Characterization of NOD2 agonists in vitro and in vivo in the context of Alzhaeimer's Disease

Mémoire

Morgan McLaughlin

Maîtrise en neurosciences - avec mémoire
Maître ès sciences (M. Sc.)

Québec, Canada

Characterization of NOD2 agonists *in vitro* and *in vivo* in the context of Alzheimer's Disease

Mémoire

Morgan McLaughlin

Sous la direction de :

Serge Rivest, directeur de recherche

Résumé

La maladie d'Alzheimer (MA) et l'angiopathie amyloïde cérébrale (AAC) sont les deux formes les plus courantes de démence liée à l'âge qui partagent de nombreuses caractéristiques moléculaires, notamment l'accumulation de bêta-amyloïde (Aβ) dans les parois des vaisseaux sanguins cérébraux. Les cellules du système immunitaire inné, telles que les monocytes patrouilleurs, sont capables de surveiller les vaisseaux sanguins cérébraux et de phagocyter la bêta-amyloïde vasculaire ainsi que d'autres substances. Les monocytes patrouilleurs sont devenus une cible thérapeutique dans la MA, et leur phagocytose de l'Aβ permettrait une redistribution à l'équilibre entre le parenchyme et les espaces périvasculaires et vasculaires, ce qui réduirait ensuite la charge dans le parenchyme. Des recherches antérieures ont démontré que les monocytes peuvent être convertis du phénotype inflammatoire au phénotype de patrouille en utilisant la liaison du MDP à NOD2 (Lessard et al., 2017). Nous émettons l'hypothèse que le développement d'analogues de la MDP ayant des effets immunomodulateurs similaires à ceux de la MDP pourrait conduire à un médicament préventif dans la MA. Nous avons utilisé les lignées cellulaires HEK-Blue NOD2 et HEK-Blue TLR2 pour détecter les analogues qui se lient à NOD2. Nous avons également utilisé le test MTS sur des PBMC et des cellules HepG2 pour évaluer la viabilité cellulaire et la cytométrie perlée pour caractériser les cytokines et les interférons libérés par les cellules exposées aux analogues du MDP. Nous avons effectué des tests de phagocytose pour évaluer si les analogues du MDP modifiaient le taux de phagocytose par les monocytes. De plus, nous avons effectué des tests *in vivo* sur des souris WT pour évaluer si les analogues de la MDP pouvaient provoquer des changements phénotypiques dans les monocytes et si ces changements phénotypiques se produisaient chez les souris NOD2 KO. Les analogues de la MDP ont le potentiel de devenir un médicament préventif de la MA en augmentant la phagocytose de l'Aβ et en diminuant l'Aβ vasculaire. D'autres recherches sont nécessaires pour mieux comprendre le rôle exact des monocytes patrouillant dans la MA.

Mots clés : Maladie d'Alzheimer, monocytes, NOD2, analogue

Abstract

Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA) are the two most common forms of age-related dementia which share many molecular features including the accumulation of amyloid beta (Aβ) in the walls of cerebral blood vessels. Cells of the innate immune system such as patrolling monocytes are able to survey cerebral blood vessels and can phagocytose vascular Aβ along with other substances. Patrolling monocytes have become a therapeutic target in AD, and their phagocytosis of Aβ would allow for an equilibrium-driven redistribution between parenchyma and perivascular and vascular spaces, subsequently reducing the burden in the parenchyma. Previous research has demonstrated that monocytes can be converted from the inflammatory phenotype to the patrolling phenotype using the binding of MDP to NOD2 (Lessard et al., 2017). We hypothesize that the development of MDP analogs similar immunomodulatory effects as MDP could lead to a preventative medication in AD. We used cell culture lines to determine which MDP analogs bind to NOD2. We also used the MTS assay to assess cell viability and cytometric bead characterize the inflammatory response of cells exposed to MDP analogs. We conducted phagocytosis assays to assess if MDP analogs modified the rate of phagocytosis in monocytes. We then conducted *in vivo* testing and found that MDP analogs were able to convert inflammatory monocytes to patrolling monocytes in WT mice, but this effect was not observed in NOD2 KO mice. MDP analogs have the potential to become a preventative medication in AD but further research is needed to better understand the exact role of patrolling monocytes in AD.

Keywords: Alzheimer's disease, monocytes, NOD2, analog

Table of Contents

List of figures

Introduction

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of tables

List of abbreviations

Aβ	Amyloid Beta
ABCA1	ABC transporter subfamily member A family 1
ABCB1	ABC transporter subfamily member B family 1
AD	Alzheimer's disease
ALR	AIM2-like receptor
APC	Antigen presenting cell
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCR	B-cell receptor
C3dg	Complement component 3dg
CAA	Cerebral amyloid angiopathy
CARD	Caspase recruitment domains
CBA	Cytometric bead array
CCL2	C-C motif chemokine ligand 2
CCL3	C-C motif chemokine ligand 3
CCL5	C-C motif chemokine ligand 5
CCR1	C-C motif chemokine receptor 1
CCR2	C-C motif chemokine receptor 2
CCR5	C-C motif chemokine receptor 5
CD	Clusters of differentiation or Chron's disease, context dependent
C/EBPβ	CCAAT/enhancer-binding protein beta
Cepbp	CCAAT/enhancer-binding protein beta gene

CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCR1	C-X-C motif chemokine receptor 1
CXCR2	C-X-C motif chemokine receptor 2
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBNA2	The Epstein-Barr virus-encoded transcriptional transactivator
EMEM	Eagle's minimum essential medium
EOAD	Early onset Alzheimer's disease
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated single-cell sorting
FBS	Fetal bovine serum
GlcNAc	N-acetylglucosamine
Gly	Glycine
GMDP	GlcNAc-MurNAc-$_{L}$-Ala-$_{D}$-*iso*-Gln
GMP	Granulocyte and macrophage progenitors
GPCR	G-coupled protein receptor
HepG2	Hepatoma G2
IgA	Immunoglobin A
IgG	Immunoglobin G
IgM	Immunoglobin M
IKBa	NF-κB inhibitor a

IKKα	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IRF	Interferon-regulatory factors
IRF3	Interferon-regulatory factor 3
IRF8	Interferon-regulatory factor 8
KLF2	Kruppel-like factor 2
L-Ala	L-Alanine
LIP	Liver-enriched transcriptional inhibitory protein
LOAD	Late-onset Alzheimer's disease
LPS	Lipopolysaccharide
LRP-1	Lipoprotein receptor-related protein 1
LRR	Leucine rich repeat
$Ly6C^{high}$	Lymphocyte antigen 6 complex high expression
$Ly6C^{low}$	Lymphocyte antigen 6 complex low expression
MAP	Mitogen activated protein
MAVS	Mitochondrial antiviral-signaling protein
mCSF	Macrophage colony stimulating factor
MDA-5	Melanoma differentiation-associated protein 5
MDP	Muramyl Dipeptide
MDP	Macrophage and dendritic cell precursor
MHC	Major histocompatibility complex

MHC II	Major histocompatibility complex class II
MMSE	Mini mental state examination
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium
MurNAc	N-acetlymuramic acid
NEMO	NF-kappa-B essential modulator
NFAT	Nuclear factor of activated T cells
NFT	Neurofibrillary tangle
NF-κB	Nuclear factor κB
NLR	Nucleotide-binding and oligomerization domain containing receptor
NOD	Nucleotide oligomerization domain
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOR	Novel Object Recognition
NR	Nuclear receptor
Nr4a1/Nurr77	Nuclear receptor subfamily 4 group A member 1
Nr4a2/Nurr1	Nuclear receptor subfamily 4 group A member 2
NT	Neurotransmitter
NVU	Neurovascular unit
ONR	Orphan nuclear receptor
PAMP	Pathogen associated molecular pattern
PART	Primary age-related taupathy
PBMCs	Peripheral blood mononuclear cells
PHF-TAU	Paired helical filaments tau
PRR	Pattern recognition receptor

