regulation through

targeting the inflammasome components or the inflammasome-related signaling (such as DNA methylation, histone modification and non-coding RNA (miRNAs and lncRNAs) (68,69).

The priming is an initial stage for the subsequent assembly event of NLRP3 inflammasome, and its usually achieved by stimuli-induced NF-κB activation leading to transcriptional upregulation of NLRP3, pro-IL-1β and pro-IL-18. Several TLR ligands and cytokines are identified as priming factors, such as Pam3CSK4/TLR2, poly(I:C)/TLR3, LPS/TLR4 and HMGB1/TLR2,-4,-9; as well as cytokines such as TNF, IL-1 and IFN (70). Interestingly, in certain cells (like monocytes), NLRP3 inflammasome can be activated by extracellular LPS alone, where LPS provides both the priming and the activation signals (71). In addition, besides NF-κB, several other signaling pathways are involved in NLRP3 priming, such as Erk/c-Jun/AP-1, CD36/PKCδ pathway, as well as lipid or glycolytic metabolism-mediated SREBP2 activation (72). After gene transcription, the inflammasome components need to be licensed for oligomerization or subsequent processes through post-translational modifications, which are also regulated by several signaling pathways. Since NLRP3 and the other components (ASC, NEK7) as well as the target proteins (pro-IL-1β and caspase-1) contain multiple post-translational modification sites, the licensing event could also be attributed to the effect of the second signal (72,73).

The second signal triggers the assembly of the NLRP3 inflammasome complex, and is induced by diverse stimuli mediating cellular stress/injury such as PAMPs and DAMPs. Generally, the activation step is mainly associated with the disruption of intracellular ions homeostasis (such as K^+ and Cl^- efflux, cytosolic Ca^{2+} flux), mitochondrial dysfunction, excessive ROS production, metabolic remodeling, lysosomal damage, endoplasmic reticulum stress and disrupted Golgi network integrity (Figure 6) (74) .

Low intracellular potassium (K^+) is a common and required upstream trigger of NLRP3 inflammasome activation. Several NLRP3 activators have been described that induce intracellular K^+ efflux, including bacterial pore-forming toxins (nigericin, gramicidin) and extracellular ATP-mediated gating of the P2X7 and TWIK2 channels. Chloride (Cl^-) efflux through chloride intracellular channel proteins (CLIC1) and (CLIC4) is another inducer for NLRP3 activation. CLIC-mediated Cl^- efflux is downstream of K^+ efflux and needed for optimal NLRP3 assembly. K^+ efflux facilitates NLRP3-NEK7 complex formation, and Cl^- efflux mediates ASC oligomerization; however, Cl^- efflux alone is unable to trigger functional inflammasome. Ca^{2+} mobilization, either when it releases from the ER or enters from extracellular sources, can also

induce the activation of NLRP3 inflammasome. For example, uncontrolled Ca^{2+} influx into mitochondria can lead to mitochondrial damage that triggers inflammasome activation (64,74).

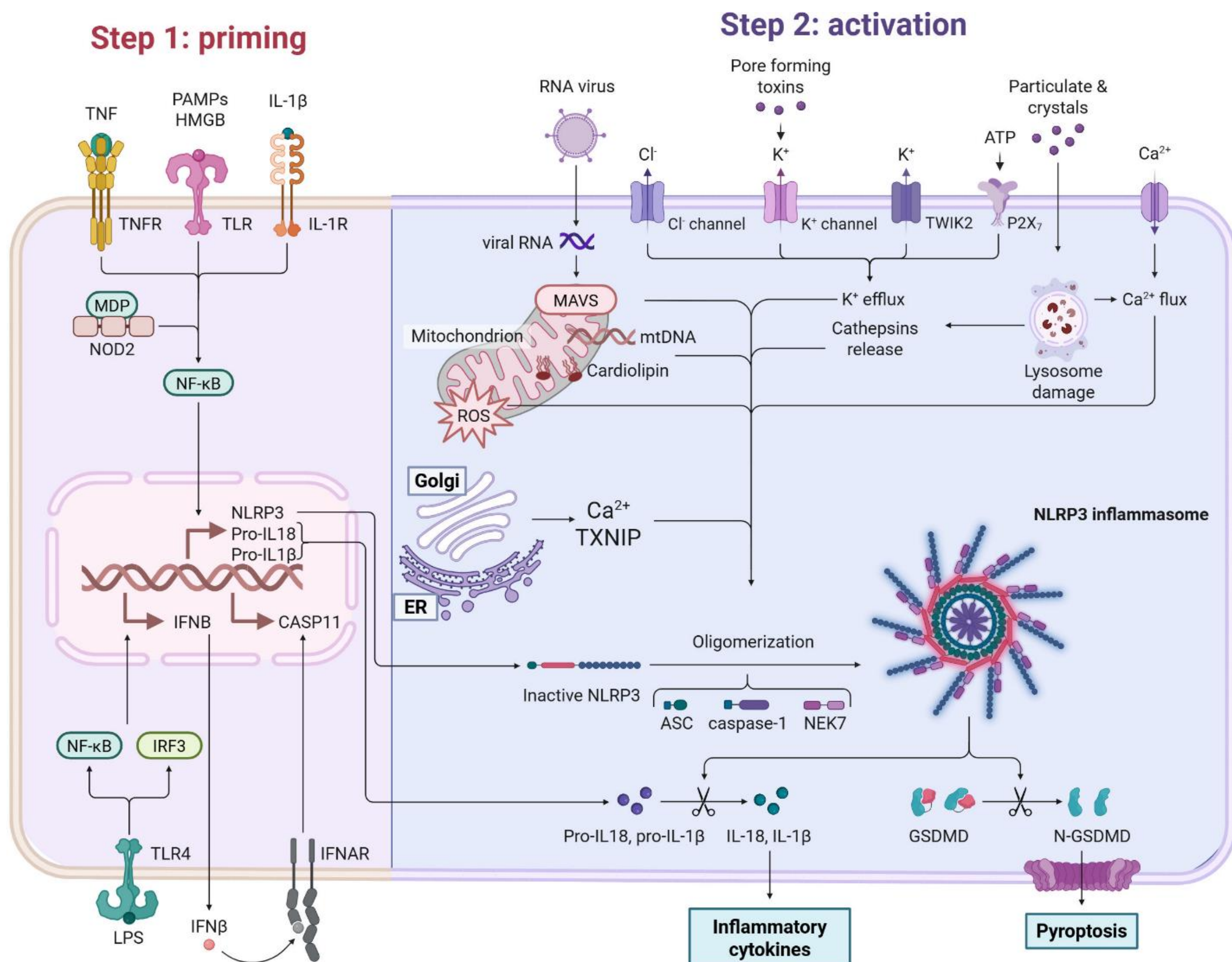


Figure 6. Schematic overview of priming and activation signals of NLRP3 inflammasome. *NLRP3 inflammasome is regulated by priming and activation events. The priming induces the expression of NLRP3, pro-IL-1β and pro-IL-18, and licensing the NLRP3 protein, while the actitation step mediates the NLRP3 complex assembly and the subsequent release of IL-1β and IL-18 cytokines. BioRender.com template with modification.*

Lysosomal disruption upon phagocytosis of crystals or aggregated particulates is another mechanism for NLRP3 inflammasome activation. Groups of endogenous substances (such as MSU crystals, cholesterol/lipid crystals and amyloid-β aggregates) or exogenous (including silica, asbestos, poly(I:C) and alum) have been shown to induce lysosomal rupture. This rupture leads to release the lysosomal contents into the cytosol, particularly cathepsin enzymes, thus inducing inflammasome activation. Inhibition of cathepsins by pharmacological inhibitors suppresses

NLRP3 activation under lysosomal disruptors, suggesting involvement of cathepsins in NLRP3 activation (75).

Endoplasmic reticulum (ER) plays essential roles in lipid and protein biosynthesis, and its function is affected by several internal and external stress conditions, such as oxidative stress, Ca^{2+} imbalance, hypoxia, inflammation and environmental toxins. Under ER homeostasis disturbances, cells activate a mechanism called ER stress response (ERSR), including unfolded protein response (UPR), and chronic or uncontrolled ERSR has been linked to NLRP3 inflammasome activation (76).

In response to ERSR or oxidative stress, TXNIP protein is activated and triggers the formation of the NLRP3 inflammasome complex through direct binding to NLRP3. Alternatively, stress conditions could also activate CHOP-dependent TXNIP pathway leading to TXNIP mediated-mitochondrial ROS production and subsequently inflammasome activation (77). ER associates with mitochondria in connecting sites called mitochondria-associated membranes (MAMs). Besides their role in regulating Ca^{2+} homeostasis, lipid metabolism and ROS production, these sites have been reported to act as a platform for inflammasome assembly under stress conditions (78). The ER-associated proteins (SREBP2 and SCAP) translocate to Golgi apparatus and recruit NLRP3 through direct interaction to drive NLRP3 localization and activity. Furthermore, disassembly or dispersed *trans*-Golgi network in response to NLRP3 activation has been reported to serve as a scaffold for NLRP3 assembly. In addition, the negatively charged PtdIns4P phospholipid of Golgi membrane provides the contact site for NLRP3 recruitment (74).

Mitochondrial dysfunction is a source of various NLRP3 inflammasome activators, including mtDNA, mtROS and OxPhos byproducts. Under certain circumstances, mitochondria-associated proteins (such as cardiolipin, MFN2 and MAVS) direct NLRP3 localization to the mitochondria that acts as scaffold for NLRP3 inflammasome assembly. In addition, cellular processes involved in mitochondrial homeostasis (autophagy, mitophagy and mitochondrial dynamics) have also been linked to inflammasome activation. To meet bioenergetic and biosynthetic demands, immune cells adopt distinct metabolic programs to sustain their function in response to microenvironment factors. Metabolic perturbations or alteration of metabolic pathways, including OxPhos, glycolysis, fatty acid or amino acid metabolism have been also implicated in the regulation of the NLRP3 inflammasome (79,80). One of the features of the immune cells (including MΦs) is to undergo a metabolic shift from OxPhos to glycolysis in

response to an inflammatory condition. Glycolytic flux, as well as several regulators of glycolysis and glycolysis-related by- and end-products have been found to modulate the activity of the NLRP3 inflammasome (81).

3.7. Interleukin-1β (IL-1β)

IL-1β, together with IL-18 and IL-1α, belongs to IL-1 family cytokines that share common structure and receptor binding mode. Unlike IL-18 and IL-1α, the expression of IL-1β is highly inducible. While several non-immune cells have been reported to produce IL-1β, such as keratinocytes, fibroblasts, synoviocytes, endothelial and neuronal cells (83), the main sources of IL-1β are monocytes and MΦs (82). IL-1β plays a central regulatory role in innate immunity, functions as a leukocytic pyrogen and shapes the adaptive immune responses. In response to tissue damage, IL-1β drives local inflammation at the inflammatory sites, and can mediate systemic inflammation as well. IL-1β is required for various homeostatic functions under normal condition, including the regulation of feeding, sleep, pain and temperature. However, dysregulated IL-1β is implicated in various acute and chronic inflammatory conditions, such as arthritis, neuropathic pain, cancer and vascular disease (83–85).

Nevertheless, the activity of IL-1β is tightly regulated at different levels by several mechanisms; the pro-IL-1β transcript is regulated at transcription and post transcriptional level, and IL-1β production in its mature form requires caspase-1-dependent processing in inflammasome-dependent manure. The IL-1β receptor (IL-1R1) is also regulated by number of antagonists and dominant negative receptor complexes; and the presence of soluble decoy receptors (lacking the functional domain, such as IL-1R2) that bind to IL-1β in the cytosol or in the extracellular matrix to limit its activity. Furthermore, the intracellular signaling pathways of activated receptor are also under control of several negative regulators (82,84,86).

IL-1R1 contains intracellular TIR domain, and upon activation recruits MyD88 that induces the downstream signaling NF-kB and MAPKs; and mediates the transcription of IFNβ and IFN-inducible genes in MyD88-independent signaling (87). On target cells, the IL-1β-mediated signaling can induce the expression of a wide range of genes including its own gene, that provide positive feedback loop to boost the inflammatory response. Furthermore, IL-1β through IL-1R also induces the expression of several negative feedback regulators to control and terminate its mediated-effects (82,88). In line, defective IL-1β pathway has been reported to increase the susceptibility to infection and impaired adaptive immunity; or severe systemic and local

inflammation under uncontrolled over-activation (84,89). Thus, targeting IL-1β signaling have been introduced as a therapeutic strategy to challenge a broad spectrum of inflammation-associated diseases (90).

3.8. NLRP3 as a target of pharmaceutical companies for drug design

Although NLRP3 inflammasome activation is crucial in host protection against microbial infections and host-derived danger signals, aberrant activation of the NLRP3 inflammasome has been implicated in the pathogenesis of a wide range of autoinflammatory, chronic inflammatory and metabolic disorders (91). Recently, targeting NLRP3 as a novel therapeutic approach has attracted pharmacologists and the drug industry to design effective NLRP3 inhibitors in order to treat various acute and chronic inflammatory conditions (92).

Several strategies have been developed to target and modulate the NLRP3 inflammasome activity, however, up to date, there are no available approved NLRP3-targeted inhibitors in human (93). Nevertheless, indirect inflammasome inhibitors like IL-1 blockers (canakinumab and rilonacept) are approved therapies for the treatment of various autoinflammatory diseases, including cryopyrin-associated periodic syndromes (CAPS), gout and rheumatoid arthritis (94,95). However, patients under IL-1 blocker treatment have shown a higher risk of opportunistic infections and sepsis, since this approach also may halt the inflammatory response driven by other inflammasomes that are required for host defense. In addition, targeting IL-1 does not prevent other NLRP3 inflammasome downstream functions, including IL-18 release and pyroptosis (96). Another suggested strategy involves targeting the upstream pathways that regulate NLRP3 inflammasome, including oxidative stress, various metabolic and inflammatory signaling pathways. This approach may provide potential therapeutic applications not only to target the NLRP3 inflammasome but also to manage other inflammatory-related mechanisms. While it is an important strategy for inhibition of NLRP3 inflammasome, it may lead to unintended side effects, including interaction with other processes essential for cellular functions (75,97).

The recent advances in the knowledge of NLRP3 inflammasome structure and the mechanism of activation/inhibition drive researchers to develop specific NLRP3 inhibitors that could have many advantages over the indirect methods (98). This approach intends to directly inhibit the oligomerization of NLRP3 protein, NLRP3 ATPase activity or its binding with other NLRP3 inflammasome components, to prevent the formation of the inflammasome complex. A

number of small-molecule inhibitors of the NLRP3 have been reported, with some reaching clinical trials for the treatment of various inflammatory conditions. These include sulfonylurea compounds (including MCC950) that present high-affinity binding but with safety limitations, while non-sulfonylurea compounds are considered safe but with lower affinity (92). Notably, most of NLRP3 inhibitors under clinical trial belong to sulfonylurea compounds or derived from MCC950 (93). Other potential therapeutic strategies include targeting NLRP3 at the transcription level via genetic and epigenetic modulation. For instance, disrupting the NLRP3 at the genomic level using CRISPR/Cas9 leads to inhibit NLRP3 inflammasome activation, without risks of off-target effects (99). Furthermore, non-coding RNAs (including miRNAs and lncRNAs) have been reported to regulate NLRP3 inflammasome through post-transcriptional repression of NLRP3 or other inflammasome components (100). Previously, our group reviewed the regulatory roles and mechanisms that several nuclear receptors exert on NLRP3 inflammasome, including the regulation of NLRP3 transcription, in order to draw attention to the importance of nuclear receptors as potential targets to modulate NLRP3 inflammasome functions (101). A recent report suggests a combinatorial therapy of targeting both nuclear receptors and microRNAs as a promising strategy to inhibit NLRP3 (102). Despite encouraging progress in NLRP3 inflammasome-targeted therapies, there is still a concern about their safety and potential off-target effects. Therefore, the efforts to design potent and specific NLRP3 inflammasome inhibitors with improved efficacy are urgently needed for the benefit of treating various autoinflammatory and inflammatory diseases.

