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

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1. 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.

2. Theoretical background

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. 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 through circulation in partially polarized states, and are further shaped by the surrounding tissue microenvironment under either homeostasis or disease. 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 levels of proinflammatory cytokines, and antigen-presenting capacity. The alternatively activated MΦs (M2) exhibit anti-inflammatory activity and are involved in tissue repair, resolution of inflammation and parasitic infections, with poor antigen presentation.

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. 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. 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. According to their localization, PRRs may be grouped into membrane-bound receptors, such as Toll-like receptors (TLRs), or intracellular cytosolic receptors including nucleotide-binding domain leucine-rich repeat receptors (NLRs). TLRs are a family of transmembrane receptors, located in cell membrane and other membranes of intracellular compartments, such as the 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.

Plasma membrane TLRs are specialized to recognize distinct microorganisms-derived components, for instance TLR4 detects bacterial lipopolysaccharide (LPS). TLR4 is the most extensively investigated receptor among the TLRs. Besides being a potent sensor for bacterial, TLR4 this receptor can also recognize DAMPs derived from extracellular matrix molecules or intracellular factors. 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. Upon ligation of TLR4, the intracellular TLR4 domain recruits several downstream adapter molecules that trigger distinct signaling cascades to mediate gene transcription or activate other cellular functions involved in immune defense. These signaling pathways include nuclear factor-kB (NF-kB), mitogen-associated protein kinase MAPK family members, as well as Akt signaling that is involved in limiting proinflammatory cytokine secretion.

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. And importantly, number of NLRs have been described as core proteins that trigger the inflammasome formation and its subsequent events.

In response to the perturbation of cellular homeostasis, 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. Inflammasome formation requires activation of a sensor protein that recruits an adaptor called ASC. This adaptor ASC functions as a bridge between the inflammasome components, which recruits the pro-form of the cysteine protease caspase-1. The inflammasome assembly triggers autocatalytic cleavage of caspase-1. The activated caspase-1 subsequently cleaves gasdermin D which drives the formation of membrane pores, leading to inflammatory-lytic cell death called pyroptosis. In addition, caspase-1 also mediates the cleavage of pro-IL-1β and pro-IL-18 to their biologically active forms, and the matured IL-1β and IL-18 cytokines are subsequently released from the cells through the gasdermin D pores.

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 and Pyrin) that are also able to form inflammasomes. Each inflammasome formation/activation is regulated by distinct mechanisms that include different accessory proteins. Importantly, NLRP3 inflammasome is the most deeply investigated inflammasome sensor, due to its wide range of activators, and its association with several inflammatory diseases.

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. 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.

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. In addition, besides NF-κB, several other signaling pathways are involved in NLRP3 priming, such as Erk/c-Jun/AP-1 and CD36/PKCδ pathways, as well as lipid or glycolytic metabolism-mediated SREBP2 activation. After gene transcription, the inflammasome components need to be licensed for oligomerization or subsequent processes through post-translational modifications. 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, mitochondrial dysfunction, excessive ROS production, metabolic remodeling, lysosomal damage, endoplasmic reticulum stress and disrupted Golgi network integrity.

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. 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.

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. 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. This approach intends to directly inhibit the oligomerization of NLRP3 protein, to prevent the formation of the inflammasome complex. 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. Other potential therapeutic strategies include targeting NLRP3 at the transcription level via genetic and epigenetic modulation. For instance, non-coding RNAs have been reported to regulate NLRP3 inflammasome through post-transcriptional repression of NLRP3 or other inflammasome components. 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. 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.

Unlike NLRP3, some members of NLR have been reported to positively regulate signaling cascades, instead of forming inflammasome, such as NOD1 and NOD2. Following activation, NOD1 and NOD2 have been described to recruit RIPK2 kinase through CARD domains to mediate downstream signaling cascades, including NF-κB signaling, MAPK signaling and the IRF3-/IRF7-dependent expression of IFNs. NOD1/NOD2 are specialized sensors to detect intracellular bacterial peptidoglycan (PGN), however, they recognize different conserved motifs of PGN. 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.