PSEN1	Presenilin 1
PSEN2	Presenilin 2
PU.1	Purine-rich box 1
PXR	Pregnane X receptor
RAGE	Receptor for advanced glycation end products
RIG-I	Retinoic acid inducible gene 1
RICK/RIP2	Rip-like interacting caspase-like apoptosis-regulatory protein kinase
RLR	Retinoic acid inducible gene 1 like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEAP	Secreted embryonic alkaline phosphatase
Ser	Serine
SV	Sindbis virus
TAB1	TAK1-binding protein
TAB2/3	TAK2/3 binding protein
TAI	Tau aggregation inhibitors
TAK1	Transforming growth factor beta-activated kinase 1
TBK-1	TANK-binding kinase 1
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha

TREM	Triggering receptors expressed on myeloid cells
TREM1	Triggering receptors expressed on myeloid cells 1
TREM2	Triggering receptors expressed on myeloid cells 2
UV	Ultraviolet
WT	Wild type

This thesis is dedicated to my parents who had supported me throughout my master's program.

Science and everyday life cannot and should not be separated. – Rosalind Franklin

We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the Universe. That makes us something very special. – Stephen Hawking

Acknowledgments

I would like to thank my family, mom, dad and sister for their unconditional support throughout this journey. Without their support completing this degree would not have been possible. I would like to thank my supervisor Dr. Serge Rivest for his mentorship and insightful commentary. I am grateful to him for accepting me into his lab and providing me with an exciting research project that has led to collaboration with a pharmaceutical company that will hopefully lead to a preventative treatment to Alzheimer's disease.

I would like to thank my current and former lab members Nataly, Marie-Michèle, Paul, Vincent, Majid and Pierre-Alexandre for their support during my master's. I would like to thank Paul, Marie-Michele, André, Majid, and Vincent for their technical support. I am grateful for friends that I have made at CHUL and their support was essential to my success: Melanie, Laura, Raquel, Camille, André, Sara, Nesrine, Sarah, Maxime, and Irshad. I am thankful for my committee members Drs. David Gosselin and Ayman El Ali for accepting to review my thesis.

Authors' Contributions

Morgan McLaughlin conducted the HEK-Blue NOD2 and HEK-Blue TLR2 assays in addition to the MTS assays with peripheral blood mononuclear cells and the HepG2 cell line. Paul Préfontaine conducted the Cytometric Bead Array (CBA) assays in addition to the phagocytosis assays. Pierre-Alexandre Piec conducted the *in vivo* work in WT and NOD2 KO mice. All analysis in this work were conducted by Morgan McLaughlin.

Introduction

1.1 Adaptive and Innate Immune Systems

1.1.1 History and Early Theories

Nearly all, if not all multicellular organisms face life-threatening infections throughout their lifetimes. These infections can be caused by bacteria, fungi, and viruses and in order for these organisms to survive they must initiate an appropriate response to eliminate or neutralize these pathogens.

Immunity is an early evolutionarily developed response to infection and allows for multicellular organism survival. These immune responses are mediated by two main branches of the immune system: the innate immune system and the adaptive immune system. In an evolutionary sense, innate immunity is much older than adaptive immunity. The same modules of host defense mechanisms are found in plants and animals, meaning that this was developed before the two kingdoms split (Janeway & Medzhitov, 2000). Adaptive immunity is only found in vertebrae, the only phylum known to mount an adaptive immune response. Vertebrates have also developed other mechanisms to inhibit the activation of innate immune responses (Janeway & Medzhitov, 2000).

The adaptive immune system recognizes foreign antigens, provides pathogen-specific adaptor proteins and retains immunological memory of infection. The cells in this branch are T and B lymphocytes. Furthermore, the adaptive immune system does not respond immediately but has responses that are long-lasting, very specific to the pathogen, and sustained in the long-term by memory T cells. Adaptive immune responses are characterized by two main types: antibody responses and cell-mediated immune responses (Alberts et al., 2002). In antibody responses, B cells are triggered to release antibodies called immunoglobulins. The antibodies circulate throughout the bloodstream and bind to the foreign antigen that caused its production. When the antibody binds to the antigen, it inactivates viruses and microbial toxins by rendering them unable to bind to receptors on host cells (Alberts et al., 2002). In cell mediated responses, T cells react directly with the foreign antigen

that is presented to them on outside of the host cell. The T cell then either kills the virus-infected host cell which eliminates the infected cell before it can replicate or it produces molecules that activate macrophages to eliminate the microbes that they have phagocytosed (Alberts et al., 2002).

The immune responses from the innate immune system are immediate. The cells within the innate immune are monocytes, macrophages, neutrophils, natural killer cells, mast cells, dendritic cells, eosinophils and basophils. The innate immune system provides baseline protection against many pathogens and foreign proteins. Innate immune system responses are characterized by inflammation and the release of cytokines and interferons.

Both branches of the immune system are able to recognize specific structural and functional components associated with specific classes of microorganisms. When recognition of a pathogen occurs, this elicits responses that determine the location, viability, replication, and pathogenicity of the microorganism. In addition, the innate immune system must be able to recognize ‘self’ and ‘non-self’. This theory was first proposed by Frank MacFarlane Burnet in 1959 (Gong et al., 2020) Burnet has six categories of body constituents, parts of the body that to not elicit immunological responses when they come into contact with each other:

1. Any body component that is either directly or broken up expendable cells can be present in blood or lymph are non-antigenic to the individual organism.
2. Cells with different genetic backgrounds can implant during embryonic life can become non-antigenic and survive indefinitely.
3. Certain components of the body are antigenic in mammals this is thyroglobulin and parts of the lens and spermatozoa. This is likely related to two factors (a) these components are normally inaccessible to blood and/or lymph (b) they were not present in the body when tolerance to self was established during embryonic life.

4. Blood, tissue cells, or tissue extracts from animals that are not genetically identical to the host will provoke some type of immune response. The intensity of the response is likely to widely vary.
5. Tolerance is best demonstrated with implanted foreign cells can persist from embryonic to adult life. Through appropriate *in utero* and *in ovo* injections, partial tolerance to non-cellular antigens can be produces sometimes. It is more effective when the injections are done at short regular intervals during the early weeks of the free life of the animal. With some antigens this procedure does not achieve immunological tolerance.
6. Many conditions can lead to abnormal destruction of body components, ie. red blood cells and platelets is caused by activity of antibody-like agents produced by the body. There is no abnormal antigenicity in the 'target' cells, in these cases the auto-antibody is product of a disordered set of antibody-producing cells (Burnet, 1959).