3.9. NOD1 and NOD2

NOD1 and NOD2 belong to the NLRC subfamily of NLRs, and have been introduced as NLRC1 and NLRC2, respectively, in the new nomenclature system. NOD1 is widely expressed in various tissues, however NOD2 expression is limited mainly to hematopoietic cells and intestinal compartment cells (such as goblet cells, Paneth cells and enterocytes) (103). NOD1/NOD2 share similar structural arrangements, however NOD1 has a single N-terminal CARD domain, whereas NOD2 contains two tandem CARDs (Figure 4) (104). NOD1/NOD2 are specialized sensors to detect intracellular bacterial peptidoglycan (PGN), however, they recognize different conserved motifs of PGN. NOD1 senses g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) of Gram-negative bacteria, while NOD2 senses muramyl dipeptide (MDP) motif which is found in both Gram-positive and Gram-negative bacteria. In addition, NOD1/NOD2 are also involved in

the recognition of other pathogens (including viruses, parasites and fungi), as well as DAMPs-related to ER stress, perturbed calcium homeostasis and disruption of the cytoskeleton dynamics (105).

Upon ligand binding, NOD1/NOD2 undergo conformational changes to release autoinhibition, leading to oligomerization of NOD proteins and activation of downstream signaling effectors. NOD1/NOD2 recruit RIPK2 through CARD-CARD interactions that facilitates the activation of both MAPKs and NF-κB pathways. Since PGNs are internalized into the cytosol via phagocytosis, endocytosis or bacterial secretion systems, the activation of NODs requires the translocation to the sites nearby intracellular membranes of PGNs delivery (106). Activation of NOD1/NOD2 also involves induction of autophagy and type I interferon signaling in cells- and context-dependent manner (107). Since NOD1/NOD2 contain CARD domain, it has been proposed that these proteins may bind to various caspases to induce cell death. Furthermore, NOD1/NOD2 have been implicated in IL-1β processing through activation of caspase-1, as well as regulation of several cytokines, including IL-6, IL-8 and IL-10 (103,108,109). Although NOD1/NOD2 share common structure and signaling pathways, they differ in their activation mode and regulation at the cellular level. For instance, NOD1/NOD2 exhibit different oligomerization mechanisms and are regulated by distinct auxiliary control proteins for only NOD1 or NOD2, such as Erbb2-interacting protein that only binds to NOD2 (110,111). NOD signaling is essential in maintaining immune and tissue homeostasis, and is involved in mediating adaptive and trained immune responses. In line with this, dysfunctional NOD1or NOD2 are linked to impaired host defense and associated with several chronic inflammatory diseases (such as Crohn's disease, IBD and asthma) (112,113).

3.10. Vitamin A and retinoic acid

Vitamin A is a lipophilic micronutrient and represents a group of chemically related and biologically active retinoids (retinol, retinal, retinoic acid (RA) and their isomers). Since animals are unable to *de novo* synthesize vitamin A, they obtain it solely through their food as provitamin A. In the body, vitamin A is required to be metabolized to its biologically active RAs through two-step reaction, the alcohol form of vitamin A (retinol) is oxidized to retinaldehyde (retinal) by alcohol- or retinol dehydrogenases (ADHs, RDHs); subsequently retinal is converted to RAs by the “rate-limiting enzymes” called retinaldehyde dehydrogenases (RALDHs) (Figure 7) (114).

Several RA isomers have been identified *in vivo*; however all-trans RA (ATRA) is the most functionally active form in the body. Tissue-specific and context-dependent expression of retinol-oxidizing enzymes has been reported, which makes RA synthesis restricted to certain tissues and cells. In addition to immune cells (DCs and MΦs), epithelial cells, stromal cells and hematopoietic progenitor cells can also produce RA (115).

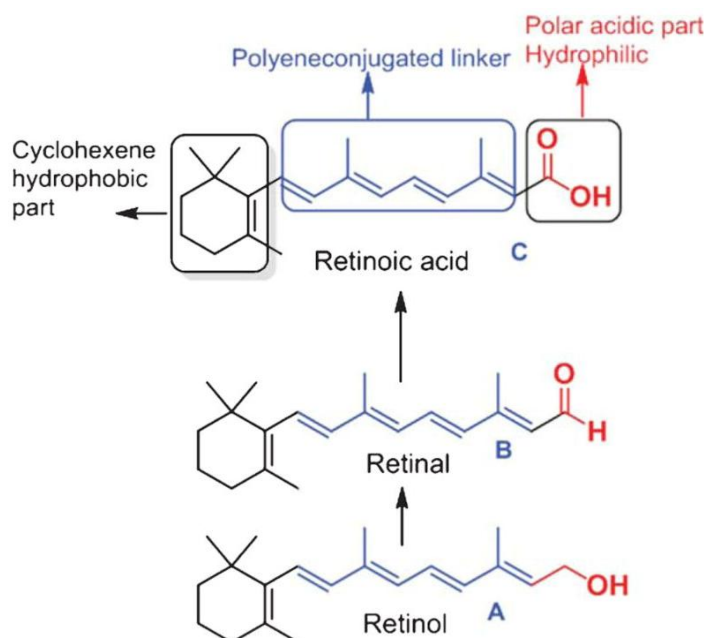


Figure 7. Schematic representation of retinoid structures. *Retinol is oxidized to retinal by alcohol- or retinol dehydrogenases, then retinal is converted to RA by retinaldehyde dehydrogenases* (116).

The predominant route for retinoid delivery is through circulating retinol conjugated to retinol binding protein (RBP4), that binds STRA6 receptor (stimulated by retinoic acid 6) on target tissues to mediate the intracellular delivery. However, there are other direct routes of transportation such as packaging retinyl esters in lipoproteins or chylomicrons, or binding of retinoic acid to albumin (117). Furthermore, RA can act in an autocrine or paracrine manner in the surrounding microenvironment to drive signaling processes.

Nevertheless, RA synthesis and availability are tightly regulated by a set of RA-degrading enzymes (such as cytochrome p450 subfamily 26 (CYP26) enzymes), that function in negative feedback mechanisms. Besides that, RA itself transcriptionally regulates its synthesizing and degrading enzymes (115). RA functions through ligand-dependent transcription factors that belong to class II nuclear receptors or RXR heterodimers. Two subfamilies of nuclear receptors are

activated by RA, retinoic acid receptors (RARα,β,γ) and to a lesser extent peroxisome proliferator-activated receptors (PPARβ,δ). Notably, RA-mediated effect through PPARβ/δ have been investigated in HaCaT human keratinocyte cell line and NaF cells (derived from tumors that arise in MMTV*neu* mice), that exhibit a high fatty acid-binding protein 5 (FABP5)/CRABP-II ratio. While CRABII drives RA through RAR to induce growth inhibition, FABP5 delivers RA to PPARβ/δ to mediate cellular proliferation (118,119). Furthermore, the 9-cis-13,14-dihydroretinoic acid is an isomer that acts as a ligand for RXRs (120).

Upon ligation, these receptors heterodimerize with RXRs (RXRα,β,γ) and bind to retinoic acid response elements (RAREs) in the genome to regulate gene transcription of retinoid-responsive genes (119). Generally, in the presence of the ligand, the heterodimer complex recruits coactivator proteins and drives gene expression; while in the absence of ligand, the corepressors are recruited leading to the inhibition of gene expression (121). Furthermore, RA-activated nuclear receptors and their isoforms exhibit tissue-specific expression patterns, functions that depend on the cellular contexts. In addition, RA-activated nuclear receptors are regulated by various post-translational modifications. For example, phosphorylation of RAR in certain sites in N-terminal domain promotes DNA binding, however phosphorylation of other sites in RAR could lead to RAR degradation (101,122).

In addition to this classical mechanism, RA also has non-canonical or non-genomic functions mediated by direct interaction with various cytosolic signaling networks in receptor-dependent and -independent manner. In response to RA, RARs localized to membrane lipid rafts can activate RhoGTPase leading to the activation of downstream effector pathways, like MAPKs (Erk and p38). RA has also been shown to stimulate several other signaling pathways, including PI3K and/or PKB/Akt and protein kinase A (PKA). The non-canonical actions of RA in the cytosol can also be mediated by other RA partners, such as retinoic acid binding proteins (CRABPs) (122,123). RA has been shown to directly and indirectly regulate various miRNAs and their biogenesis in different cellular processes and the pathogenesis of disease conditions (124).

So far, RA emerges as a key multi-functional signaling molecule which is involved in a wide spectrum of biological events including cell differentiation, metabolism, cell death, oxidative stress; and regulation of nervous system, immune response and inflammation (125). RA is an essential component of tissue microenvironment that is necessary for immune homeostasis and proper immune functions. It regulates the innate and adaptive immune cells (especially T cells),

and mediates their crosstalk, particularly in intestinal mucosal immunity and other mucosal tissues. Since gut mucosa is rich in RA and immune cells, under the steady state microenvironment, RA shapes the polarization of intestinal MΦ and DC subsets to drive the differentiation of naïve T cells into gut-homing T cells (such as Tregs). In addition, RA potentiates the expression of gut-homing proteins α4β7 integrin and C-C chemokine receptor 9 (CCR9) on T and B cells, supporting the immune tolerance of the mucosal gut. However, during the inflammatory response, RA induces the polarization of proinflammatory DCs and MΦs phenotypes and promotes effector T cell differentiation (Th1 and Th17), potentiating the immune responses instead of tolerogenic conditions (126,127).

In monocytes/ MΦs, the production of RA is affected by the immunological context and the availability of RA itself in certain microenvironments. In addition, the expression of RA-synthesizing enzymes is also influenced by other factors such as PAMPs and cytokines under an inflammatory condition (127). Growing body of evidence suggests that RA and RAR signaling mediate MΦs polarization through regulation of several transcription factors in tissue-specific manner, including *Arg1, GATA6*, *Irf4* and *Zbtb46* (128,129). Furthermore, RA and RAR signaling are involved in the modulation of a range of MΦs functional processes such as cytokine production, phagocytosis and efferocytosis, and other intracellular mechanisms including autophagy, cholesterol efflux and metabolism (130–133). Importantly, the immune regulatory roles of RA have also been linked to several PRR functions (such as TLRs and RLRs) (134,135). Since RA is an essential component of tissue homeostasis and inflammatory host responses, dysregulated RA signaling or RA synthesis have been associated with a broad range of immunological disorders, including inflammatory bowel diseases and autoimmune diseases (127). In addition, several inflammatory conditions have been reported to negatively modulate vitamin A balance, which results in altered immunological responses in human and animal models (125). Several evidence suggest a link between vitamin A deficiency/ hyporetinolemia and infectious diseases (such as tuberculosis, measles, malaria, diarrhea and HIV) (125,133). Therefore, understanding the potential mechanisms and therapeutic targeting of complex and multifaceted RA-signaling based on the context and microenvironmental conditions, could provide a window to control a number of inflammatory and infectious diseases.

4. Aims of the study

Macrophages (MΦs) are crucial effector cells of the innate immune system and exhibit high plasticity and heterogeneity in phenotype and function, which are determined by the activating stimuli and their tissue microenvironment. In response to NLRs activation, MΦs mediate distinct host immune or/and tissue repair responses. In human, *in vitro* differentiation of monocytes with M-CSF or GM-CSF are widely used models for studying MΦs polarization and functions. This experimental differentiation provides different MΦ populations with different phenotypic states and characteristics. While GM-CSF promotes monocytes to differentiate to classically polarized MΦs, M-CSF is associated with alternative polarization of MΦs (136,137). Since RA represents a tissue-derived signal that assumed to regulate the MΦs functions, the main objective of this study was to investigate the potential modulatory effects of ATRA on human monocyte-derived MΦs, particularly upon NLRP3, NOD1 and NOD2 activation, and delineate possible mechanisms of action.

The specific aims of this study were as follows:

- To investigate the effect of ATRA on cytokine secretion following LPS activation of M-MΦs
- To study whether ATRA modifies NLRP3 inflammasome priming or activation in M-MΦs
- To study potential molecular mechanisms (signaling pathways, metabolism) behind the modulatory effect of ATRA
- To investigate the modulatory effect of ATRA on NOD1-induced cytokine secretion in M-MΦs and GM-MΦs
- To investigate the modulatory effect of ATRA on NOD2-induced cytokine secretion in M-MΦs and GM-MΦs

5. Materials and methods

5.1. Reagents

MCC950 (NLRP3-selective inflammasome inhibitor) and Ultrapure LPS from Escherichia coli were purchased from InvivoGen (San Diego, CA, USA). All-trans retinoic acid, adenosine triphosphate (ATP) and 3-bromopyruvate (3BP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IL-10 was obtained from PeproTech (Rocky Hill, NJ, USA). Z-YVAD-FMK (caspase-1 inhibitor) was purchased from BioVision Technologies (Milpitas, CA, USA). C14-Tri-LAN-Gly (NOD1 specific agonist) and L18-MDP (NOD2 specific agonist) were obtained from InvivoGen (SanDiego, CA, USA).

5.2. Ethics statement

Leukocyte-enriched buffy coats were obtained from healthy blood donors, through National Blood Transfusion Service. The procedure was documentary approved by the Director of the National Blood Transfusion Service. The study and all experimental protocols were in accordance with, and approved by the Regional and Institutional Ethics Committee of the University of Debrecen.

5.3. Monocyte isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte-enriched buffy coats. Briefly, the blood samples were diluted in physiological saline solution (PSS), and submitted to density-gradient centrifugation using Ficoll Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). The PBMC layer was collected and washed with PSS and MACS buffer (phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2mM EDTA). Monocytes were purified from PBMCs using immunomagnetic positive selection with anti-CD14-conjugated microbeads as reported by the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

5.4. Macrophage differentiation

The obtained monocytes were suspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, and 500 U/mL of penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the

suspended monocytes were cultured in 24-well plates at a density of 1.1 × 106 cells/mL in either 50 ng/mL M-CSF or 80 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ, USA) containing media and incubated at 37 °C and 5% CO2. After 48 h, half of the culture media was carefully removed and replaced with fresh media containing the same amounts of the cytokines. On day 5, the cells were used for the experiments.

5.5. Macrophage treatment

On day 5, the MΦs were treated with ATRA (1 µM) alone or pretreated with ATRA for 4 h and stimulated with LPS (100 ng/mL) for different time points. For IL-1β induction, MΦs were treated with ATP (5 mM) for 45 min. Where indicated, cultures were pretreated with an inhibitor for 1 h, and then LPS was applied. The control (mock) was treated with 0.1% DMSO/ethanol. In NOD1/NOD2 experiments, cells were treated with 500 ng/ml C14-Tri-LAN-Gly the NOD1-specific agonist or 100 ng/ml L18-MDP the NOD2-specific agonist for the indicated time points. Where indicated, cells were pretreated with ATRA (1µM) for 4 hours before adding NOD1/NOD2 agonists. For each experiment, data was obtained from at least four parallel analyses.

5.6. RNA preparation

Total RNA was extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) in accordance with the manufacturer's instructions. The RNA quantity and quality were determined using a spectrophotometer (NanoDrop ND1000; Promega Biosciences, Madison, WI, USA). The isolated RNA was treated with DNase and RNase inhibitor (Ambion, Austin, TX, USA). cDNA synthesis was achieved using random hexamers and the SuperScript II First-strand Reverse Transcriptase system (Thermo Fisher Scientific, Waltham, MA, USA).

5.7. Quantitative Real-Time PCR

For quantitative RT-PCR, Taqman Gene Expression Assays were used with the Taqman™ Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). The amplification was performed using a QuantStudio12K Flex qPCR instrument (ABI). Human Taqman gene expression assays were purchased from Thermo Fisher Scientific (Waltham, MA, USA), NLRP3 (Hs00918082_m1), and IL-1β (Hs01555410_m1). The amplification program was, 10 min at 95 °C followed by 40 cycles of 10 s at 95 °C, and 1 min at 60 °C. The relative expression values for

each transcript of interest were calculated by the comparative Ct method, and human cyclophilin (Ppia) was used for normalization.