Upon ligand binding, NOD1/NOD2 undergo conformational changes to release autoinhibition, leading to oligomerization of NOD proteins and activation of downstream signaling effectors. 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. Although NOD1/NOD2 share common

structure and signaling pathways, they differ in their activation mode and regulation at the cellular level. 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.

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, and metabolize it to retinoic acid (RA). Several RA isomers have been identified *in vivo*; however all-trans RA is the most functionally active form in the body. RA can act in an autocrine or paracrine manner in the surrounding microenvironment to drive signaling processes. RA functions through ligand-dependent transcription factors that belong to class II nuclear receptors or RXR heterodimers. RA-activated nuclear receptors and their isoforms exhibit tissue-specific expression patterns, functions that depend on the cellular contexts. 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. 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. RA is an essential component of tissue microenvironment that is necessary for immune homeostasis and proper immune functions. RA shapes the polarization of intestinal MΦ and DC subsets to drive the differentiation of naïve T cells into gut-homing T cells. However, during the inflammatory response, RA induces the polarization of proinflammatory DCs and MΦs phenotypes and promotes effector T cell differentiation, potentiating the immune responses instead of tolerogenic conditions.

Growing body of evidence suggests that RA and RAR signaling mediate MΦs polarization through regulation of several transcription factors in tissue-specific manner. 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. Importantly, the immune regulatory roles of RA have also been linked to several PRR functions (such as TLRs and RLRs).

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. 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. Several evidence suggest a link between vitamin A deficiency/ hyporetinolemia and infectious diseases (such as tuberculosis, measles, malaria, diarrhea and HIV). 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.

3. 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. 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.

4. Materials and methods

Reagents

Unless otherwise stated, all reagents used in the experiments were purchased from InvivoGen (San Diego, CA, USA).

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.

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. The PBMC layer was collected and washed with PSS and MACS buffer. Monocytes were purified from PBMCs using immunomagnetic positive selection with anti-CD14-conjugated microbeads as reported by the manufacturer's instructions.

Macrophage differentiation

The obtained monocytes were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, and 500 U/mL of penicillin-streptomycin. Finally, the suspended monocytes were cultured in 24-well plates in either 50 ng/mL M-CSF or 80 ng/mL GM-CSF, 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.

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 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.

RNA preparation

Total RNA was extracted using TriReagent in accordance with the manufacturer's instructions. The RNA quantity and quality were determined using a spectrophotometer. The isolated RNA was treated with DNase and RNase inhibitor. cDNA synthesis was achieved using random hexamers and the SuperScript II First-strand Reverse Transcriptase system.

Quantitative Real-Time PCR

For quantitative RT-PCR, Taqman Gene Expression Assays were used with the Taqman™ Gene Expression Master Mix. The amplification was performed using a QuantStudio12K Flex qPCR instrument (ABI). The relative expression values for each transcript of interest were calculated by the comparative Ct method, and human cyclophilin (Ppia) was used for normalization.

Western blot analysis

Cells were harvested and washed with PBS; directly lysed in 2X Laemmli sample buffer and boiled for 10 min. Protein lysate were separated using SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk diluted in TBS-Tween buffer. The membrane was incubated overnight at 4 °C with primary antibodies in 1:1000 dilution. After washing step, the membrane was incubated for 1 h at room temperature with a corresponding HRP-conjugated secondary Abs in 1:5000 dilution. Membrane-bound peroxidase proteins were detected on X-ray films using the ECL system. β-Actin was used as the internal control.

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. The isolated monocytes were plated and differentiated in Seahorse XF96 cell culture microplates. After treatment, MΦs subjected to the metabolic assay tests. For mitochondrial

stress test, cells were washed and incubated in XF assay medium 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). 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.

Cytokine measurements

The concentration of cytokine secretion was determined from the obtained cell culture supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits. The protocol was according to the manufacturer's instructions. The quantifications were performed using a FlexStation 3 Microplate Reader.

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 test; or unpaired student's t-tests. Differences between groups were considered significant at p values less than 0.05.