Burnet (1959) continued his theory by stating that immune reactions including antibody production, occur (a) if antigens associated with foreign substances enter the body, (b) if parts of the body that are normally inaccessible become accessible or (c) if antibody producing cells deviate from its limitations established during embryonic life. He posed that embryonic elimination of self-reactive patterns are eliminated during development leading to residues that would be able to react with and recognize foreign configurations that enter the organism post-embryonic development (Burnet, 1960). He finally argues that long-lasting retention of information such as antigen recognition, must be mediated by a form of genetic transmission from cell to descendent cell (Burnet, 1959). The self and non-self theory evolved into the 'pattern recognition' theory proposed by Charles Janeway in 1989 (Janeway, 1989). Janeway goes on to say that specific T-cell activation is required for self/non-self discrimination. Janeway (1989) later concludes that self/non-self discrimination evolutionarily came before development of specific recognition and that many immune responses are non-antigen specific responses. These non-antigen specific defense mechanisms may have evolved under heavy selection pressures to recognize elements unique to microorganisms that are not found in the

host (Janeway, 1989). In January 1989, Janeway concludes that nonclonally distributed pattern recognition receptors are on lymphocytes and antigen presenting cells (APCs). Janeway also found that these receptors are present in organisms that we study today such as mice, rats, chickens, and humans. He also concludes that the two signals required for lymphocyte activation are likely to have been included in early immune responses (Janeway, January 1989). Pattern recognition receptors (PRRs) recognize conserved components of pathogen associated molecular patterns (PAMPs). PAMPs are a diverse class of microbial molecules that share recognizable microbial features, sometimes it is the entire molecule and sometimes it is a part of a molecule/polymeric assemblages. These molecules alert the organism to intruding pathogens (Bianchi, 2007). This theory was supported through the discovery of toll-like receptors (TLRs) which are present on APCs and act as pattern recognition receptors in sterile inflammation. Sterile inflammation occurs from trauma, ischemia-reperfusion injury, or from chemically induced injuries and is characterized by the absence of microorganisms (Chen & Nuñez, 2010). Sterile inflammation is usually denoted by the recruitment of neutrophils and macrophages in addition to the production of cytokines and chemokines like tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Chen & Nuñez, 2010). TLRs were also found to indirectly activate the adaptive immune response by promoting the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules (Gong et al., 2020).

Unfortunately, TLRs were unable to explain infection-dependent immune responses which caused Polly Matzinger to propose the ‘danger’ theory. This theory postulated that danger signals were released by damaged cells which initiated immune responses (Matzinger, 2007). This theory also proposes that the immune system is generally concerned more with what does damage rather than destroying everything that is foreign (Matzinger, 2007). The danger theory also proposes that the immune system response has different levels based off of the severity of the signal (Matzinger, 2007). This led to the discovery of endogenous molecules that were released during tissue damage and were called danger associated molecular

patterns (DAMPs) by Walter Land in 2003 (Land, 2003). DAMPs can be molecules like extracellular ATP and extracellular DNA, fragmented cell walls and extracellular matrices. There are also many other danger signals that consist of delocalized molecules and fragments of macromolecules (Heil & Land, 2014). This theory help explain why even in the absence of harmful bacteria or viruses, immune responses still occur. DAMPs can be recognized not only by PRRs but also by a second class of receptors known as non-PRR DAMP receptors. These receptors are the receptor for advanced glycation end products (RAGE), triggering receptors expressed on myeloid cells (TREMs), many G-protein-coupled receptors (GPCRs) and ion channels. The danger theory has generated controversy but currently it is widely accepted that PAMPs and DAMPS are able to initiate immune responses through the activation of PRRs.

1.1.2 Pattern Recognition Receptors

PRRs are a superfamily that contain toll-like receptors (TLRs), nucleotide-binding, and oligomerization domain containing receptors (NLRs), retinoic acid inducible gene I-like RNA helicases, C-type lectins, and AIM2-like receptors (ALRs) (Saxena & Yeretssian, 2014) Each of these PRR families are classified based on their structural homology and different adaptor proteins required for the receptor's function and signal transduction (Hansen et al., 2011). PRRs are not created equal as they are broadly sorted into three classes: secreted, transmembrane and cytosolic. Secreted PRRs include collectins, ficolins, and pentraxins. These receptors bind to microbial cell surfaces thereby initiating classical and lectin pathways within the complement system. They are also responsible for opsonizing pathogens for phagocytosis by macrophages and neutrophils (Iwasaki & Medzhitov, 2010). Opsonization is a process of the immune system that uses opsins to mark foreign pathogens for elimination (Lausen et al., 2020). Transmembrane PRRs include TLRs and C-type lectins. In mammals, TLRs are expressed on either the plasma or endosomal membrane. TLRs that are found on the cells surface recognize conserved microbial patterns such as lipopolysaccharide (LPS), lipoteichoic acids, bacterial lipoproteins and flagellin (Iwasaki & Medzhitov, 2010). Endosomal TLRs

recognize microbial and nucleic acids such as double-stranded RNA, single-stranded RNA, and double-stranded DNA (Iwasaki & Medzhitov, 2010). The expression of TLRs is cell-type specific and allows scientists to differentiated cells based on their TLR expression (Iwasaki & Medzhitov, 2004). Dectin-1 and Dectin-2 within the C-type lectin family recognize β-glucans and mannan which are found on fungal cell walls (Robinson et al., 2009). Cytosolic PRRs include retinoic acid inducible gene 1 (RIG-I) like receptors (RLRs) and NLRs. RLRs are responsible for detecting viral pathogens and are commonly expressed on most cell types. RLRs use a common adaptor molecule mitochondria antiviral signaling protein (MAVS) which leads to the activation of transcription factor NF-κB and interferon regulatory factor 3 (IRF3) (Iwasaki & Medzhitov, 2010). RIG-1 also recognizes PAMPs associated with any single stranded RNA viruses (Yoneyama & Fujita, 2008). RIG-I is a sensor for single stranded RNA viruses and some double stranded DNA viruses (Iwasaki & Medzhitov, 2010). NLRs are a large family of intracellular sensors that detect pathogens and stress signals. They are divided into three subfamilies based on their N-terminal domains. In most cases, NLRs indirectly detect degradation of products of peptidoglycans, forms of stress like UV radiation, microbial products, and non-infectious crystal particles (Martinon & Tschopp, 2009). Nearly all PRRs that activate transcription factors NF- κB, IRF, or nuclear factor of activated T cells (NFAT) are sufficient to induce T- and B-cell responses. Secreted PRRs and endocytic PRRs (scavenger receptors and mannose receptor) are not capable of inducing an adaptive immune response alone. TLRs are known to trigger the activation of adaptive immune responses in many effector classes which include immunoglobulin (Ig)M, IgG and IgA antibody responses, T helper (Th)1, Th17, and $CD4^+$ T cell responses, and $CD8^+$ T cell responses (Iwasaki & Medzhitov, 2004). Cytosolic DNA sensor pathways are sufficient to activate Th1, cytotoxic CD8+ T and antibody responses via TANK-binding kinase-1 (TBK-1) (Ishii et al., 2008). While only some PRRs can activate the adaptive immune system, all are able to trigger a biochemical cascade via the innate immune system. Detection of a microorganism leads to cytokine release from dendritic cells which recruits lymphocytes who release different cytokines to activate effector responses (Iwasaki & Medzhitov, 2015).