5.8. Western blot analysis

Cells were harvested and washed with PBS; directly lysed in 2X Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), containing 25% glycerol, 2% SDS, 1% b-mercaptoethanol, and 1% bromophenol blue); and boiled for 10 min. Protein lysate were separated using SDS-PAGE and different percentage gels were used depending on the molecular weight of the protein analyzed. Then, the protein lysate transferred onto a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was blocked with 5% non-fat dry milk diluted in TBS-Tween buffer (50 mM Tris, 0.5 M NaCl and 0.05% Tween-20, pH 7.6). The membrane was incubated overnight at 4 °C with primary antibodies in 1:1000 dilution. ASC (sc-30153) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), NLRP3 (Cat. No. 15101), caspase-1 (Cat. No. 3866), IL-1β (Cat. No. 12703), cleaved caspase-1 (Cat. No. 4199), cleaved-IL-1β (Cat. No. 83186), p-Akt/(S473) (Cat. No. 9271), p-mTOR (Ser2448) (Cat. No. 2971), p-p70S6 Kinase (Thr389) (Cat. No. 9234), p70S6 Kinase (Cat. No. 2708), p-Stat3 (Tyr705) (Cat. No. 9145), p-p38 MAPK (Thr180/Tyr182) (Cat. No. 9211), p-SAPK/JNK (Thr183/Tyr185) (Cat. No. 9251), p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cat. No. 9101), and p-IκBα (Ser32) (Cat. No. 2859) were obtained from Cell Signaling technology (Danvers, MA, USA). After washing step, the membrane was incubated for 1 h at room temperature with a corresponding HRP-conjugated secondary Abs in 1:5000 dilution (goat anti-rabbit IgG, No. 170-6515) from Bio-Rad Laboratories (Hercules, CA, USA). Membrane-bound peroxidase proteins were detected on X-ray films using the ECL system (SuperSignal West Pico/Femto chemiluminescent substrate; (Thermo Fisher Scientific, Waltham, MA, USA). β-Actin (8457) (Cell Signaling technology, USA) was used as the internal control. The ImageJ software was used for analysis and quantification of protein bands.

5.9. Metabolic assays and extracellular flux analysis

The investigation of real-time alterations in the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of MΦs were conducted using a Seahorse XF 96 Analyzer (Seahorse Biosciences, North Billerica, MA, USA). The isolated monocytes (50,000 cell/well) were plated and differentiated in Seahorse XF96 cell culture microplates (Seahorse Biosciences, North Billerica, MA, USA). After treatment, MΦs subjected to the metabolic assay tests. For

mitochondrial stress test, cells were washed and incubated in XF assay medium (Seahorse Bioscience, North Billerica, MA, USA) supplemented with 10 mM glucose and 2 mM L-glutamine and incubated for one hour at 37 °C in a CO2-free incubator. The baseline OCR was recorded, and the cells were then subjected to the following compounds: Oligomycin (Oligo), an ATP synthetase inhibitor (1µM); carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), an uncoupling agent (1µM); and rotenone and antimycin A (R + A) as mitochondrial complex I and III inhibitors (1:1µM), respectively. Real-time changes in the OCR were recorded every 6 min (1 min mixing, 5 min measurement) for five loops. For the glycolytic stress test, the RPMI media was replaced by XF media supplemented with 2 mM L-glutamine and incubated for 1 h at 37 °C in CO2-free conditions. After equilibration, the real-time changes in the ECAR were recorded every 9 min (1 min mixing, 8 min measure) for 5 loops, during sequential treatment of the following compounds: 10 mM glucose (Glu), 1 µM oligomycin (Oligo), and 50 mM 2-deoxy-D-glucose (2-DG). 2-DG is a glucose analog that acts as glycolysis inhibitor through competitive binding to glucose hexokinase. The background control was determined by the testing media. The test was run for 90 min according to the manufacturer's protocol and the injection time for each compound is indicated in the graphs. The protein concentration was determined using the Bradford protein assay. The obtained values were normalized to the corresponding total protein content. Wave 2.3 Agilent Seahorse Desktop software was used for the data analysis.

5.10. Cytokine measurements

The concentration of cytokine secretion was determined from the obtained cell culture supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits. IL-1β, IL-6, IL-10, IL-8 and TNFα were measured using ELISA kits from BD Biosciences (San Diego, CA, USA). IL-18, IL-6, IL-10 and IFNβ were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA). The protocol was according to the manufacturer's instructions. The quantifications were performed using a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The minimum detectable levels were 3.9 pg/mL for IL-1β, 3.1 pg/mL for IL-8, 7.8 pg/mL for TNF-α, 11.7 pg/ml for IL-18, 9.38 pg/mL for IL-6, 31.3 pg/mL for IL-10 and 7.81 pg/mL for IFNβ

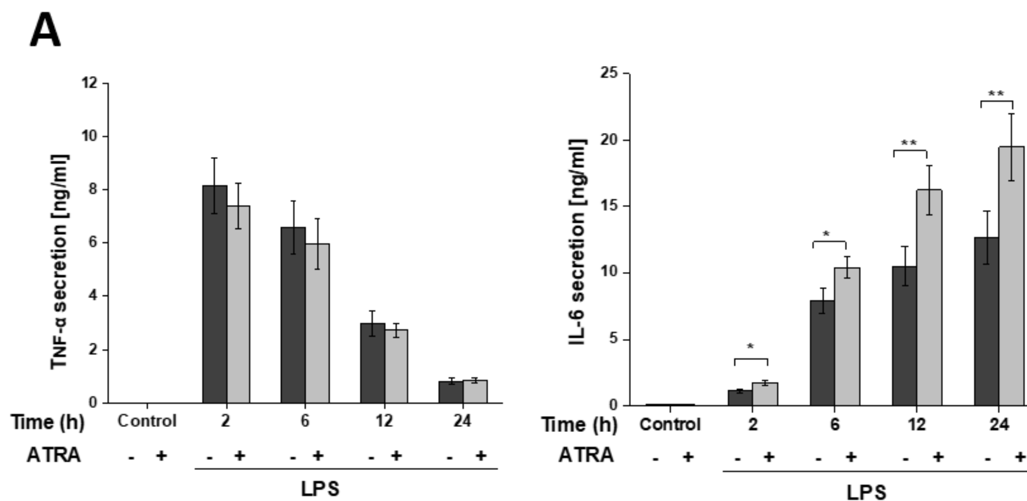
5.11. Statistical analysis

Experimental results were presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical significance was determined using One-way ANOVA followed by Tukey–Kramer, Tukey's HSD and Dunnett's post-hoc tests; or unpaired student's t-tests. Differences between groups were considered significant at p values less than 0.05.

6. Results

6.1. ATRA modifies LPS-induced proinflammatory cytokine secretion in M-MΦs

IL-1β, TNF-α, and IL-6 cytokines are considered as key proinflammatory cytokines that are secreted by MΦs and DCs (138). To address whether ATRA has effects on the secretion of proinflammatory cytokines upon activation of human monocyte-derived macrophages (M-MΦs), we treated M-MΦs with LPS in the presence or absence of ATRA for different time points. Using the ELISA method, cytokine production was quantified from cell culture supernatants. The results showed that treatment with ATRA did not influence the TNF-α secretion, while significantly upregulated IL-6 secretion of LPS-activated M-MΦs (Figure 8A). Activation of these cells with LPS and ATP results in rapid induction of IL-1β secretion at 2 h, then gradually decreases over time, which is consistent with a previous report (139). Treatment with ATRA significantly enhanced and prolonged IL-1β secretion of LPS/ATP-treated M-MΦs. However, no effect was observed on the secretion of IL-1β or other cytokines following only ATRA treatment of non-primed cells (Figure 8B). Concentration-dependent effect of ATRA was detected in the induction of IL-1β secretion of LPS/ATP-treated M-MΦs (Figure 8C). Since IL-1β requires specific mechanisms for maturation and secretion that involve NLRP3 inflammasome (51), we treated the primed cells with NLRP3 inhibitor MCC950. The results showed that MCC950 abolished IL-1β secretion (Figure 8D), indicating that ATRA effect on IL-1β secretion is mediated via an NLRP3 inflammasome-dependent pathway.



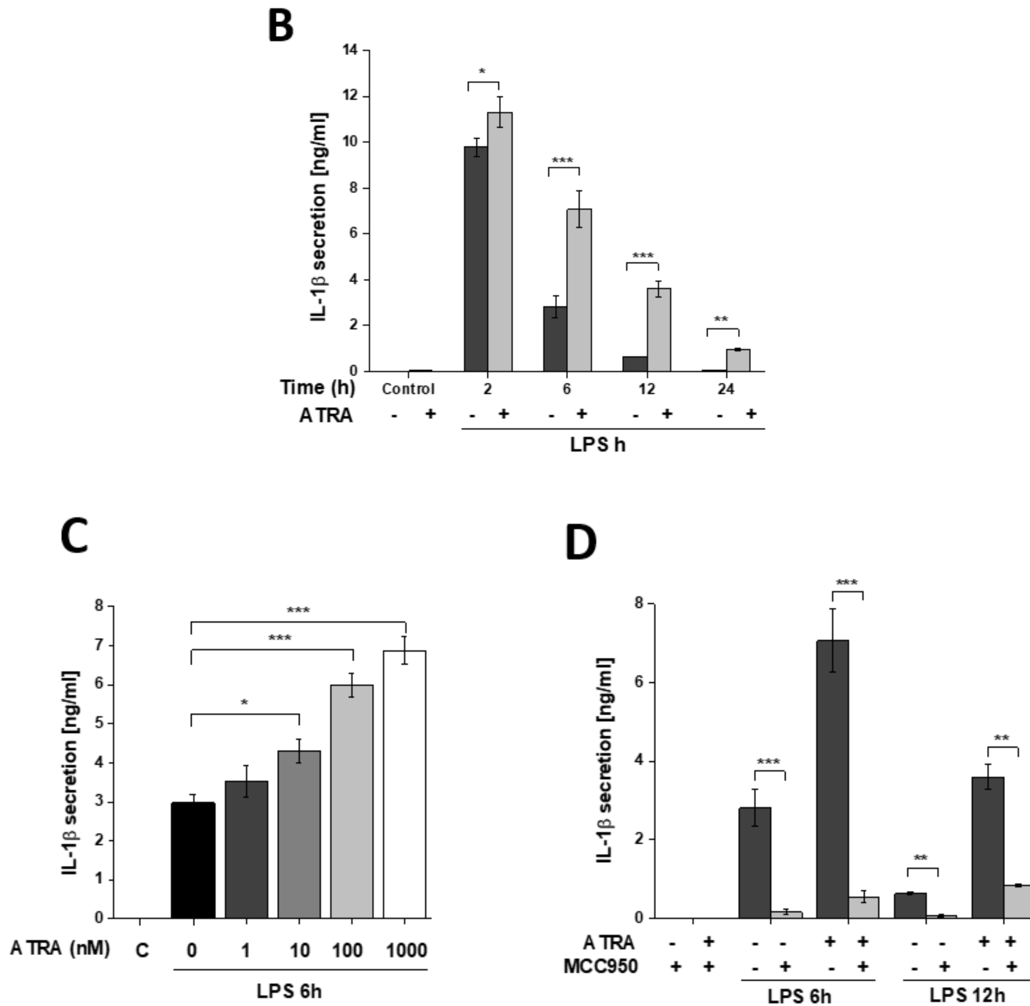
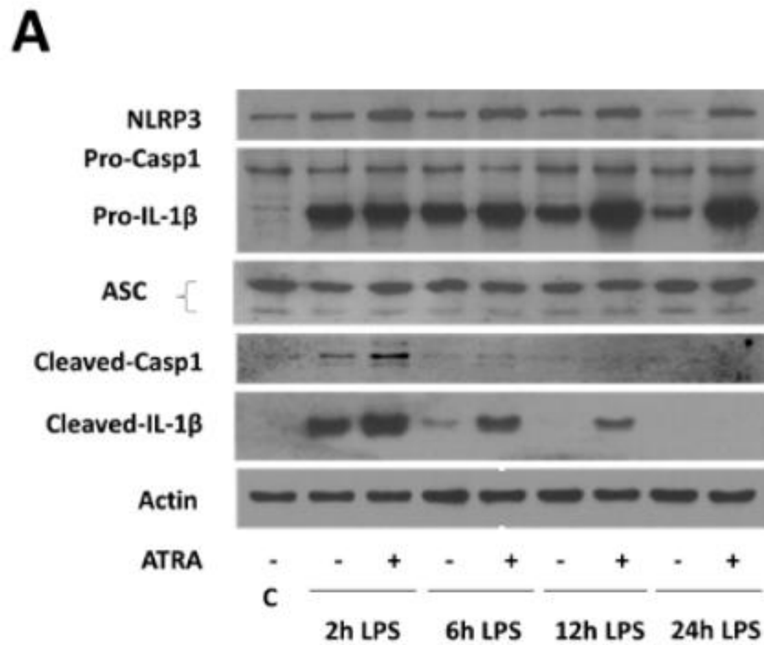


Figure 8. ATRA differentially modulates proinflammatory cytokine secretion of LPS-activated MΦs. *MΦs were pre-incubated with ATRA (1 μM) where indicated, and then stimulated with LPS (100 ng/mL) for the indicated time. Cell culture supernatants were collected, and the secretion of (A) TNFα and IL-6 were measured by ELISA. (B) For IL-1β induction, cells were subsequently incubated with ATP (5 mM) for 45 min. (C) Cells were pre-treated with increasing concentrations of ATRA as indicated, and IL-β secretion was measured 6 h following treatment. (D) Cells were pretreated with MCC950 (1 μM) 1 h before ATP treatment. C, control (6 h mock-treated cells). All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). +, −, presence or absence of indicated substance, respectively.*

6.2. ATRA prolongs LPS-induced IL-1β cytokine secretion by augmenting LPS-induced NLRP3 and pro-IL-1β expression

In activated M-MΦs, NLRP3 inflammasome-mediated IL-1β secretion involves a two-step activation process. The priming step is typically induced by TLRs that trigger the expression of

the NLRP3 inflammasome components and NLRP3 post-translational licensing. The second step is required for the assembly of the NLRP3 inflammasome complex, leading to the activation of the caspase-1 enzyme, and subsequently IL-1β maturation (140). To determine whether ATRA interacts with the priming signal of LPS-treated cells, the protein expression of the inflammasome components was evaluated using western blot method. Our results showed that while ATRA did not affect the adaptor ASC and the pro-form of caspase-1 enzyme in LPS-treated cells, it significantly upregulated the expression of NLRP3 sensor and pro-IL-1β substrate. In addition, the expression of cleaved caspase-1 and IL-1β was significantly enhanced in ATRA/LPS-treated cells compared to only LPS-primed cells, suggesting that ATRA may also augment caspase-1 activity (Figure 9A). To investigate whether ATRA transcriptionally modulates the expression of *pro-IL-1β* and *NLRP3* of LPS-activated M-MΦs, the total RNA isolated from treated cells and control samples was subjected to RT-PCR. The results show that ATRA enhanced the mRNA expression of *NLRP3* and *pro-IL-1β* of LPS-treated M-MΦs (Figure 9B). These results indicate that ATRA prolongs LPS-induced IL-1β secretion in part by potentiating the priming step through upregulating of the expression of *NLRP3* and *pro-IL-1β* in LPS-activated cells.