5. Results

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

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. 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. 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. Concentration-dependent effect of ATRA was detected in the induction of IL-1β secretion of LPS/ATP-treated M-MΦs. Since IL-1β requires specific mechanisms for maturation and secretion that involve NLRP3 inflammasome, we treated the primed cells with NLRP3 inhibitor MCC950. The results showed that MCC950 abolished IL-1β secretion, indicating that ATRA effect on IL-1β secretion is mediated via an NLRP3 inflammasome-dependent pathway.

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

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. 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. 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.

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*, however ATRA significantly and time-dependently upregulated both the mRNA and protein levels of NLRP3. 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*.

ATRA modifies signal transduction pathways required for inflammasome priming

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. 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. These results indicate that ATRA modulates the signaling pathways that are implicated in the priming event of NLRP3 inflammasome.

ATRA inhibits LPS-induced Akt/mTOR signaling pathway

Upon activation, TLR4 induces the Akt/mTOR signaling pathway that plays a key role in limiting the inflammatory response and mediating the anti-inflammatory action of activated MΦs. 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. 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.

ATRA attenuates the secretion of LPS-induced IL-10

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. In addition, the result showed that ATRA significantly downregulated LPS-induced IL-10 secretion at different time points. 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.

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 metabolism. 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. 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. 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. Consistent with these results, we found that ATRA treatment significantly upregulated hexokinase 2 (HK2) of LPS-activated M-MΦs. HK2 is a key enzyme in the glycolysis pathway that catalyzes the first step in glucose metabolism. To investigate whether HK2 is involved in NLRP3 inflammasome-mediated IL-

1β, we subjected 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. 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.

NOD1 differently activates human MΦ subpopulations

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, 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. 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.

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

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

Next, 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. 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.

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. 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.

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

Next, 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. 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. These results suggest that NOD1 induces IL-1β and IL-18 secretion in human MΦ subpopulation, and ATRA is capable to modulate this secretion.

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. 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. These results indicate that IL-1β and IL-18 secretion are inducible in NOD2-activated MΦs, and ATRA is capable to modulate this secretion.

6. 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. 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. 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.

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. Importantly, ATRA has been reported to modulate the secretion of IL-1β in both primary MΦs and myeloid cell lines. 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. 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. In line with other reports, our sequence analysis of the NLRP3 promoter region has revealed the presence of potential binding sites for RXR heterodimers. 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. 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. 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. 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. 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. 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. 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. 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. 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. 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.

Activated MΦs undergo a rapid metabolic shift toward glycolysis, while alternatively activated MΦs are characterized by increased mitochondrial respiration. Several metabolic pathways, and their associated enzymes and metabolites have been reported to differentially regulate NLRP3 inflammasome. 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. 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. Furthermore, pharmacological inhibition of HK2 activity attenuates LPS-induced IL-1β production in RAW MΦs cell line. 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. Importantly, IL-10 has been shown to limit the glycolytic flux and inhibit the expression of several genes in the glycolytic pathway in LPS-stimulated BMDMs. 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.

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. Surprisingly, we detected high induction of IL-8 secretion triggered by NOD1/NOD2, similar to the results previously obtained from LPS-stimulated MΦs. IL-8 is a potent proinflammatory chemokine that functions as a neutrophil chemoattractant and a mediator for angiogenesis. 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. 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. 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. 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. IRF1 is an upstream regulator of IRF3, which is a main inducer of IFNβ expression. 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 and RIG-like helicases. 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.

ATRA has been reported in several contexts to differentially modulate NF-κB pathway, PI3K/Akt and MAPKs signaling. 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. 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. 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 with a wide range of inflammatory and autoinflammatory disorders. In line, vitamin A deficiency can lead to increased susceptibility to various infectious and inflammatory diseases such as tuberculosis, malaria, asthma and colitis. 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.

7. 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.

8. List of publications

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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.
   DOI: http://dx.doi.org/10.1371/journal.pbio.3001526
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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.
   Front. Immunol. 12, 630569, 2021.
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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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