1.1.3 Innate Immune Responses to Pathogens

Microbial pathogens are able to enter the host through three different ways: mucosal surfaces, a break in the skin (epithelial barrier) or through bites of insect vectors. In order for the host to effectively respond to the invasion, innate sensors are strategically placed in distinct anatomical, tissue, cellular, and subcellular compartments. When a pathogen crosses an anatomical compartment, it informs the host how threatening it is (Iwasaki & Medzhitov, 2015). For example, microorganisms in the lumen of the gut does not trigger inflammation; however, when microorganisms cross the epithelial layer it induces a local inflammatory response. When pathogens enter the bloodstream, this signals a systemic response because if the infection is not contained, the immune system becomes stressed and put into overdrive, leading to sepsis (Iwasaki & Medzhitov, 2015). The epithelium is able to recognize pathogens and is able to inform the host when it is breached. Epithelial cells have PRRs that induce chemokine release that then recruit leukocytes circulating in the bloodstream to initiate an innate defense. Epithelial cells have a small number of TLR2, TLR3, TLR4, and TLR5 expressed which ensures that only invasive and not local bacteria are recognized (Abreu, 2010). Underneath the epithelial layer is the lamina propria. This is where macrophages, dendritic cells (DCs), and mast cells reside. They are another layer of defense and are able to recognize invading pathogens and elicit an appropriate response. These cells have PRRs that initiate the secretion of inflammatory cytokines and chemokines that specifically recruit monocytes, neutrophils, eosinophils, and basophils from the bloodstream to the site of infection (Iwasaki & Medzhitov, 2015). When circulating leukocytes are able to quickly arrive at a site of infection, they are able to stop a replicating pathogen in three ways: phagocytosis of the pathogen, secretion of toxic granules, and lysis of the pathogen and infected cells (Iwasaki & Medzhitov, 2015). If this second layer of response is insufficient to contain a given pathogen, the immune system begins a systemic level response upon recognition of the invading pathogen by TLRs, NLRs, and C-type lectin receptors (CLRs) expressed on Neutrophils, monocytes and eosinophils. When PRRs are activated, enhanced phagocytosis occurs in addition to degranulation, respiratory burst, and death of

bacterial, fungal, and protozoan pathogens (Thomas et Schroder, 2013). The specific activation of TLRs initiates the generation of neutrophil extracellular traps that contain extruded DNA which are able to trap bacteria and begin the rapid induction of inflammatory cytokines and chemokines (Brinkmann et al., 2004). Inflammatory cytokines such as IL-1β, TNF, and IL-6 act on the liver and central nervous system. In the liver, these cytokines cause the production of acute-phase response proteins which promote pathogen clearance through complement activation and phagocytosis (Gabay & Kusher 1999). In the brain, these cytokines cause fevers and general discomfort/illness in the host (Pecchi et al., 2009).

This generic response to pathogens is driven by the innate immune system, which is the focus of this memoire, can be subdivided into two main kinds of responses: intrinsic and extrinsic innate immune recognition. The difference between the two kinds of responses is whether this response is mediated by infected or non-infected cells (Stetson, 2009). Extrinsic innate immune recognition is performed by transmembrane receptors like TLRs and Dectins and does not require the cells expressing these receptors to be infected. Intrinsic innate immune recognition is performed by intracellular receptors like NLRs and RLRs and in general requires the cell to be infected. PRRS involved in Intrinsic innate immune responses are broadly expressed on most cells because they are susceptible to pathogens, especially viruses (Iwasaki & Medzhitov, 2010). Extrinsic recognition is primarily mediated by specialized cells within the innate immune system like macrophages and dendritic cells (DCs). Both kinds of recognition are able to induce antimicrobial effects when they are activated and are even able to induce adaptive immunity through various mechanisms (Iwasaki & Medzhitov, 2010). For example, the recognition of a microbial cell or viral particle by a TLR on a DC is followed by endocytosis or phagocytosis of the pathogen and processing of the microbial cell. Antigens of the microbial cell are then given to T cells through major histocompatibility complex (MHC) molecules. The origin of the antigen is determined through the physical association between the antigen and the PAMP that triggered the TLR. This association is determined through the co-delivery of the antigen and the TLR ligand

(PAMP) to the same phagosome and endosome. Antigens are then processed and selected to be presented by MHC II (Blander & Medzhitov, 2006). When antigens and PAMPS are within the same phagosome/endosome, this indicates to the dendritic cell that they have a common origin. Furthermore, TLR activation leads to the induction of costimulatory molecules and cytokines that are necessary for differentiation and activation of T lymphocytes (Iwasaki & Medzhitov, 2010). Another important component of extrinsic innate immune recognition is associative recognition which is mainly conducted by B cells. The co-engagement of the B-cell receptor (BCR) and one of many innate immune signaling pathways, like the C3dg complement component, results in a large enhancement of the antibody response. The C3dg is able to 'flag' the antigen as foreign, in addition to telling B cells the origin of this antigen. In addition, when BCRs and TLRs are co-stimulated, this enhances the antibody response (Dempsey et al., 1996). The extrinsic innate immune recognition is able to enhance adaptive immune responses.

The mechanism for internal innate immune recognition is less clear than external innate immune recognition. Cytosolic receptors such as RIG-I and melanoma differentiation-associated protein 5 (MDA-5) are able to detect viral nucleic acids in infected cells. But in most cases these cells are not antigen presenting cells (APCs) (Iwasaki & Medzhitov, 2010). In external innate immune recognition cells are marked through their physical association with the microbial PAMPs but in intrinsic innate immune recognition detection of viral nucleic acid is not known to be coupled with viral antigens. It is unknown if even when intracellular sensors are activated within an APC how the microbial antigens are preferentially targeted to be presented to T cells. A theory to how this occurs is that the innate recognition event is somehow able to direct the microbial antigens to autophagic degradation followed by major histocompatibility complex (MHC) presentation. This is due to the association between MHC class II antigen presentation and autophagy (Schmid & Münz, 2007). Another theory is that intrinsic innate immune recognition may be coupled with the induction of adaptive immunity through a mechanism that is independent of a

physical association and relies on another kind of coincidence detection (Iwasaki & Medzhitov, 2010).

1.2 Monocytes

Monocytes, the cells that will be focused on in this memoire, are within the innate immune system and have roles in tissue development, host defense, and homeostasis. Monocytes originate in the fetal liver from late yolk-sac derived erythromyeloid progenitors in the last wave of hematopoiesis (Hoeffel & Ginhoux, 2018). Once the immature hematopoietic stem cells settle and further develop in the fetal liver, they invade the bone marrow and only become fully functional after birth (Wolf et al., 2019). Multipotent hematopoietic stem cells, a subclass of hematopoietic stem cells, give rise to common lymphoid progenitors and common myeloid progenitors. Monocyte lineage and differentiation is controlled by many transcription factors such as macrophage colony stimulating factor (mCSF), C/EBPβ, purine-rich box 1 (PU.1) and interferon regulatory factor 8 (IRF8) (Dahl et al., 2003). PU.1 influences the fate of progenitor cells and is essential to induce the development of microglia and monocytes (Dahl et al., 2003). $PU.1^{-/-}$ mice present a stark drop in macrophages, resident macrophages, and monocytes (Freidman 2007). PU.1 and IRF8 give rise to megakaryocyte-erythrocyte progenitors in addition to granulocyte and macrophage progenitors (GMPs) (Pons & Rivest, 2022). This leads to macrophage and DC precursor (MDP) production (Yáñez et al., 2017).

GMPs and MDPs are critical for monocyte production both groups can generate classical monocytes. MDPs are responsible for generating patrolling/nonclassical and intermediate monocytes. In mice, the surface marker expression that specifically characterizes these cells are $Ly6C^{high}$ (inflammatory/classical) and $LyC6^{low}$ (patrolling/non-classical) and in humans these cells are characterized as $CD14^{++}CD16^{-}$ (inflammatory/classical), $CD14^{++}CD16^{+}$ (intermediate), and $CD14^{+}$ $CD16^{++}$ (patrolling/non-classical).