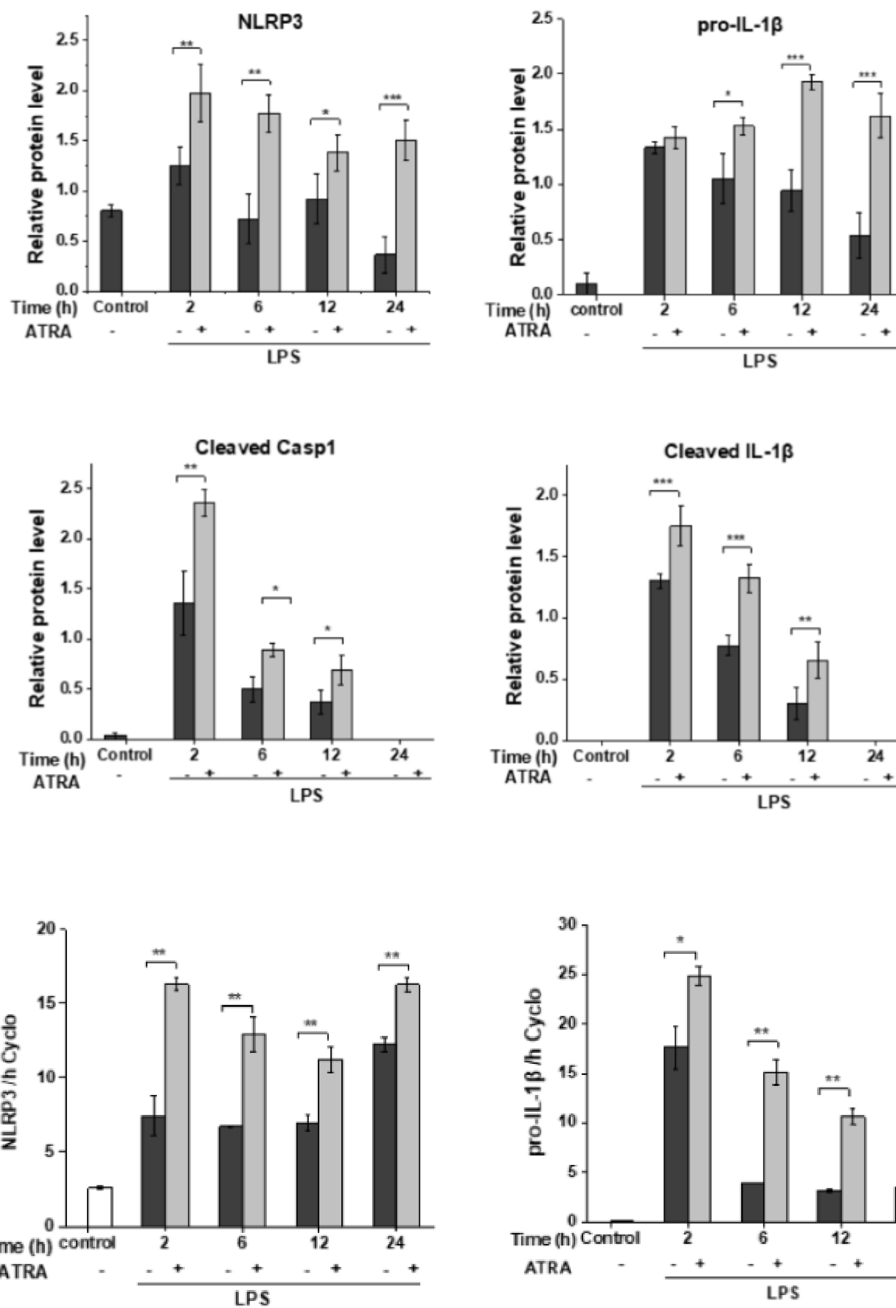


Figure 9. ATRA enhances LPS-induced pro-IL-1β and NLRP3 expression in human MΦs. *MΦs were pre-incubated with ATRA (1 µM) where indicated, prior to stimulation with LPS (100 ng/mL), then 5 mM ATP was applied for 45 min. (A) Representative immunoblot of NLRP3, pro-caspase-1, pro-IL-1β, and ASC from the cell lysates; and released caspase-1 and IL-1β in supernatant. Bar graphs represent the relative protein expression of pro-IL-1β and NLRP3 determined by densitometry. β-actin was used as internal control. (**B**) The relative gene expression of pro-IL-1β and NLRP3 was measured by qPCR. The expression was normalized to the reference gene (human cyclophilin; Cyclo) expression. C, control (6 h mock-treated cells). All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). +, −, presence or absence of indicated substance, respectively.*

6.3. ATRA alone enhances NLRP3 but not Pro-IL-1β expression

Next, we aimed to elucidate whether ATRA influences the expression of *NLRP3* and *pro-IL-1β* in M-MΦs. To address this, we treated M-MΦs with ATRA alone, and the expression of RNA and protein of NLRP3 and pro-IL-1β was investigated using RT-PCR and western blot methods. Our findings show that ATRA did not change the expression of *pro-IL-1β* (Figure 10A), however ATRA significantly and time-dependently upregulated both the mRNA (Figure 10B) and protein levels of NLRP3 (Figure 10C). These results suggest that ATRA alone has partial ability to affect the priming signal of NLRP3 inflammasome, although ATRA enhances the NLRP3 expression as a key component of the inflammasome, it is not capable of inducing the expression of the inflammasome substrate *pro-IL-1β*.

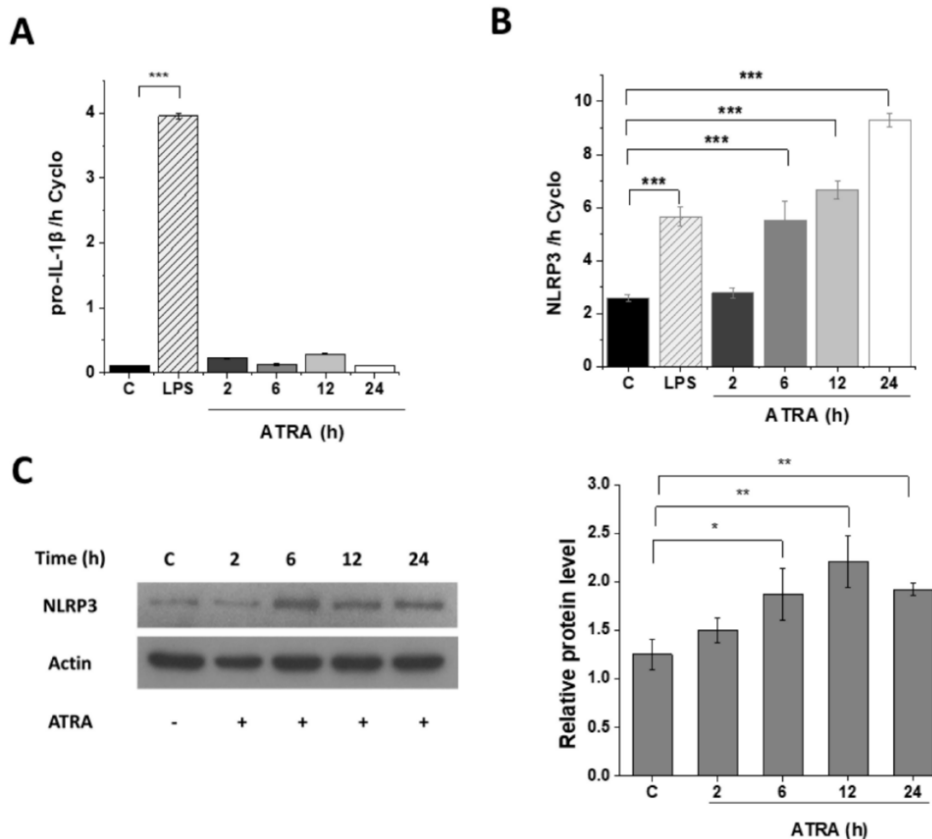
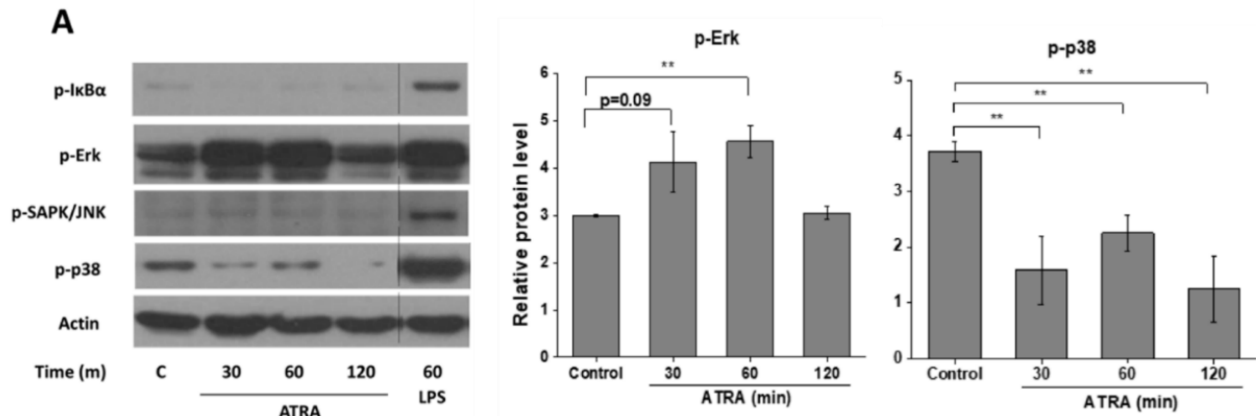


Figure 10. Induction of NLRP3 by ATRA. *Relative gene expression of pro-IL-1β and NLRP3 were measured by quantitative RT-PCR. (A, B) MΦs were treated with ATRA for different time intervals. Here, 6-h LPS-primed cells served as a positive control.* ***(C)*** *Representative immunoblot of NLRP3 protein expression of ATRA-treated MΦs as indicated. β-actin was used as internal control. C, control (6 h mock-treated cells). All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). +, −, presence or absence of indicated substance, respectively.*

6.4. ATRA modifies signal transduction pathways required for inflammasome priming

The canonical action of ATRA is mediated via RAR nuclear receptors, which trigger genomic effects by regulating the transcription of target genes. However, ATRA has non-genomic activities that involve rapid induction of signaling cascades, including MAPKs (Erk, p38) (141–144). To further investigate whether ATRA interacts with those signaling pathways involved in NLRP3 inflammasome priming; M-MΦs were subjected to either ATRA treatment or in combination with LPS. Western blot was used to detect the expression of signal transduction pathways obtained from cell lysates. ATRA treatment displayed significant enhanced Erk phosphorylation and attenuated phosphorylation of p38, nevertheless no changes were detected in IkB-α and SAPK/JNK pathways (Figure 11A). Next, we aimed to investigate whether ATRA modulates the signaling pathways under LPS stimulation. The results show that ATRA slightly increased the LPS-induced phosphorylation of IkB-α, and significantly prolonged LPS-induced Erk and SAPK/JNK phosphorylation, while significantly downregulated the p38 phosphorylation (Figure 11B). These results indicate that ATRA modulates the signaling pathways that are implicated in the priming event of NLRP3 inflammasome.



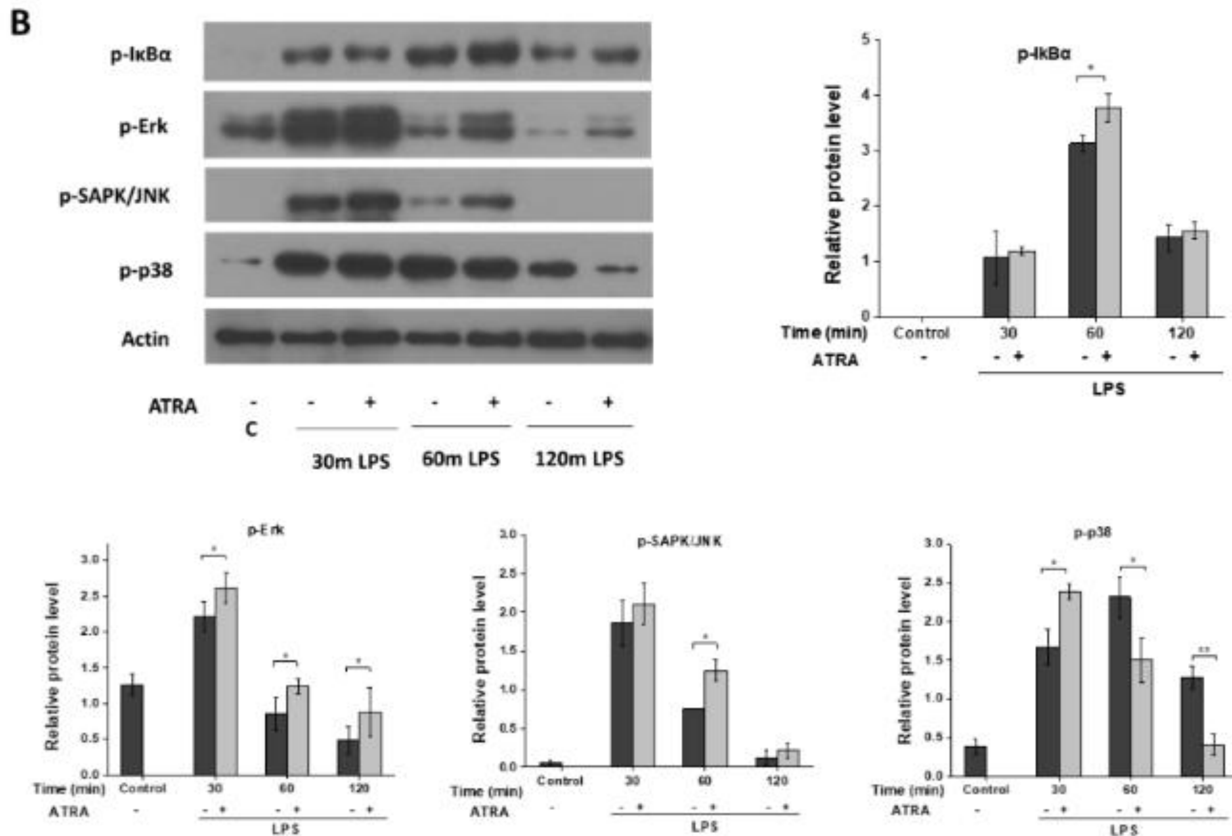


Figure 11. ATRA mediates signal transduction changes in MΦs. *(A) Representative immunoblots of phosphorylated IκBα, Erk, SAPK/JNK, and p38 from whole-cell lysates after ATRA treatment for the indicated time points. Here, 60-min LPS-primed cells served as a positive control. (B) MΦs were pre-incubated with ATRA (1 µM) before LPS priming for the indicated time, and phosphorylated IkBα, Erk, SAPK/JNK, and p38. β-actin was used as internal control. C, control (mock-treated cells). All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$). +, −, presence or absence of indicated substance, respectively.*

6.5. ATRA inhibits LPS-induced Akt/mTOR signaling pathway

Upon activation, TLR4 initiates intracellular signaling cascades and activates a transcription program to mediate upregulation of proinflammatory cytokines. The Akt/mTOR signaling pathway plays a key role in limiting the inflammatory response and mediating the anti-inflammatory action of activated MΦs (29,145). In addition, this pathway has been reported to negatively regulate the IL-1β production by suppressing the caspase-1 activation (146). Thus, we aimed to see whether ATRA modulates LPS-activated Akt/mTOR signaling pathway in M-MΦs. As expected, LPS stimulation resulted in the induction of Akt and mTOR phosphorylation, as well

as p70S6K as a downstream effector of mTOR (Figure 12). However, the phosphorylation of Akt is completely suppressed by ATRA treatment; in addition, mTOR and p70S6K as downstream target were also attenuated. These results suggest that the Akt/mTOR pathway is in part involved in ATRA modulation of NLRP3 inflammasome function.

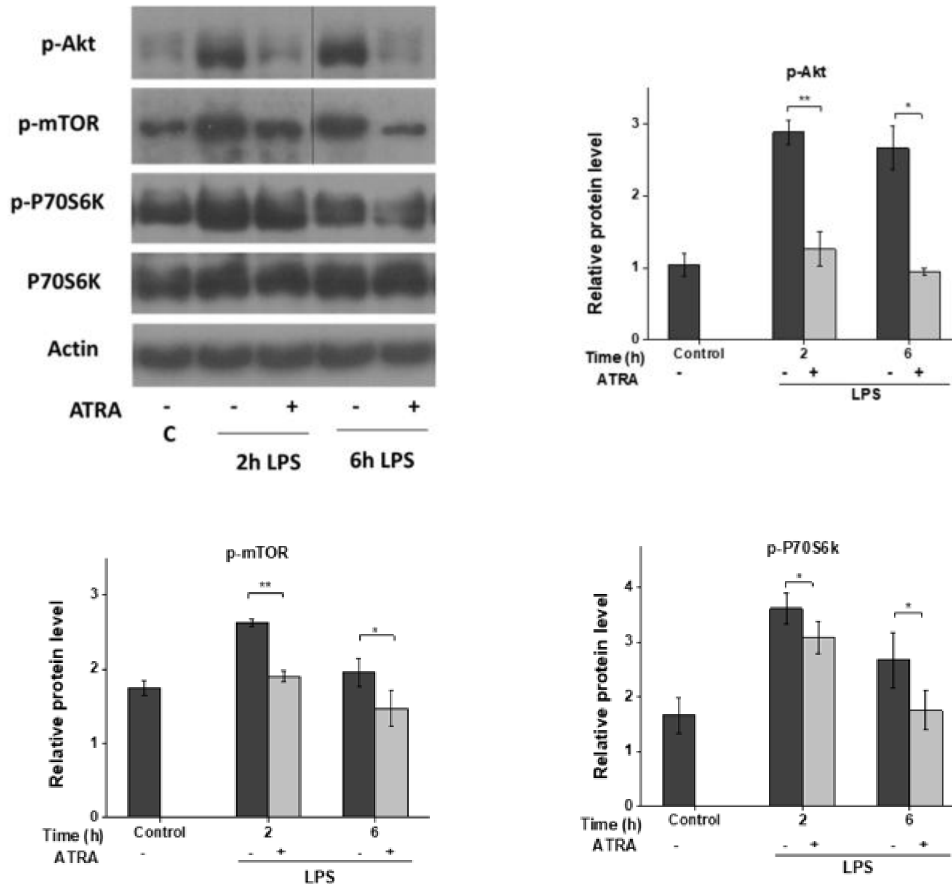


Figure 12. ***ATRA mediates signal transduction changes in MΦs.*** *Representative immunoblots of phosphorylated Akt, mTOR, P70S6K, and total P70S6K from whole-cell lysates. MΦs were pre-incubated with ATRA (1 μM) before LPS priming for the indicated time; then, whole-cell lysates were used for Western blot. β-actin was used as internal control. C, control (mock-treated cells. All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$). +, −, presence or absence of indicated substance, respectively.*

6.6. ATRA attenuates the secretion of LPS-induced IL-10

Akt/mTOR pathway regulates the activity of downstream target STAT3, a key mediator for the expression of IL-10. In addition, IL-10 is a master anti-inflammatory cytokine that restricts the inflammatory response and cytokine production (146–148). To address this, we sought to examine

whether ATRA-mediated Akt/mTOR pathway inhibition affects the STAT3 signaling and IL-10 production in LPS-activated cells. Stimulation of M-MΦs with LPS activated STAT3 protein as indicated by its phosphorylation, however ATRA significantly attenuated this activation (Figure 13A). In addition, the result showed that ATRA significantly downregulated LPS-induced IL-10 secretion at different time points (Figure 13B). Next, we investigated whether exogenous IL-10 can inverse the role of ATRA in upregulated IL-1β secretion. Treatment of M-MΦs with recombinant human IL-10 in the presence of LPS or ATRA/LPS significantly downregulated IL-1β production. These results indicate that STAT3/IL-10 signaling is involved, in part, in ATRA-enhanced IL-1β secretion of LPS-activated M-MΦs. Furthermore, ATRA drives the activated M-MΦs to exhibit more proinflammatory features over the anti-inflammatory ones.

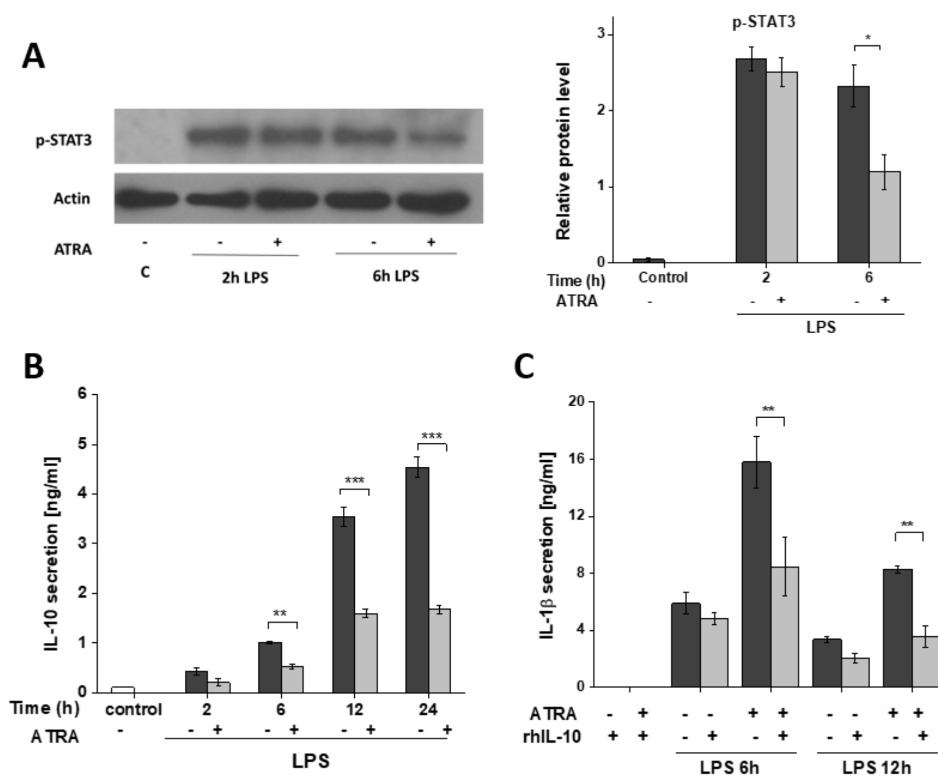
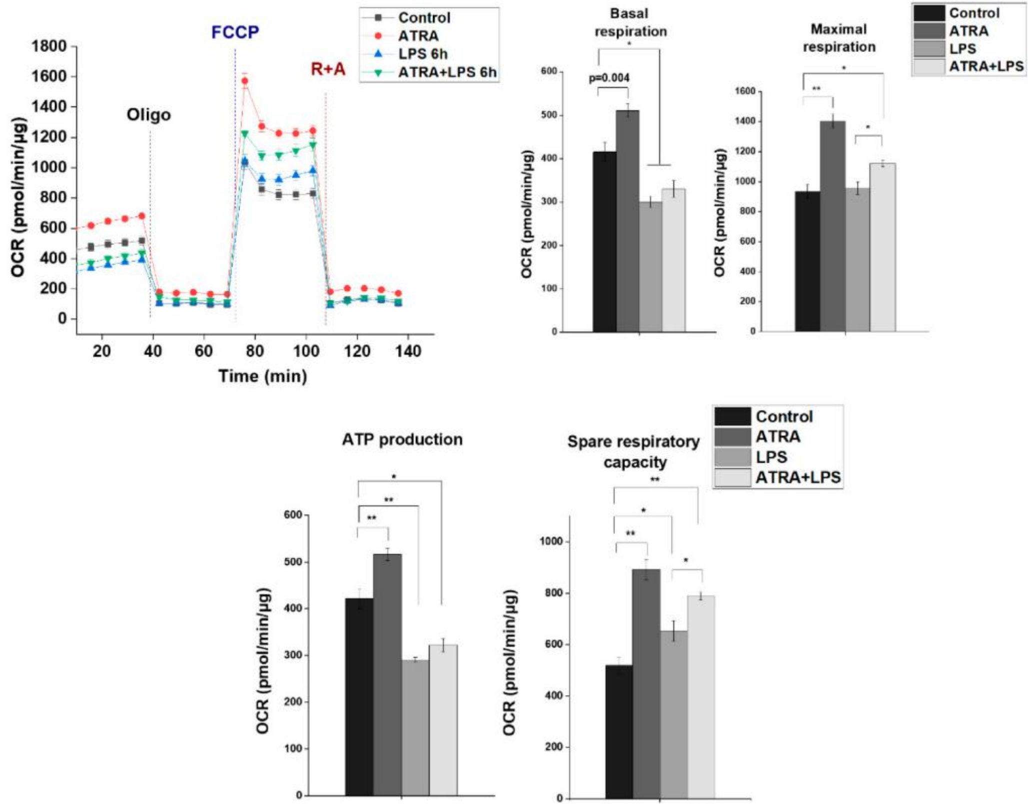
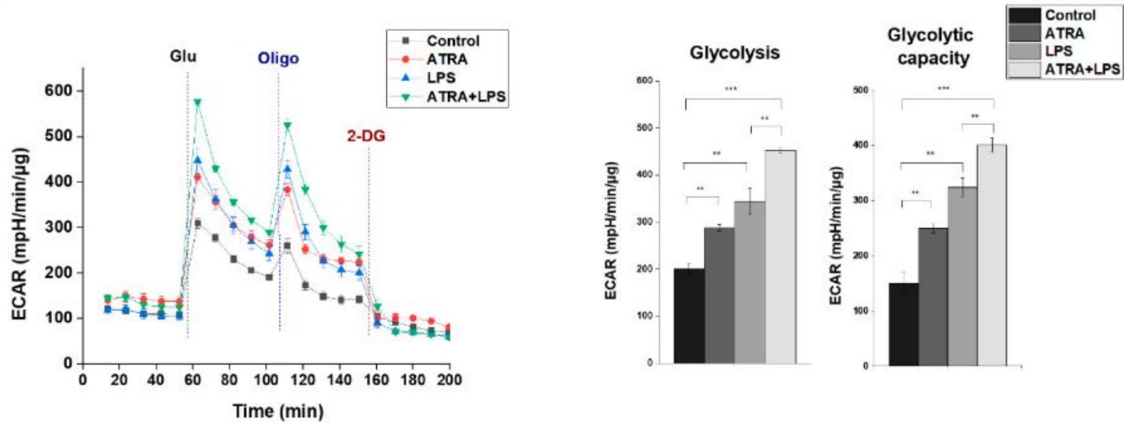


*Figure 13. **ATRA modulates LPS-induced IL-10 secretion and STAT3 activation in MΦs.** MΦs were pre-incubated with ATRA (1 μM) prior to stimulation with LPS (100 ng/mL) for the indicated time. (**A**) Representative immunoblots of phosphorylated STAT3 from whole-cell lysates. β-actin was used as internal control. (**B**) The secretion of IL-10 was assessed by ELISA from cell culture supernatants. (**C**) The cells were pre-treated with recombinant human IL-10 (rhIL-10) (100 ng/mL) 1 h before LPS priming and subsequently incubated with ATP (5 mM) for 45 min. Then, the cell culture supernatants were collected, and the secretion of IL-1β was assessed by ELISA. C, control (mock-treated cells). All results are shown as means ± SEM. (* $p< 0.05$, ** $p < 0.001$, *** $p < 0.001$). +, −, presence or absence of indicated substance, respectively.*

6.7. ATRA mediates a metabolic shift towards glycolysis in LPS-stimulated M-MΦs

mTOR pathway is a key regulator of a broad range of cellular processes, including growth, survival, aging and metabolism (147,149). Thus, we hypothesized that ATRA may affect the mitochondrial functions of LPS-stimulated cells. To test this, M-MΦs were subjected to LPS stimulation in the absence or presence of ATRA, and the mitochondrial oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were evaluated using a Seahorse analyzer. OCR is an indicator of mitochondrial respiration, while ECAR mainly represents anaerobic glycolysis. Our results showed that treatment with ATRA alone significantly increased the OCR, indicating enhanced mitochondrial respiration. However, under LPS challenge, the basal respiration and ATP production were downregulated and ATRA treatment did not recover the LPS effects (Figure 14A). Nevertheless, ATRA treatment significantly restored the LPS-induced downregulation of maximal respiration and spare respiratory capacity, parameters that measure the mitochondria fitness under increased energy demands. Hence, these results suggest that ATRA enhances the mitochondrial function and has a protective role of LPS-treated M-MΦs (Figure 14A). The glycolysis analysis showed that ATRA alone significantly upregulated ECAR for glycolysis and the glycolytic capacity, and the same effect was observed in LPS-treated M-MΦs. Furthermore, ATRA treatment of LPS-stimulated cells significantly potentiated the glycolysis and glycolytic capacity compared to the LPS-treated or ATRA-treated M-MΦs (Figure 14B). Consistent with these results, we found that ATRA treatment significantly upregulated hexokinase 2 (HK2) of LPS-activated M-MΦs (Figure 14C). HK2 is a key enzyme in the glycolysis pathway that catalyzes the first step in glucose metabolism (150). To investigate whether HK2 is involved in NLRP3 inflammasome-mediated IL-1β secretion, we subjected LPS-treated M-MΦs to 3-bromopyruvate (3BP), a specific inhibitor for HK2. The results showed that 3BP significantly downregulated IL-1β secretion of both LPS-treated and ATRA/LPS treated-M-MΦs (Figure 14D). Altogether, these results suggest that ATRA potentiates the glycolytic activity of M-MΦs, that participates in the elevated NLRP3 inflammasome-mediated IL-1β secretion under LPS challenge.


A
Control
ATRA
LPS 6h
ATRA+LPS 6h
FCCP
R+A
Oligo
OCR (pmol/min/µg)
Time (min)
Basal respiration
p=0.004
Maximal respiration
Control
ATRA
LPS
ATRA+LPS
ATP production
Spare respiratory capacity


B
Control
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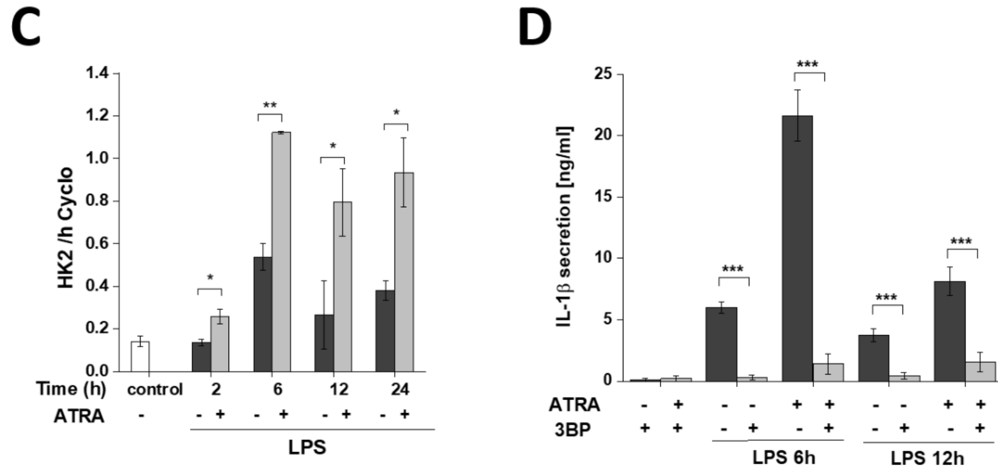
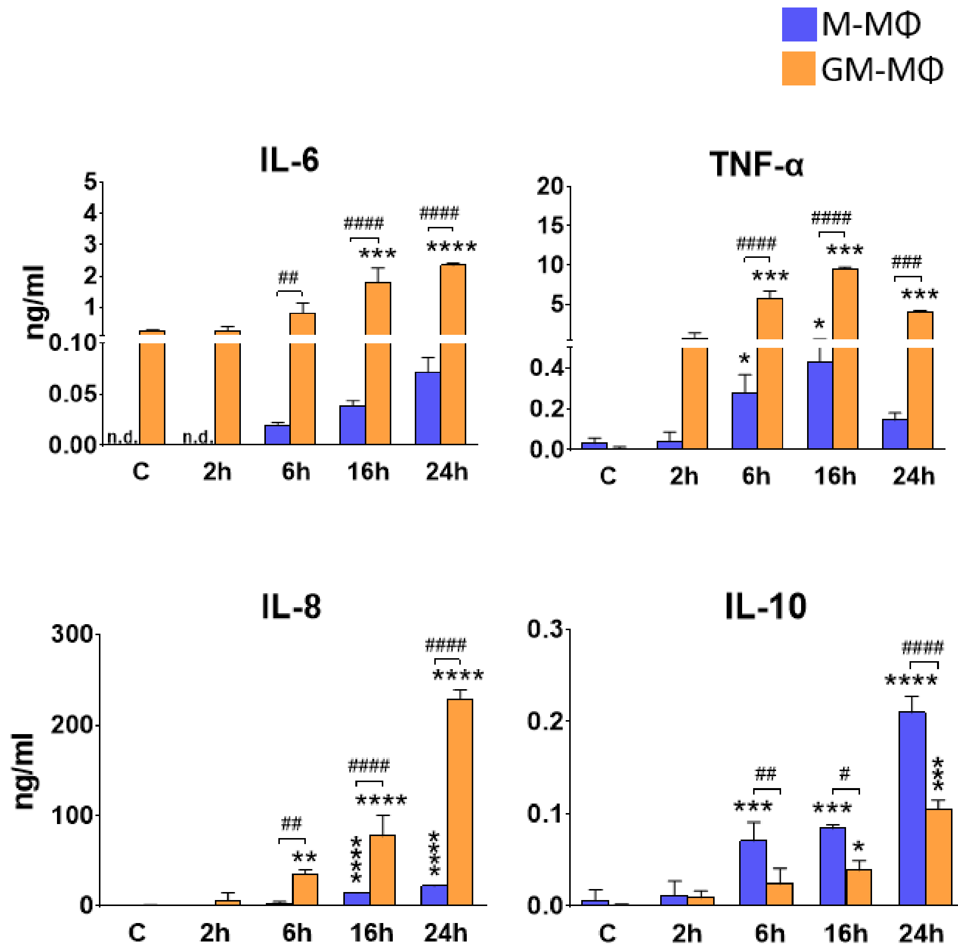