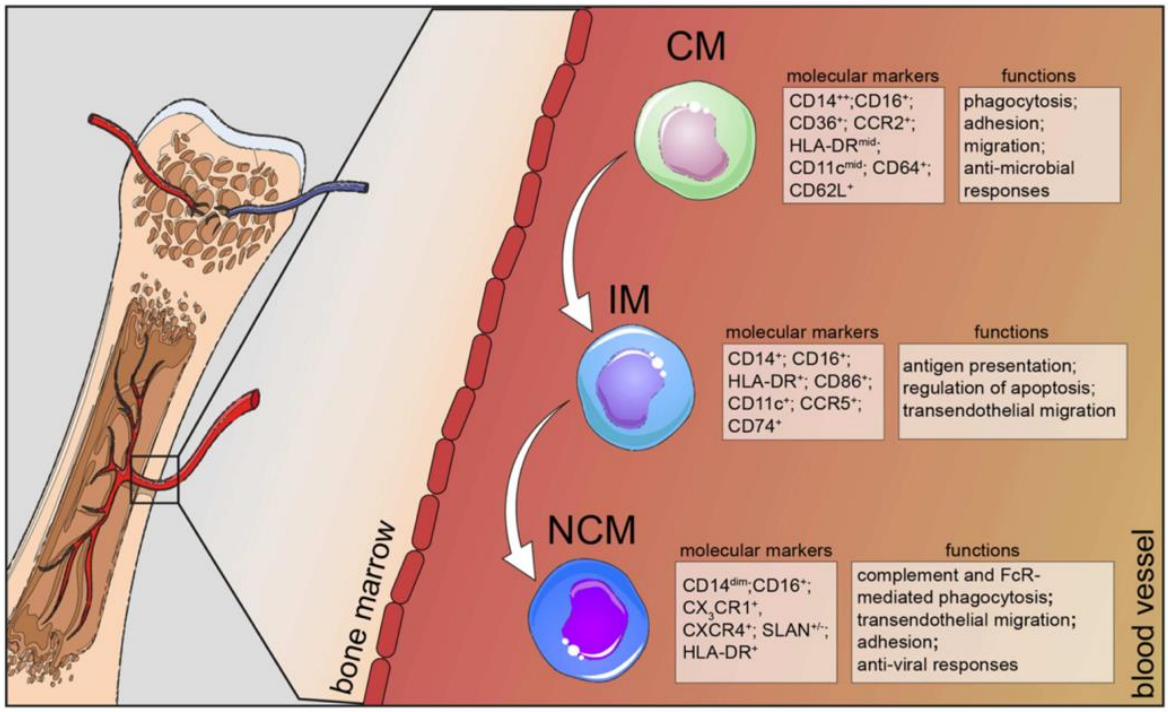


Figure 1. 1 Human monocyte subsets
Human monocytes develop and mature in the bone marrow and are released into circulation as $CD14^+$ classical monocytes. Classical monocytes give rise to non-classical monocytes ($CD14^-$ $CD16^+$) via in intermediate step of $CD14^+$ $CD16^+$ monocytes (Kapellos et al., 2019). **Abbreviations:** CCR, C-C motif chemokine receptor; CD, cluster of differentiation; CM, classical monocyte; CXCR, C-X-C chemokine receptor; CX3CR, C-X3-C motif chemokine receptor; HLA-DR, human leukocyte antigen DR; IM, intermediate monocyte; NCM, non-classical monocyte (patrolling) monocyte; SLAN, 6-sulfo LacNAc.

Monocyte subsets tend to display different functional properties which partially relies on the methylation status of immune-related genes (Kapellos et al., 2019). Classical monocytes migrate to C-C motif chemokine ligand 2 (CCL2) and C-C motif chemokine ligand 3 (CCL3). They then produce reactive oxygen species (ROS) in addition to constraining fungi better than intermediate monocytes (Serbina et al., 2009). Classical monocytes quickly respond to infection or injury and are able to infiltrate different tissues and then differentiate into macrophages or dendritic cells. They actively participate in inflammatory responses due to their ability to release a wide range of cytokines, chemokines and adhesion molecules. Their ability to produce a wide variety of inflammatory molecules may come from their high

expression of critical innate immune receptors like TLR4, TREM1, CCR2, NLRP3 among many others (Anbazhagan et al., 2014). In general, $CD14^+$ human monocytes have higher levels of chemokine receptors such as CCR1, CCR2, CCR5, CXCR1, and CXCR2. The expression of these receptors highlights the cell's potential to migrate to sites of injured or inflamed tissue as they are strongly implicated in neutrophil and leukocyte migration (Reichel et al., 2006; Richardson et al., 2003). Classical monocytes are also characterized by their ability to secrete proinflammatory molecules such as Il-6, IL-8, CCL2, CCL3, and CCL5 (Wong et al., 2011). Furthermore, it is currently widely accepted that classical monocytes have the ability to differentiate into monocyte-derived macrophages and dendritic cells (DCs) which play important roles in inflammation and the resolution of inflammation (Kapellos et al., 2019).

Of the three monocyte subtypes, intermediate monocytes express the highest levels of antigen presentation-related molecules. Intermediate monocytes can release TNF-α, IL-1β, IL-6 and CCL3 when TLRs are stimulated. Intermediate monocytes are increased in patients with systemic infections which implies that they have an important role in the immediate defense against pathogens (Kapellos et al., 2019). However, the exact role of intermediate monocytes is unknown, as a different report suggested that activation led to the production of IL-10, an anti-inflammatory cytokine, when TLRs were stimulated. Whether or not these cells are capable of producing pro- and anti-inflammatory cytokines at the same time requires further investigation (Skrzeczynska-Moncznik et al., 2008).

Non-classical monocytes have a distinct metabolic and transcriptomic profiles as compared to non-classical monocytes. Key differences include reliance on carbohydrate metabolism over respiratory chain metabolism, antigen processing capabilities which focus on wound healing (Kapellos et al., 2019) as well as production of TNF-α to promote neutrophil adhesion to the endothelial surface (Boyette et al., 2017). Patrolling monocytes have the ability to modulate inflammation, can survey vasculature, and find particles. Patrolling monocytes and

inflammatory monocytes has distinct gene signatures. Patrolling monocytes express high levels of genes involved in cytoskeletal dynamics like CDC42 effector protein-4, creatine kinase B, and EML4 and their corresponding receptors CX3CR1, CD115, and Siglec10 (Pons & Rivest, 2022). A distinct feature of patrolling monocytes is that they do not produce reactive oxygen species (ROS) a hallmark of inflammation, but it does not exclude that they can participate indirectly.

Classical monocytes found in mice, known as Ly6C^{high}, originate in the bone marrow and make up 90% of all monocytes. Once they are in the bloodstream, they have a high level of plasticity and are able to differentiate into many cell types (Mildner et al., 2017). These cells specifically respond to injury and are able to extravasate into the tissue. Once in the tissue, they differentiate into classical tissue-resident mononuclear phagocyte populations ie. macrophages and DCs (Mildner et al., 2013). Typically, Ly6C^{low} patrolling monocytes remain in the vasculature while only a small fraction of the population patrol cell walls and act as scavengers that orchestrate tissue repair (Mildner et al., 2017). Monocytes can be converted from the inflammatory to the patrolling phenotype through the binding of muramyl dipeptide (MDP) to nucleotide-binding oligomerization domain-containing protein 2 (NOD2).