Figure 14. ATRA mediates metabolic changes in LPS-primed MΦs. *The cells were pre-treated with or without ATRA, then primed with LPS for 6 h, and subsequently subjected to the mitochondria stress test using a Seahorse XF96 Analyzer. (**A**) Real-time kinetics measurement of the oxygen consumption rate (OCR) during sequential treatment with oligomycin (Oligo), carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and antimycin A + rotenone (A+R). Representative results are shown. Bar graphs represent the calculated basal and maximal OCR, and ATP-coupled respiration and spare respiratory capacity. (**B**) Real-time kinetics measurement of the extracellular acidification rate (ECAR) after sequential treatment of glucose (Glu), oligomycin (Oligo), and 2-deoxyglucose (2-DG). Representative results are shown. Bar graphs represent calculated ECAR and glycolytic capacity obtained from the glycolytic stress test. Wave Desktop software was used for data analysis. (**C**) Relative gene expression of HK2 was measured by qPCR. The expression was normalized to the reference gene (human cyclophilin) expression. (**D**) MΦs were pretreated with 3-bromopyruvate (3BP) (80 µM) 1 h before LPS priming and subsequently incubated with ATP (5 mM) for 45 min., and then cell culture supernatants were collected, and the secretion of IL-1β was assessed by ELISA. C, control (mock-treated cells). All results are shown as means ± SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). +, −, presence or absence of indicated substance, respectively.*

6.8. NOD1 differently activates human MΦ subpopulations

Once stimulated, NOD1 undergoes a conformational change and recruits RIPK2, resulting in activation of downstream signaling cascades that induce the secretion of various inflammatory cytokines (151). To investigate the effect of NOD1 activation on cytokine secretion by the human monocyte-derived MΦ subpopulations; the cells were stimulated with NOD1-specific activator (C14-Tri-LAN-Gly), and the time dynamics of cytokine secretion were assessed from cell culture supernatants using ELISA method. Our results showed time-dependent variations in the cytokine secretion in MΦ subpopulations, in addition significant differences in the quantities of tested

cytokines between M-MΦs and GM-MΦs were observed (Figure 15) (152). IL-6 secretion gradually increased with time that reaching a peak at 24 h, and the peak of TNF-α secretion was at 16 h in both subpopulations. Furthermore, increased dynamic of IL-8 chemokine secretion was found to peak at 24 h after NOD1 stimulation. Nevertheless, GM-MΦs significantly exhibited higher cytokine secretion compared to M-MΦs in the tested cytokines. Surprisingly, although both MΦ subpopulations had the same dynamic pattern of anti-inflammatory IL-10 release, M-MΦs significantly showed higher IL-10 secretion compared to GM-MΦs. We also found that IFNβ shared the same tendency of IL-10 secretion, but M-MΦs secreted significantly less IFNβ than GM-MΦs. These results suggest that M-MΦs and GM-MΦs exhibit a different profile of cytokine secretion under NOD1 agonist treatment.



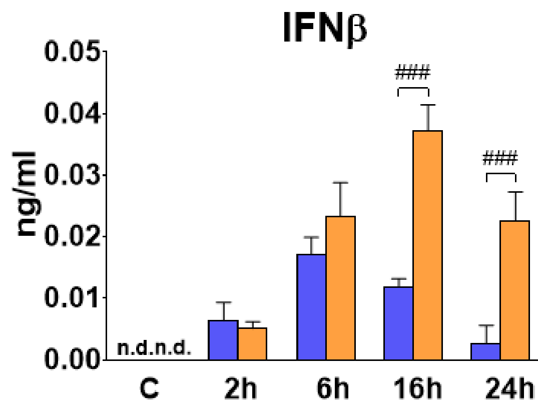


Figure 15. Time-kinetics of cytokine secretion of IL-6, TNF-α, IL-8, IL-10 and IFNβ following NOD1 activation. *M-MΦ and GM-MΦ were treated with C14-Tri-LAN-Gly (NOD1 agonist, 500 ng/ml) for the indicated time points. Control cells were treated with the same amount of vehicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

6.9. NOD1-induced cytokine secretion is differently modified by ATRA in the MΦ subpopulations

Next, we investigated whether ATRA modulates the cytokine secretion of MΦ subpopulations under NOD1 challenge. We therefore pretreated the cells with ATRA followed by NOD1 stimulation for 6 h and 24 h, and the cytokine secretion levels were measured using ELISA. Our results showed that ATRA significantly downregulated IL-6 secretion in both NOD1-stimulated MΦ subsets, however combined treatment of ATRA/NOD1 resulted in significantly increased TNF-α secretion in M-MΦs, while decreased IL-6 secretion was observed in GM-MΦs compared to only NOD1-treated cells (Figure 16). Surprisingly, in contrast, IL-8 production was differently affected upon combined treatment of ATRA/NOD1; while IL-8 release was significantly attenuated in M-MΦs, the secretion of this cytokine was upregulated in GM-MΦs. Similar results were obtained by examining the production of IL-10 and IFNβ; ATRA/NOD1 combination treatment resulted in significantly lower IL-10 and IFNβ secretion than NOD1 agonist alone in M-MΦs, and significantly higher secretion of these cytokines in GM-MΦs. Notably, treatment with ATRA alone did not affect the secretion of any tested cytokines compared to the

control group. Together, these findings suggest that ATRA differently modulates the release of pro- and anti-inflammatory cytokines of NOD1-activated MΦ subpopulations.

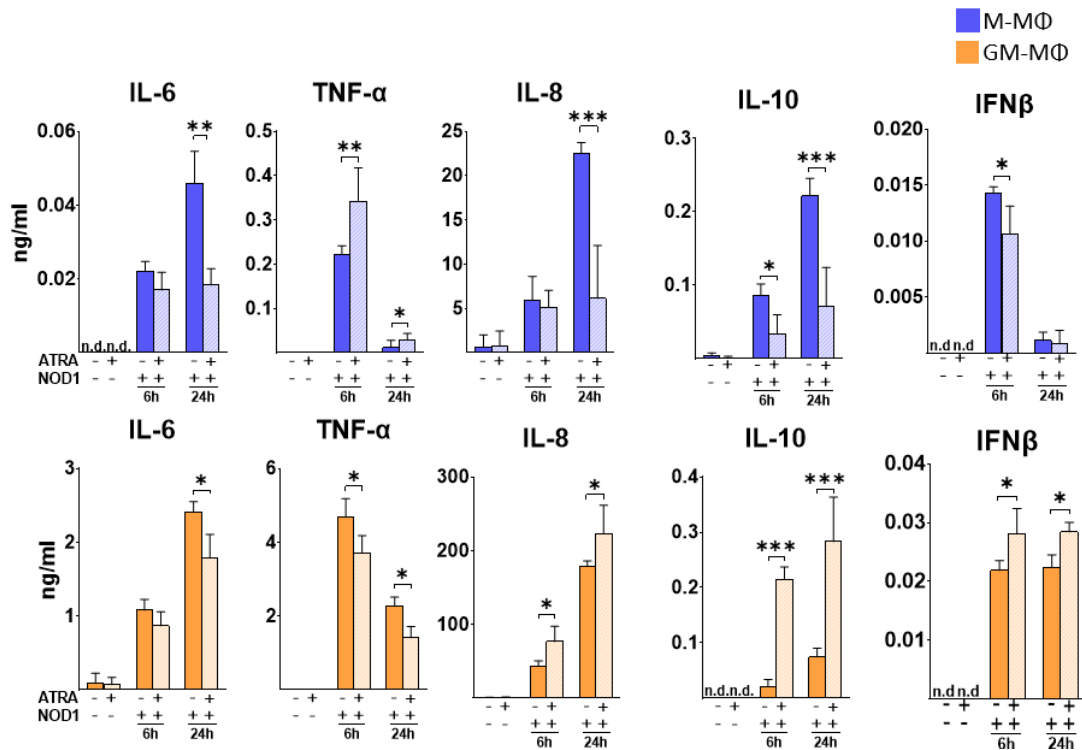
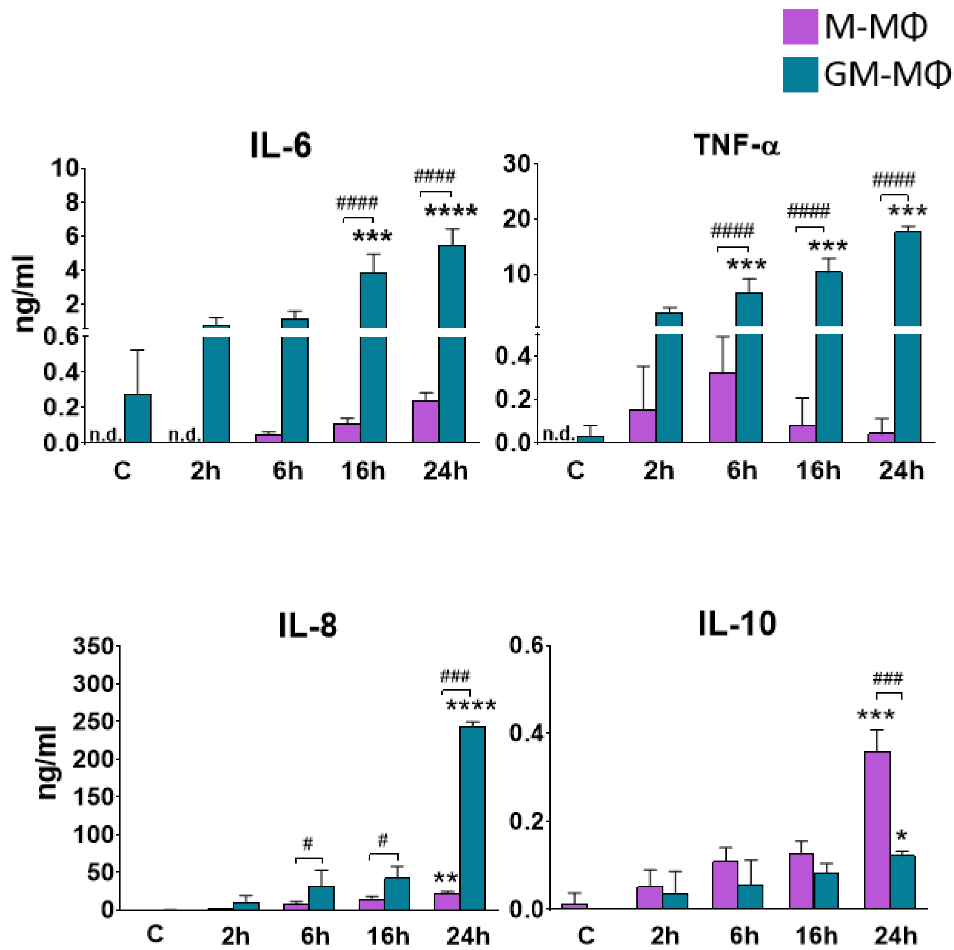


Figure 16. ATRA effect on the secretion of IL-6, TNF-α, IL-8, IL-10 and IFNβ following NOD1 activation. *M-MΦ and GM-MΦ were pretreated with ATRA (1µM) for 4 hours then stimulated with C14-Tri-LAN-Gly (NOD1 agonist, 500 ng/ml) for 6 and 24 hours. Control cells were treated with the same amount of vehicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

6.10. NOD2 induces cytokine secretion by different human MΦ subpopulations with a similar tendency as NOD1

Although NOD1 and NOD2 share a common structure, they recognize different patterns of peptidoglycan (PGN), while NOD1 senses iE-DAP that is predominantly found in Gram-negative bacteria, NOD2 senses MDP motif of both Gram-positive and Gram-negative bacteria (153). Therefore, we sought to examine the effect of NOD2 stimulation on the cytokine secretion profiles of both M-MΦs and GM-MΦs. To investigate this, cells were activated with NOD2 agonist L18-MDP and time-dependent cytokine changes were evaluated from cell culture supernatant using ELISA (Figure 17). The results showed that GM-MΦs secreted significantly

higher levels of IL-6, TNF-α and IL-8 compared to M-MΦs. The secretion of these cytokines was significantly increased and reached a peak at 24 h in NOD2-treated GM-MΦs. However, moderate secretion of IL-6 and TNF-α was observed in NOD2-treated M-MΦs, while IL-8 peaked significantly at 24 h. The secretion of IL-10 was significantly higher in M-MΦs than GM-MΦs under NOD2 activation, similar to results obtained from NOD1-activated MΦs. A gradual increase of IFNβ release was observed in both NOD2-treated MΦ subpopulations that peaked at 24 h; nevertheless, the secretion of IFNβ at 24 h was significantly higher in GM-MΦ than M-MΦ under NOD2 activation. These results indicate that NOD2 stimulation affects the cytokine secretion profiles of M-MΦs and GM-MΦs, consistent with the results obtained with NOD1 stimulation.



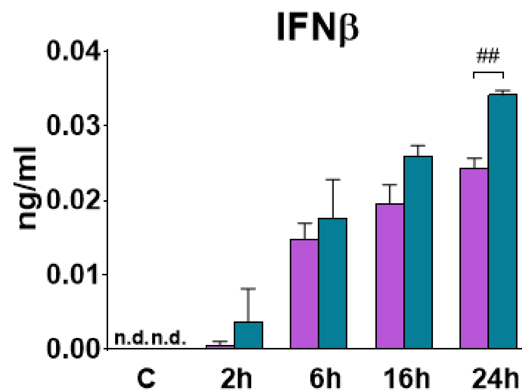


Figure 17. Time kinetics of cytokine secretion of IL-6, TNF-α, IL-8, IL-10 and IFNβ following NOD2 activation. *M-MΦ and GM-MΦ were treated with L-18 MDP (NOD2 agonist, 100 ng/ml) for the indicated time points. Control cells were treated with the same amount of vehicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

6.11. In M-MΦs, ATRA differently modifies NOD2-induced IL-8 secretion compared to induction by NOD1

Next, we investigated whether ATRA affects the cytokine secretion of MΦ subpopulations under NOD2 activation. Treatment with ATRA significantly downregulated IL-6 in both NOD2-activated MΦs. TNF-α secretion was significantly increased in M-MΦs and decreased in GM-MΦs under combined treatment of ATRAR/NOD2 compared to only NOD2-stimulated cells (Figure 18). Opposite effect was observed for IL-10 secretion, while ATRA significantly attenuated NOD2-mediated IL-10 release in M-MΦs, the secretion of this cytokine was upregulated in GM-MΦs. Of note, these results are similar to the results obtained from NOD1-activated MΦs. However, surprisingly, NOD2-mediated IL-8 secretion was significantly enhanced in both MΦs subpopulations by ATRA treatment, opposite to the results obtained from NOD1-treated M-MΦs. Furthermore, similar to IL-10, ATRA significantly attenuated NOD2-mediated IFNβ secretion in M-MΦs, while significantly enhanced NOD2-mediated IFNβ secretion in GM-MΦs. These results indicate that the modulatory effect of ATRA on the cytokine secretion profiles of MΦs subpopulation is affected by the activated NOD.

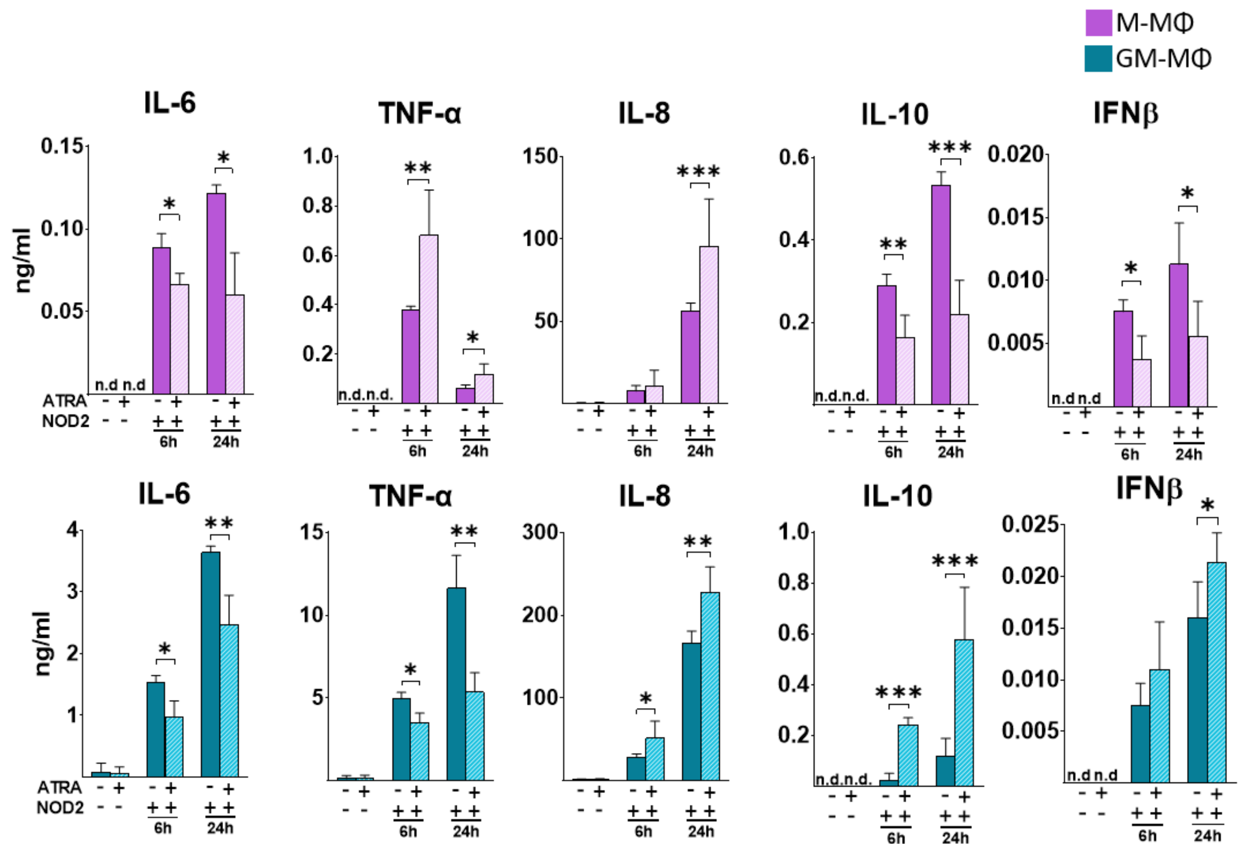


Figure 18. ATRA effect on the secretion of IL-6, TNF-α, IL-8, IL-10 and IFNβ following NOD2 activation. *M-MΦ and GM-MΦ were pretreated with ATRA (1µM) for 4 hours then stimulated with L-18 MDP (NOD2 agonist, 100 ng/ml) for 6 and 24 hours. Control cells were treated with the same amount of vehicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

6.12. ATRA differently modifies NOD1 ligand-induced IL-1β and IL-18 secretion in the human MΦ subpopulations

Unlike other cytokines, the precursors of IL-1β and IL-18 remain in inactive forms within the cytosol, and require proteolytic cleavage by caspase enzymes for their maturation. Both IL-1β and IL-18 are proinflammatory cytokines, and have been reported to be involved in the NOD1-mediated immune response (104). Thus, we sought to investigate whether NOD1 activation mediates the secretion of these cytokines in MΦs subpopulations. To achieve this, cells were induced by NOD1 for different time points, and the cell culture supernatants were subjected to ELISA measurement. We found that NOD1 induced a significant level of IL-1β release in GM-MΦs that peaked at 6 h, while mild IL-1β release was observed in NOD1-induced M-MΦs.

Nevertheless, comparable levels of IL-18 release were detected between the two NOD1-activated MΦ populations, and the peak of IL-18 release was at 16 h in both MΦs (Figure 19A). However, surprisingly, ATRA treatment significantly upregulated IL-1β and IL-18 secretion in NOD1-activated M-MΦs, while significantly attenuated the secretion of these cytokines in NOD1-activated GM-MΦs (Figure 19B). These results suggest that NOD1 induces IL-1β and IL-18 secretion in human MΦ subpopulation, and ATRA is capable to modulate this secretion.

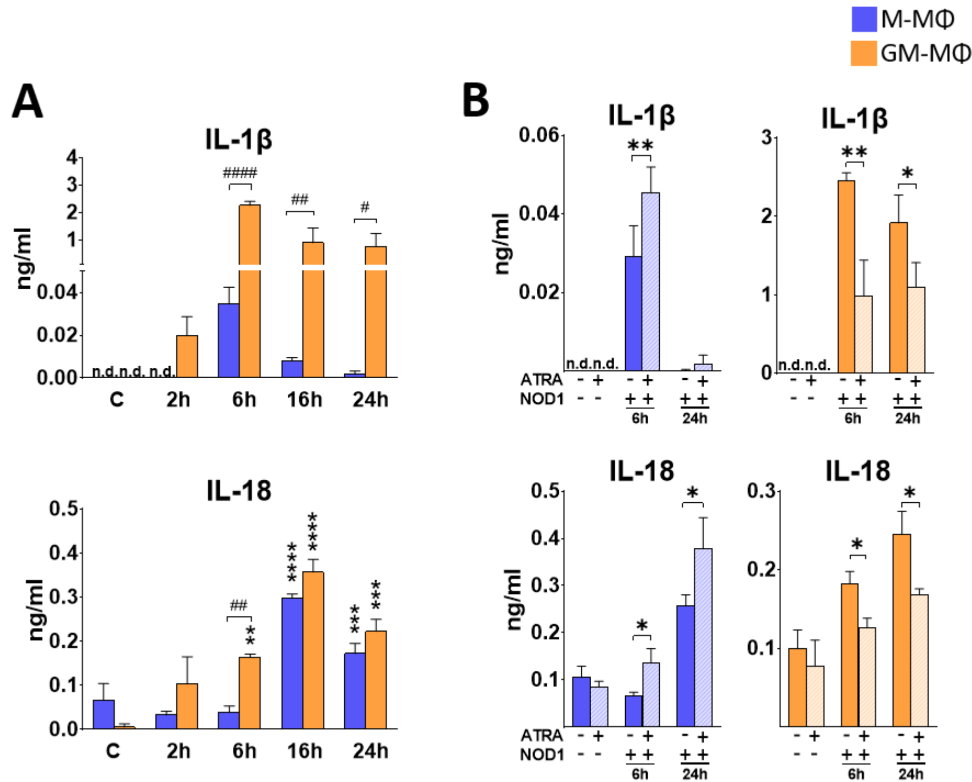


Figure 19. Secretion of IL-1β and IL-18 following NOD1 activation. *M-MΦ and GM-MΦ were treated with C14-Tri-LAN-Gly (NOD1 agonist, 500 ng/ml) for the indicated time points. Control cells were treated with the same amount of vehicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. (**A**) Time-kinetics of cytokine secretion. (**B**) Cells were pretreated with ATRA (1μM) for 4 hours and then stimulated with NOD1 agonist for 6 and 24 hours. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

6.13. NOD2-induced IL-1β and IL-18 secretion are differently modified by ATRA in the human MΦ subpopulations

Next, we investigated whether IL-1β and IL-18 secretion is induced by activation of NOD2 in MΦs subpopulations. As expected, NOD2 stimulation resulted in a significant release of IL-1β

in GM-MΦs, that reached a peak at 24 h, while IL-1β secretion in M-MΦs was slightly detectable, similar to the results obtained from NOD1-activated MΦs. IL-18 secretion was also inducible by NOD2 activation, the secretion peak for M-MΦs was at 24 h, while for GM-MΦs was at 16 h (Figure 20A). In addition, ATRA treatment resulted in upregulated levels of both IL-1β and IL-18 cytokines in NOD2-activated M-MΦs, however the secretion of these cytokines was downregulated by ATRA in NOD2-activated GM-MΦs, similar to the results obtained from NOD1-activated MΦs (Figure 20B). These results indicate that IL-1β and IL-18 secretion are inducible in NOD2-activated MΦs, and ATRA is capable to modulate this secretion in the same manner as NOD1-activated MΦs.

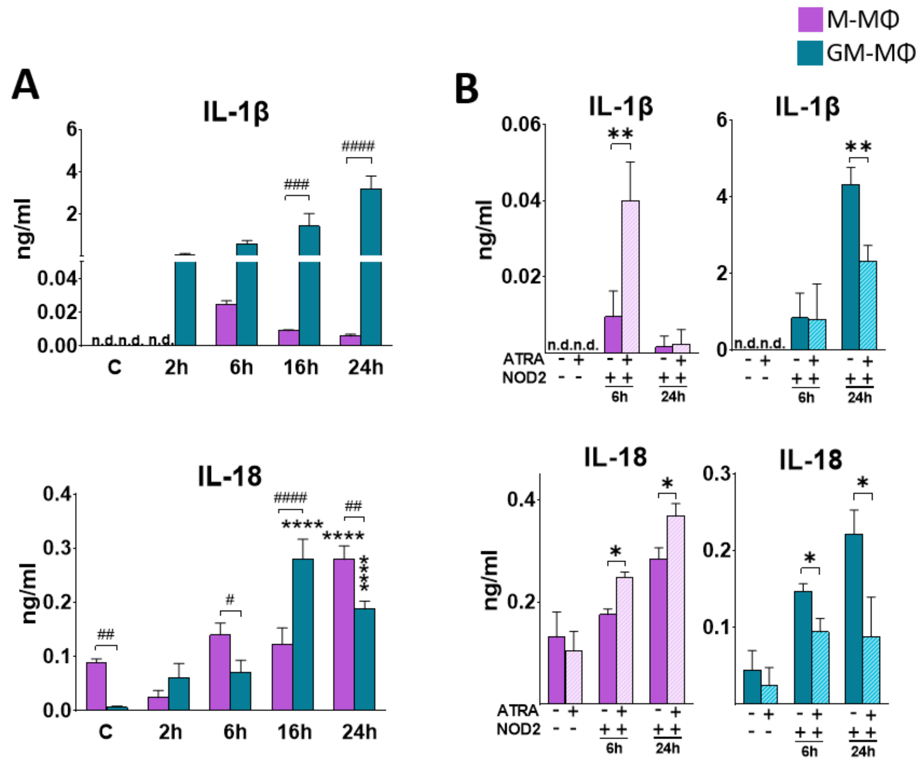


Figure 20. Secretion of IL-1β and IL-18 following NOD2 activation. *M-MΦ and GM-MΦ were treated with L-18 MDP (NOD2 agonist, 100 ng/ml) for the indicated time points. Control cells were treated with the same amount of vechicle as the activated cells. Cytokine secretion was measured from the supernatant using ELISA. (**A**) Time-kinetics of cytokine secretion. (**B**) Cells were pretreated with ATRA (1µM) for 4 hours and then stimulated with NOD2 agoinst for 6 and 24 hours. All results are shown as means ± SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; n.d. – not detected).*

7. Discussion

Macrophages (MΦs) are heterogenous and multifunctional innate immune cells, that play a key role in host immune defense, surveillance and maintain tissue hemostasis. To monitor the surrounding environment, MΦs express a wide range of sensing receptors, such as PRRs, cytokine receptors and nuclear hormone receptors, that drive MΦs polarization and their effector functions following activation. In response to environmental cues, MΦs adopt a wide spectrum of activation states ranging from classically activated to alternatively activated MΦs, that develop distinctive functional programs, including phagocytosis, antigen presentation, tissue repair and regeneration, and production and release of various cytokines and chemokines (128,154). ATRA is the most physiologically active form of vitamin A, which arises as tissue-derived signal involved in the regulation of various immune cells, including lymphocytes, DCs and MΦs (127,155). Besides its role as an endogenous agonist for nuclear receptors as ligand-activated transcription factors, ATRA is also believed to function by interacting with several cellular signal transduction pathways (123). Specifically, since ATRA presents in the local tissue microenvironments, it has been involved in maintaining and regulating various functions of MΦs, in context- and stimulus-dependent manners (127,130). Importantly, ATRA has been reported to modulate the secretion of IL-1β in both primary MΦs and myeloid cell lines (156–159). However, while NLRP3 inflammasome is involved in IL-1β secretion in myeloid cells, the possible effect of ATRA on the NLRP3 inflammasome and its associated mechanisms has not been characterized. Therefore, in our study, we aimed to investigate the potential modulatory role of ATRA on NLRP3 inflammasome-mediated IL-1β production in human MΦs. Furthermore, the effect of ATRA on other NLR-mediated inflammatory responses has not been addressed before. Thus, we extended our investigations to studying the potential effects of ATRA on NOD1 and NOD2-mediated responses in two different MΦs subpopulations. In our current research, we used two distinct human MΦ subtypes, a widely accepted model to study MΦs polarization and functions, where primary monocytes are differentiated by M-CSF or GM-CSF cytokines to generate M-MΦs or GM-MΦs that exhibit proinflammatory or anti-inflammatory characteristics, respectively.

Our data reveal that ATRA upregulates the secretion of proinflammatory cytokines IL-1β and IL-6 in LPS-treated M-MΦs. IL-1β is a crucial proinflammatory cytokine and implicated in various inflammatory and physiological events. Nevertheless, the production of IL-1β in LPS-primed MΦs is a tightly regulated process and driven by activating stimuli of NLRP3

inflammasome (83). Our results revealed that ATRA upregulates NLRP3 inflammasome-mediated subsequent production of IL-1β in LPS-primed M-MΦs through modulating both the priming and the activation events of NLRP3 inflammasome. We found that ATRA alone induces the expression of NLRP3 sensor, furthermore ATRA enhances the expression of both NLRP3 and IL-1β in LPS-primed M-MΦs. To directly regulate gene transcription, ATRA mediates its function through RAR, a ligand-dependent transcription factor that binds to RAR-response elements (RE) in the target genes (160). In line with other reports, our sequence analysis of the NLRP3 promoter region has revealed the presence of potential binding sites for RXR heterodimers, such as DR1 or DR2 elements (161). Furthermore, previous reports have identified several putative binding motifs in *NLRP3* and *IL-1β* promoters for other nuclear receptors, such as PXR and REV-ERB, that regulate these genes at the transcription level (162–164). In agreement with our findings, after our publication, it has been reported that in Kupffer cells, ATRA induces IL-1β release through promoting transcriptional priming of NLRP3 inflammasome in a RAR-dependent manner. In addition, ATRA mediates excessive accumulation of ROS, leading to further activation of NLRP3 inflammasome (165). Thus, our results suggest that ATRA may directly induce the expression of *NLRP3* via RAR- or PPAR-dependent gene regulation, however this requires further complex genomic studies.

Nevertheless, non-genomically, ATRA also modulates cytoplasmic signaling pathways in receptor-dependent and -independent mechanisms. Previous studies have reported that ATRA is capable of directly inducing non-canonical modulation of protein kinases such PI3K/Akt and MAPKs pathways (123,142). In this study, we have shown that ATRA alone enhances Erk signaling, moreover ATRA mediates upregulation of NF-κB, Erk and JNK pathways in LPS-treated M-MΦs, however, p38 signaling is downregulated in both cases. These findings raise the possibility that ATRA effects are mediated via interaction with TLR4-triggered signaling, that leads to the enhanced priming event of NLRP3 inflammasome. Previously, our group, along with others, has already reported the importance of these signaling pathways in regulating NLRP3 inflammasome-mediated cytokine secretion (139,166). Nevertheless, further studies are needed to elucidate potential mechanisms involved in detail.

Although the priming event is required to induce the expression of the NLRP3 inflammasome components and licensing the sensor protein, the assembly and activation of NLRP3 inflammasome is triggered by a second signal which includes a wide range of microbial

and host-derived danger stimuli (167). To avoid any detrimental effect of excessive NLRP3 inflammasome-induced responses, myeloid phagocytes activate Akt/mTOR signaling to limit proinflammatory responses and production of proinflammatory cytokines (145,147). Here, we found that ATRA significantly downregulates Akt/mTOR signaling in the LPS-activated M-MΦs. A previously published study has suggested that Akt inhibits NLRP3 and ASC oligomerization, therefore preventing excess inflammatory cytokine production in MΦs (168). Importantly, in myeloid cells, Akt/mTOR signaling limits the activity of NF-κB signaling and caspase-1-mediated IL-1β maturation, and functions as a positive regulator for the production of IL-10 anti-inflammatory cytokine through STAT3 pathway, providing a feedback loop to control excessive inflammation (146,147). In line with this, our findings showed a downregulation of STAT3 phosphorylation and attenuated IL-10 secretion following ATRA treatment in the LPS-primed M-MΦs. Mechanistically, we found that treatment with exogenous IL-10 results in abolished ATRA-enhanced IL-1β secretion in LPS-primed MΦs. Notably, in certain types of malignant tumors, targeting mTOR as a negative regulator for proinflammatory and inducer for anti-inflammatory response has shown promising results in advanced clinical trials (169). Our results suggest that ATRA negatively modulates Akt/mTOR/STAT3 pathway, leading to boost NLRP3-mediated IL-1β secretion via modulating IL-10-derived feedback inhibition in LPS-primed M-MΦs.