1.2.1 Muramyl Dipeptide

Muramyl dipeptide (MDP) is a peptide found in the peptidoglycan component, a crystal lattice structure made through the combination of alternating amino sugars: N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) (Heijenoort, 2001), of the cell wall in both gram-positive and gram-negative bacteria. In gram-positive bacteria, the peptidoglycan is found outside the plasma membrane and forms the cell wall. In gram-negative bacteria there is an outer membrane followed by the peptidoglycan which together form the cell wall. MDP is the minimal essential structural unit that is responsible for immunological activity in many peptidoglycans. It is made up of one carbohydrate and two amino acids and primarily binds to NOD2 although confirmation of this binding through biochemical data was not done until

2012 (Grimes et al., 2012). It was first used to treat tumors in mice (Bloksma et al., 1985). Analogs of MDP and MDP have been suggested to have nonspecific properties that are important in the potentiation of endotoxin-induced necrosis and tumor regression (Bloksma et al., 1985). Furthermore, MDP-treated cells generate an inflammatory response, through the activation of NF-kB and MAP kinase pathways. Not all analogs of MDP are able to produce this response, MDP-(L) does not generate this response when it binds to NOD2 (Grimes et al., 2012). When MDP is used as an adjuvant, it increases the expression of cell adhesion molecules and antigen presentation. Due to the increase in these processes, phagocytic activity, antimicrobial activity and antibody-mediated cytotoxicity are all enhanced (Grimes et al., 2012) after treatment with MDP. In addition, MDP induces immune responses through increased cytokine production, increase in differentiation and proliferation of T lymphocytes which leads to increased protection against foreign pathogens (Kitaura et al., 2018).

Analogs of MDP have been increasingly recognized to have therapeutic potential. The structure, composition and stereochemistry of two key amino acids play essential roles in the inhibition of macrophage migration (Nagao et al., 1979). Cheng et al. (2020) found that GlcNAc-MurNAc-$_{L}$-Ala-$_{D}$-*iso*-Gln (GMDP) is a NOD2 ligand with has activity similar to MDP. In this molecule it was noted that longer glycan chains of peptidoglycan analogs substantially impaired NOD2 stimulation. GMDP has glycosyl moiety (GlcNAc) at the C4 position of MurNAc which caused Cheng et al. (2020) to further investigate the effects of substitutions in this position. C4 substitution of MDP may cause important changes to the function of NOD2 (Cheng et al., 2020). Synthesized analogs of MDP with alkyl ester moiety caused increased NF-kB activation when compared to MDP and murabutide (Cheng et al. 2020). All analogs with this modification were more active than MDP. Furthermore, MDP analogs with a longer alkyl chain reacted with the best potency at 10nM. The compound with the C12 alkyl chain reached the best potency while C16 and C20 alkyl chains did not have a clear trend of potency, this is most likely due to the hydrophobic properties of longer alkyl chains (Cheng et al. 2020).

1.2.2 Nucleotide-binding oligomerization domain-containing protein 2 (NOD2)

NOD2 is an intracellular pattern recognition receptor that is in the Nod-like receptor family. This family of receptors regulates inflammation and apoptosis. Nod-like receptors (NLRs) primarily recognize bacteria and are broadly expressed throughout the body whereas NOD2 is primarily expressed in immune and epithelial cells. NOD2 is a large protein with many domains, it contains two N-terminal caspase recruitment domains (CARDs), a central nucleotide oligomerization domain (NOD), and a C-terminal leucine rich repeat (LRR) domain (Grimes et al., 2012). The LLR domain is a microbe-associated molecular pattern (recognizing MDP) and has a molecular structure similar to LLR domains in TLRs (Strober & Watanabe, 2011). NOD2 is also involved in the recognition of peptidoglycans in the cytosol before they are integrated into the cell wall (Kitaura et al., 2018). NOD2 expression is restricted to monocytes and macrophages in basal conditions.

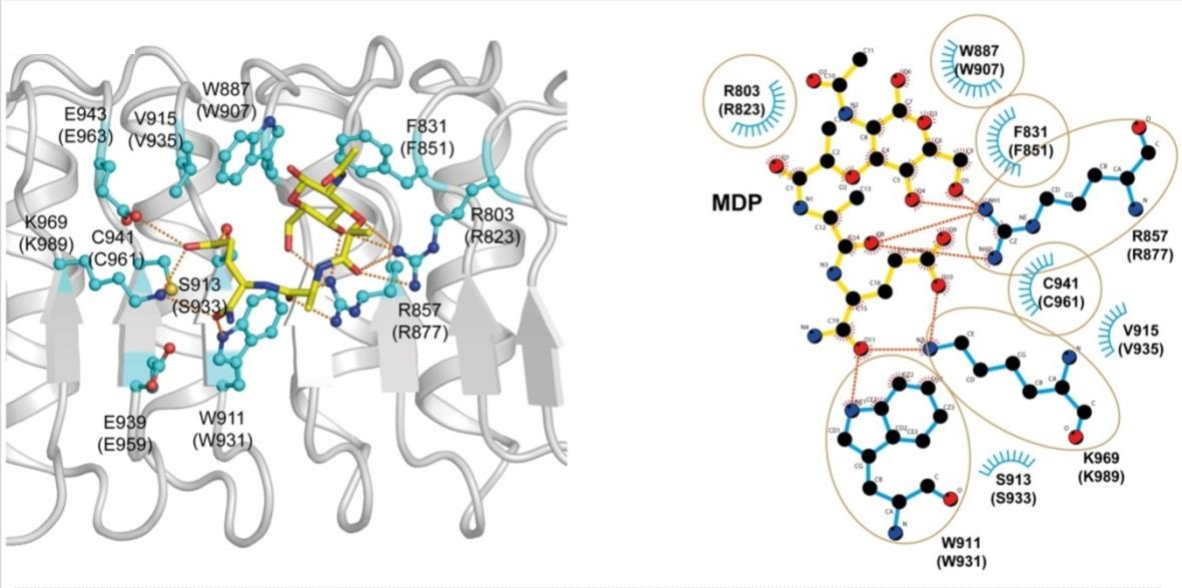


Figure 1. 2 Predicted binding of MDP in the concave surface of the LLR of NOD2.
The image on the left is the 3D interaction model where NOD2 is in grey. Key interacting residues are in ball-stick model in cyan and ligands are colored sticks (yellow). The image on the right shows the interaction of the LLR region of NOD2 and MDP in 2D. Key H-bonding residues are in the line-art model and residues with hydrophobic interactions are in half-circles. Polar contacts/H-bonds are shown by

orange dashes lines and residues in parenthesis are of human NOD2 (Cheng et al., 2020). **Abbreviations:** C, cysteine; E, glutamic acid; F, phenylalanine; K, lysine; R, arginine; S, serine; V, valine; W, tryptophan.

NOD2 is important in autophagy. Autophagy is an intracellular protein degradation mechanism where the cell forms a double-membrane vacuole that eventually combine with lysosomes to eliminate proteins that come from cellular stress responses. Autophagy is also involved in the breakdown of pathogenic bacteria and the processing of antigens during antigen-presenting immune responses, clearly demonstrating its role in immunological responses and host defense (Strober & Watanabe, 2011). NOD2 is able detect bacterial cell peptidoglycans through at least three mechanisms. The first mechanism is direct interaction with the peptidoglycan, the second is mediated interaction, and the third is a signaling relay (Strober & Watanabe, 2011). Once the peptidoglycan is detected, an inflammatory response begins through NF-kB and MAP kinase pathways. This leads to the synthesis of proinflammatory cytokines and/or chemokines. It is important to note that NOD2 activation is not nearly as robust as TLR signaling. The main MDP-activated NOD2 signaling pathway leads to NF-kB activation and the conformational change of NOD2. This results in the unfolding of the molecule which causes oligomerization and exposure of the CARD domain within NOD2. NOD2 can then bind to a downstream adaptor molecule Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK or RIP2) via a CARD-CARD interaction through the CARD domains of NOD2 and RICK (Strober & Watanabe, 2011). This interaction is critical for subsequent NOD2 induction of cytokine responses because mice without RICK do not have these responses (Strober & Watanabe, 2011). Grimes et al. (2012) were among the first to conclude that biochemically MDP binds to NOD2 without the presence of ATP and that MDP has a high affinity for NOD2. Furthermore, human NOD2 is able to recognize structurally unique MDPs. A study by Schenk et al. (2016) found that MDP from *Mycobacterium leprae*, which causes leprosy, has a distinct structure that does not affect its recognition by monocytes. This MDP has a proximal L-Ala instead of Gly which is found in the common configuration of the peptide side chain of *M. leprae*. If NOD2 is capable of recognizing naturally occurring structural

variants of MDP then it is likely that synthetic variants are recognized as well. The conversion of Ly6C^{high} to Ly6C^{low} monocytes is believed to be driven by Nr4a1.

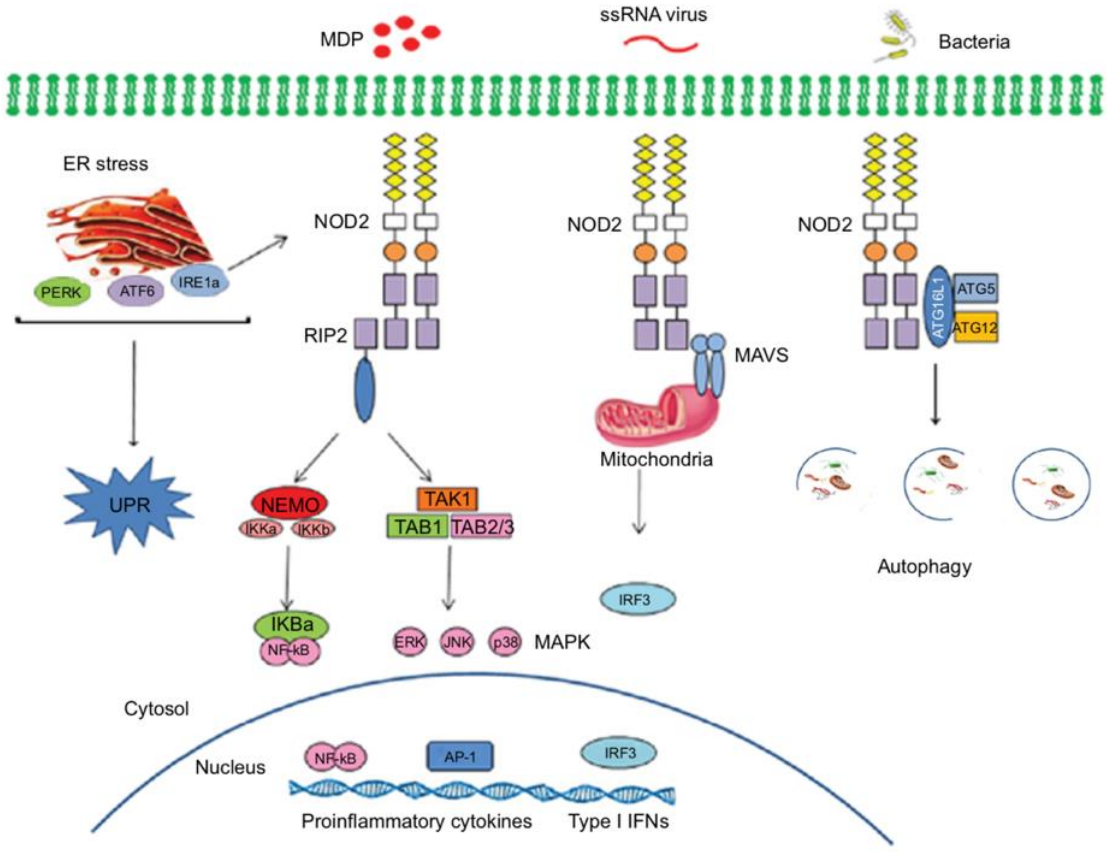


Figure 1. 3 Signaling pathways triggered by NOD2.
These are three pathways that are set in motion when NOD2 is activated. The three main stimulants of NOD2 are MDP, single-stranded RNA virus, and bacteria (Negroni et al., 2018). **Abbreviations:** AP-1, activator protein-1; ATF6, activating transcription factor 6; ATG, autophagy-related genes; ATG16L1, autophagy related 16 like 1; ER, endoplasmic reticulum; ERK, extracellular signal-related kinase; IFNs, interferons; IKB, NF-κB inhibitor; IKK, IκB kinase; IRF3, interferon response factor 3; JNK, c-Jun N-termina kinase; MACS, mitochondrial antiviral signaling; MDP, muramyl dipeptide; NEMO, NF-κB essential modulator; NOD, nucleotide-binding oligomerization domain; PERK, protein kinase RNA-like endoplasmic reticulum kinase; RIP2, receptor-interacting protein kinase 2; TAB, TGF- β activated kinase; TAK1, targets transforming growth factor- β -activated kinase 1; UPR, unfolded protein response.

1.3 Theoretical mechanisms of monocyte conversion

1.3.1 Nuclear receptor subfamily 4 group A member 1 (Nr4a1/Nurr77)

Nr4a1(Nur77) is an orphan nuclear receptor part of the Nr4a subfamily in the nuclear receptor (NR) superfamily and was first encoded by a growth-factor inducible gene that is frequently expressed in cancer cells. Nurr77 is called an orphan receptor because it does not have a specific group of ligands that is known to bind to it. Wang et al. (2003) found that Nr4a2 (Nurr1) lacked a classical binding site in the ligand binding domain in addition to a lack of cavity within the receptor due to the tight packing of the side chains. Despite the lack of a binding site and cavity, Nurr1 is still regulated within mammalian cells. It is theorized that the Nr4a subfamily's activity is regulated by transcriptional activity such as phosphorylation. Nurr77 is a nuclear receptor that can also act as a transcription factor. In response to apoptotic stimuli, Nr4a1 leaves the nucleus and targets mitochondria to release cytochrome c and apoptosis.

Nurr77 is commonly expressed in lung, prostate, breast and colon cancers. Nr4a1 expression is quickly induced by many growth factors and mitogens, implying that it is crucial for the survival of cancer cells (Hanna et al., 2011). On the contrary, Rajpal et al. (2003) found that expression of Nur77 in T cells led to a novel apoptotic pathway. Furthermore, Lee et al. (2002) used the Epstein-Barr virus in vitro which immortalizes primary B cells. The Epstein-Barr virus-encoded transcriptional transactivator (EBNA2) is responsible for this antiapoptotic activity. Lee et al. (2002) transfected B cells with the Epstein-Barr virus and then used the Sindbis virus (SV) which induces apoptosis. Lee et al. (2002) found that EBNA2 mutants bound to Nur77 prevented Nurr77 from leaving the nucleus, leaving Nurr77 unable to elicit mitochondrial-induced apoptosis and conserving the antiapoptotic properties initiated by the Epstein-Barr virus. Another study conducted by Lu et al. (2020) found that monocytes co-cultured with mesenchymal stem cells (MSCs) had a higher proportion of patrolling monocytes than compared to the control. These patrolling monocytes expressed high levels of Nr4a1. When these cells were then transplanted into mice, they found that the mice with monocytes co-cultured with MSCs had a higher survival rate after acute myocardial infarction (AMI). Lu et al. (2020) found that high levels of Nr4a1 was essential in myocyte repair after AMI. In this study,

Ly6C^{low} monocytes were essential in clearing necrotic cellular debris in addition to maintaining vessel wall stability. Lu et al. (2020) suggest that control of the balance and infiltration of inflammatory cells and the clearance and regression of inflammatory mediators as soon as possible after myocardial infection enhances myocardial remodeling and improves prognosis. Furthermore, this study highlights the essential role of Nr4a1 in tissue repair in addition to its essential role in monocyte differentiation.