MΦs exhibit high metabolic plasticity in response to various microenvironmental cues, enabling them to meet the energy demands effectively. For instance, activated MΦs undergo a rapid metabolic shift toward glycolysis, while alternatively activated MΦs are characterized by increased mitochondrial respiration (170). Several metabolic pathways, and their associated enzymes and metabolites have been reported to differentially regulate NLRP3 inflammasome (171). Specifically, accumulating evidence suggests that the glycolysis pathway is a critical regulator for NLRP3 inflammasome activity, however detailed mechanisms underlying the interaction between glycolytic cascade/glycolytic flux and NLRP3 inflammasome are still not clear (81,171). In our study, we have shown that ATRA upregulates glycolysis and the expression of HK2 in LPS-primed M-MΦs, and targeting HK2 with an inhibitor results in attenuation of IL-1β production.

Previous report has shown that ATRA activates the aerobic glycolysis pathway and reduces OxPhos-dependent ATP production, associated with modulation of a set of metabolic reprogramming genes in NB4 cell line (172). Furthermore, pharmacological inhibition of HK2

activity attenuates LPS-induced IL-1β production in RAW MΦs cell line (173). Consistently, targeting pyruvate kinase isoform M2 (PKM2) enzyme that catalyzes the final reaction in the glycolytic pathway, leads to a decrease in the activation of NLRP3 and AIM2 inflammasomes-mediated IL-1β release (174). Importantly, IL-10 has been shown to limit the glycolytic flux and inhibit the expression of several genes in the glycolytic pathway, including *Hk1*, *Hk3* and *Pfkp* in LPS-stimulated BMDMs (175). Based on our results and the available reports, we suggest that ATRA mediates enhanced glycolysis, promoting NLRP3 inflammasome activation and subsequently IL-1β release in LPS-primed M-MΦs.

While the function of NLRP3 inflammasome is highly regulated by signaling pathways, but NLRP3 itself has no direct effect on cellular signaling. Since our results clearly indicated that ATRA modifies LPS-activated signaling, we wanted to see whether signaling pathways activated by regulatory NLRs are affected by ATRA. The regulatory NLRs induce and modulate several signaling pathways; like NOD1 and NOD2 that upon activation by intracellular bacterial PGN bind scaffolding kinase protein RIPK2 and facilitate the activation of downstream NF-κB and MAPKs signaling pathways. These events trigger various host inflammatory responses, including secretion of cytokines/chemokines, in addition to NOD1/NOD2-dependent type I IFN response. Furthermore, NOD1/NOD2 have also been reported to induce caspase-1-mediated IL-1β and IL-18 processing through direct CARD-CARD interaction, or indirectly via triggering other inflammasome-forming NLRs (104,176,177).

In our study, we showed that the activation of M-MΦs and GM-MΦs with NOD1 and NOD2 agonists results in a characteristic pattern of cytokine secretion. Our comparative analysis reveals that GM-MΦs exhibit a higher secretion profile of proinflammatory cytokines compared to the M-MΦs in response to NOD1/NOD2 stimulation. However, comparable levels of IL-18 and IL-10 secretion between the two MΦ subpopulations were detected. These findings highlight the role of MΦ polarization states in response to inflammatory stimuli, besides that NOD1/NOD2-mediated cytokine secretion is another factor that may shape the MΦ subtypes functions and their interaction with the surrounding microenvironment, particularly the adaptive immune cells. We have previously reported that following treatment with LPS, a potent inducer of inflammation, GM-MΦs induces high levels of proinflammatory cytokines, while M-MΦs display high induction of IL-10 secretion. Furthermore, among the induced cytokines, IL-8 is the most highly secreted cytokine in LPS-activated MΦ subtypes (139,178). Surprisingly, we detected high induction of IL-

8 secretion triggered by NOD1/NOD2, similar to the results previously obtained from LPS-stimulated MΦs (139). IL-8 is a potent proinflammatory chemokine that functions as a neutrophil chemoattractant and a mediator for angiogenesis (179). It has been reported that IL-8 directs neutrophils chemotaxis to the inflammatory site at low concentration, while at high concentration it drives neutrophils to release neutrophil extracellular traps (180,181). Based on our results and the available reports in the field, we hypothesize that NOD1/NOD2-activated MΦ populations are involved in neutrophil chemotaxis, and may have the capability to promote neutrophil-mediated NET formation, particularly in NOD1/NOD2-activated GM-MΦs.

In line with our previous findings, we found that ATRA is also capable to modulate NOD1/NOD2-mediated cytokine secretion in the two different MΦ subpopulations. Here we found that although ATRA downregulated IL-6 in both MΦ subsets, opposite effects of ATRA were observed in the proinflammatory TNF-α, IL-1β, IL-18 and the anti-inflammatory IL-10 secretion between M-MΦs and GM-MΦs following NOD1/NOD2 stimulation. Importantly, IL-8 was differently regulated by ATRA in both MΦ subsets upon NOD1 activation, while ATRA enhanced this cytokine in NOD2-induced both MΦ subpopulations.

In MΦs, the release of cytokines has been reported to be involved in autocrine and paracrine signaling to activate or inhibit cellular functions, including the secretion of other cytokines (182). For instance, IFNβ can positively regulate IL-10, while both IFNβ and IL-10 are negative regulators of caspase-1-dependent IL-1β and IL-18 production (183–185). Accordingly, the negative correlation observed in our results between IFNβ/IL-10 and IL-1β/IL-18 under ATRA treatment may be attributed to autocrine/paracrine mechanisms. Furthermore, it has been reported that ATRA regulates the expression of IFNβ, IFNβ receptor and IRF1 in response to viral infection (186). IRF1 is an upstream regulator of IRF3, which is a main inducer of IFNβ expression. IRF1 either targets the phosphorylation or facilitates chromatin accessibility for IRF3 (187,188). Furthermore, ATRA has been shown to upregulate IL-8 gene expression by enhancing IL-8 promoter activity in a RARα-dependent manner. Several reports, including ours, previously showed that ATRA has context- and cell-dependent effects on the expression of TLRs (189–191), RIG-like helicases (RLHs) (134,192) and NLRP3 (193). However, in this current study, we didn’t detect changes in the mRNA expression of NOD1/NOD2 (data not included). Altogether, these suggest that ATRA functions through nuclear receptors may indirectly affect the NOD1/NOD2-

mediated responses. Nevertheless, further detailed mechanisms need to be studied, particularly ATRA-induced engagement of IRF1 in IFNβ secretion.

As we previously mentioned, ATRA function is extended to non-genomic effects through interaction with various signal transduction pathways. ATRA has been reported in several contexts to differentially modulate NF-κB pathway, PI3K/Akt and MAPKs signaling (such as Erk, JNK and p38) (101,122,194–196). Importantly, many of these pathways overlap with those that are also affected by NOD1/NOD2 activation. Besides, in line with our previous data, ATRA is capable to drive the cellular metabolism toward glycolysis, which is linked to MΦs function (14,172,197). These findings, along with our previous data raise the possibility of cross-talk mechanisms, and suggest that ATRA may modulate the downstream signaling of activated NOD1/NOD2 and/or alter the metabolic status of the MΦs (Figure 21).

In conclusion, our data demonstrate that the nuclear receptor agonist ATRA is capable to modulate the function of the investigated NLRs in human MΦs. We suggest that this effect of ATRA can be attributed to a complex interaction of this molecule with several cellular mechanisms, including signaling pathways, gene transcription and metabolism. This raises the possibility that ATRA may also be involved in the regulation of other NLR-mediated pathways. Previous reports have linked the dysregulated NLRs, specifically NLRP3, NOD1 and NOD2, with a wide range of inflammatory and autoinflammatory disorders (177,198). In line, vitamin A deficiency can lead to increased susceptibility to various infectious and inflammatory diseases such as tuberculosis, malaria, asthma and colitis (155,199). For this reason, our results highlight the potential of ATRA and ATRA-mediated signaling as a potential therapeutic strategy to target NLRP3, NOD1 and NOD2-associated diseases.

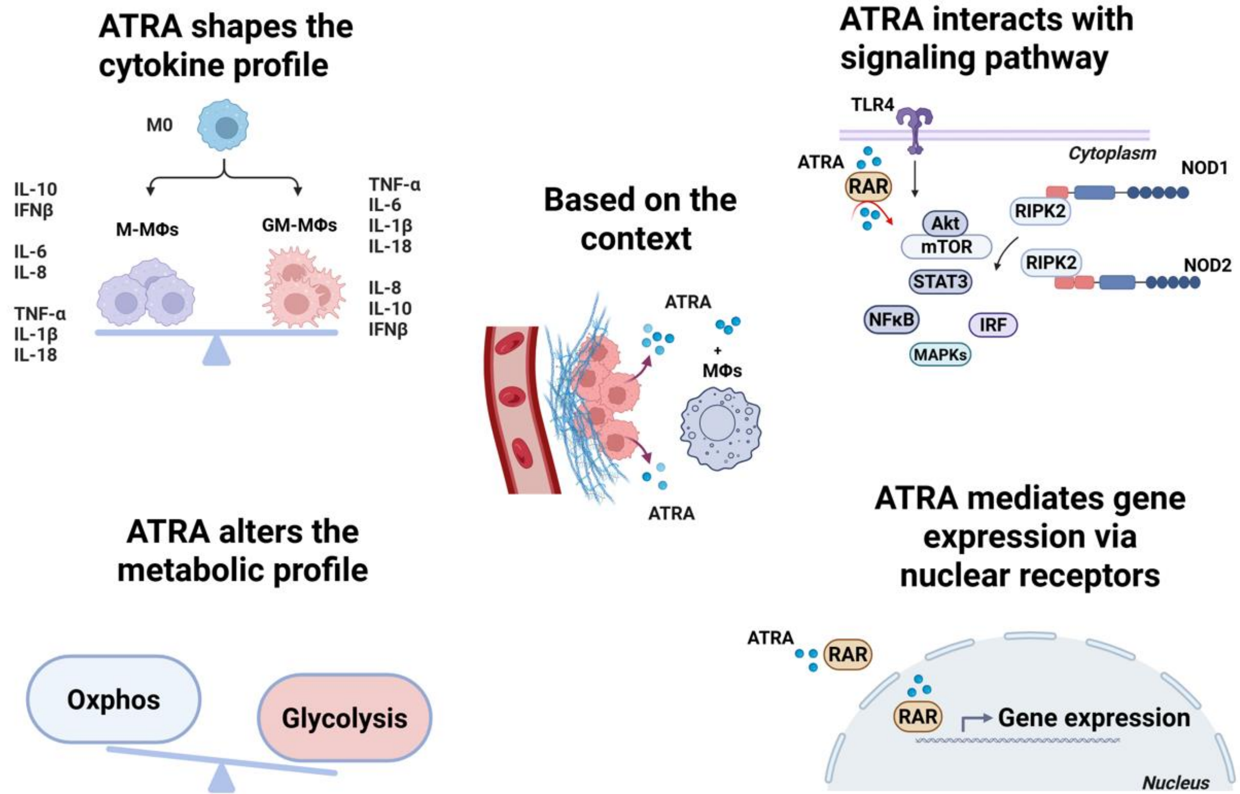


Figure 21. Proposed model for the effects of ATRA. *ATRA can modulate the MΦ cytokine profile through several mechanisms, including signaling pathways, gene expression and metabolism. Created in BioRender.com.*

8. Summary

Macrophages (MΦs) are diverse population of innate immune cells that phenotypic and functional properties are determined by thier tissue-microenvironment and developmental origin. MΦs depend on PRRs to recognize various self and non-self-derived stimuli and subsequently initiate inflammatory responses for host defense and homeostasis. Upon activation, PRRs trigger a series of signaling events and transcriptional programs to regulate the MΦs associated function, including the production of cytokines and chemokines. In certain circumstances, a number of PRRs, particularly NLRs, can trigger the formation of a multi-protein complex called inflammasome. The inflammasome is required for activation of caspase-1, subsequently leading to the maturation of IL-1β and IL-18 cytokines. ATRA unequally presents in the tissues/organs and has an important modulatory role in immune responses. ATRA has been implicated in the immune maturation and tolerance of adaptive immunity, besides its role in the functional polarization and activation of MΦs. In the present study, we used human (MΦs differentiated in the presence of GM-CSF or M-CSF to generate inflammation inducing (GM-MΦs) or inflammation resolving (M-MΦs) cells, respectively. These MΦ subpopulations are commonly used models to study the MΦs activities and cellular responses. We aimed to investigate the potential modulatory effect of ATRA on MΦs upon activation of NLRs, including NLRP3 NOD1 and NOD2. In first part, in M-MΦ subset, our results show that ATRA treatment significantly modulates both the priming and the activation of NLRP3 inflammasome of LPS-activated cells. ATRA enhances the expression of NLPR3 and pro-IL-1β, alters TLR4-mediated signaling, and shifts the metabolism toward glycolysis, that, in part, augments NLRP3 inflammasome activity. In the second part, we conducted a comparative analysis on the NOD1- and NOD2-induced cytokine release under ATRA treatment, in both M-MΦ and GM-MΦ subsets. Our results show that the activation of NOD1 or NOD2 in the two MΦ subpopulations results in different patterns of cytokine release. Furthermore, treatment with ATRA differently modulates cytokine secretion triggered by NOD1 and NOD2 in MΦ subsets. Together, our data indicate that ATRA modulates NLRP3 inflammasome activation, and the cytokine secretion in human MΦs upon targeting regulatory NLRs. The effects of ATRA are highly context-dependent and our results highlight the importance of ATRA as tissue derived signal in shaping MΦ functions. Our results may hold therapeutic promise for conditions where MΦs-associated inflammatory conditions/diseases need to be regulated.

9. Key words

Macrophages, Retinoic acid, Vitamin A, NOD-like receptor, NLRP3 inflammasome, NOD1, NOD2, Cytokine, Signaling pathway, Metabolism

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12. List of publications

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Registry number: DEENK/485/2025.PL
Subject: PhD Publication List

Candidate: Ahmad Alatshan
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Ahmad, H., **Alatshan, A.**, Bíró, E., Benkő, S.: Retinoic acid differently modulates NOD1/NOD2-mediated inflammatory responses in human macrophage subsets.
   Front. Immunol. 16, 1-11, 2025.
   DOI: http://dx.doi.org/10.3389/fimmu.2025.1609763
   IF: 5.9 (2024)

2. **Alatshan, A.**, Kovács, G. E., Aladdin, A., Czimmerer, Z., Tar, K., Benkő, S.: All-Trans Retinoic Acid Enhances both the Signaling for Priming and the Glycolysis for Activation of NLRP3 Inflammasome in Human Macrophage.
   Cells. 9 (7), 1591, 2020.
   DOI: http://dx.doi.org/10.3390/cells9071591
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List of other publications

3. Tóth, K., Lénárt, N., Berki, P., Fekete, R., Szabadits, E., Pósfai, B., Cserép, C., **Alatshan, A.**, Benkő, S., Kiss, D., Hübner, C. A., Gulyás, A., Kaila, K., Környei, Z., Dénes, Á.: The NKCC1 ion transporter modulates microglial phenotype and inflammatory response to brain injury in a cell-autonomous manner.
   PLoS Biol. 20 (1), e3001526, 2022.
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4. Kovács, E. G., **Alatshan, A.**, Budai, M. M., Czimmerer, Z., Bíró, E., Benkő, S.: Caffeine Has Different Immunomodulatory Effect on the Cytokine Expression and NLRP3 Inflammasome Function in Various Human Macrophage Subpopulations.
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5. **Alatshan, A.**, Benkő, S.: Nuclear Receptors as Multiple Regulators of NLRP3 Inflammasome Function.
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DOI: http://dx.doi.org/10.3389/fimmu.2021.630569
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6. Csete, D., Simon, E., **Alatshan, A.**, Aradi, P., Dobó Nagy, C., Jakus, Z., Benkő, S., Győri, D., Mócsai, A.: Hematopoietic or Osteoclast-Specific Deletion of Syk Leads to Increased Bone Mass in Experimental Mice.
Front. Immunol. 10, 937, 2019.
DOI: http://dx.doi.org/10.3389/fimmu.2019.00937
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