If Nr4a1 is deleted, Ly6C^{low} monocytes are not present in these mice. Hanna et al. (2011) found that when they transplanted bone marrow from Nr4a1$^{-/-}$ mice into WT mice, those mice developed fewer patrolling monocytes than the control. Interestingly, they also found that the number of myeloid progenitor cells was normal, meaning that the cells were affected in the later stages of monocyte development. Hanna et al (2011) also found that Nr4a1$^{-/-}$Ly6Clow monocytes were arrested in predominantly the S and G2 phase of interphase in the cell cycle. The S phase is responsible for DNA replication while in the G2 phase cells continue to grow and generate proteins that prepare them to enter mitosis (Vermeulen et al., 2003). Upon further analysis Hanna et al. (2011) found increased phosphorylation when compared to controls of histone H2AX at Ser139 which is an indicator of damage because it is a marker that identifies double-stranded breaks in DNA. This damage leads to apoptosis of Ly6C^{low} cells within the bone marrow. (Hanna et al., 2011) Nr4a1 is required in Ly6C^{low} cells to properly execute monocyte differentiation.

1.3.2 CCAAT/enhancer-binding protein beta (C/EBPβ)

The conversion of monocytes is not only controlled by Nr4a1. Other transcription factors like C/EBPβ and KLF2 are implicated in the regulation of monocyte differentiation (Mildner et al., 2017). C/EBPβ is within the C/EBP transcription factor family and is expressed by the CCAAT/enhancer-binding protein beta gene (Cepbp). C/EBPβ is encoded by an intron-less gene and its distinct isoforms have varying biological functions. The liver-enriched transcriptional inhibitory protein (LIP) is the dominant-negative C/EBPβ isoform that lacks the transactivation domain.

Expression of this isoform compared to C/EBPβWT control failed to rescue the developmental defects in LY6C^{low} monocytes caused by Cebpb ablation. Furthermore, LIP mice had a reduction in all monocyte subsets (Bégay et al., 2014). These experiments demonstrate the importance in balancing the isoforms of C/EBP. In the bone marrow of C/EBPβ deficient mice, Ly6C^{high} monocytes were present in the experimental and control groups while Ly6C^{int} monocytes had a 50% reduction in population and Ly6C^{low} monocytes were completely absent from the experimental group. It is important to note that the precursors to Ly6Clow monocytes were present at similar levels between the experimental and control groups (Mildner et al., 2017). Mildner et al (2017) also determined that the elimination of C/EBPβ expression caused cell intrinsic defects and is necessary for the generation and survival of Ly6C^{low} monocytes. Furthermore, monocytes isolated from Cepbp$^{-/-}$ mice were found to downregulate the Ly6C^{low} monocyte gene signature. Even in LY6Cint monocytes the Ly6C^{low} monocyte gene signature was downregulated (Mildner et al., 2017). These findings indicate that Cepbp expression is essential for monocyte differentiation. Finally, C/EBPβ interacts with Nr4a1 and is able to induce its expression. Mechanistically speaking, C/EBPβ binds to the promoter and enhancer regions of Nr4a1 in monocyte-derived cells and activates Nr4a1 expression. It is important to recognize that although Nr4a1 is vital for monocyte conversion, it is not the only factor important to monocyte differentiation.

1.3.3 Kruppel-like factor 2 (KLF2)

Kruppel-like factor 2 (KLF2) is a member of the zinc finger transcription factor family which is involved in the regulation of function of endothelial cells, embryonic development, and maintenance of quiescence in T cells and monocytes (Jha & Das 2017). KLF2 is also plays regulatory roles in inflammatory diseases and is implicated in their pathogenesis. The KLF family is highly conserved, with 17 members reported. All members of this protein family have three Cys2/His2 zinc finger motifs in tandem which they use to bind to common DNA-binding regions of their transcriptional target sequences (Jha & Das 2017). KLF2 is the most widely studied due to its role in lymphocyte biology, regulation of proinflammatory activation in

endothelial cells and monocytes, and its regulatory role in pathological conditions and vascular diseases (Jha & Das 2017).

Although many studies have demonstrated that Nr4a1 is one of the essential factors in monocyte conversion, the exact mechanism using the NOD2 pathway is unknown. NOD2 is able to recognize MDP, single stranded RNA virus, and bacteria and each stimulus activates a different pathway. MDP NOD2 activation leads to proinflammatory cytokine and interferon expression while single stranded RNA NOD2 activation leads to mitochondrial activation while bacterial NOD2 activation leads to autophagy (Negroni et al., 2018). Generally speaking, MDP binds to NOD2 which activates RIP2. RIP2 phosphorylates NEMO, IKKa, and IKKβ or TAK1, TAB1, and TAB2/3. The NEMO-IKK complex activates IKBa and NF-kB in the cytosol which makes its way to the nucleus to promote transcription of proinflammatory cytokines and interferons. The TAK1-TAB complex activates MAP kinase and ERK in the cytosol (Negroni et al., 2018). Nr4a1 is believed to be regulated at post-translational levels and generally requires phosphorylation to be activated (McMorrow & Murphy, 2011). However recent work by Egarnes et al. (2017) found an endogenous ligand that is recognized by Nr4a1.

1.4 Implications in disease

1.4.1 Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most prevalent form of dementia and is characterized by the accumulation of amyloid beta (Aβ) in the cerebral cortex and cerebral amyloid angiopathy (CAA) (Weller et al., 2009). AD makes up two thirds of individuals with dementia (Kamboh, 2004). Clinical diagnosis of AD is done after neurological testing that excludes other forms of dementia. An official diagnosis of AD can only be done after death through examination of the patient's brain tissues (Kamboh, 2004). There are two main forms of Alzheimer's disease: early onset Alzheimer's (EOAD) and late onset Alzheimer's disease (LOAD). EOAD onset is before the age of 65 and makes up around 4-5% of all AD patients (Mendez, 2012). Around 5% of EOAD patients have an autosomal dominant mutation of amyloid

precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2) (Chi et al., 2021). EOAD is a heterogenous disorder that results in clinical symptoms that do and do not align with amnesic patients. Between 22-64% of all EOAD cases have atypical symptoms such as deficits in language, visuospatial abilities, praxis, and other non-memory cognition. LOAD has a high heritability ($h^2 \approx 60–80\%$), and it is difficult to know the genetic contribution of this disease because it is highly inheritable (Gatz et al., 1997). Variants of the apolipoprotein E (APOE) gene affect LOAD susceptibility (Naj et al., 2011). Patients with EOAD typically have a more aggressive progression than LOAD, leading to a shorter duration of the disease (Mendez, 2012).

Cognitive decline seen in EOAD and LOAD and can begin more than a decade than the onset of dementia symptoms (Wilson et al. 2012). Cognitive decline in AD can be explained by two main hypotheses: the tau hypothesis and the amyloid cascade hypothesis (Shen et al., 2018). The tau hypothesis poses that due to neuronal death, toxic oligomeric forms and tau filaments released into the extracellular environment, activate microglia, and promote a deleterious cycle causing further neuronal degeneration (Maccioni et al., 2010). The amyloid cascade hypothesis poses that neurodegeneration in AD is caused by abnormal accumulation of Aβ plaques in various regions of the brain (Barage & Sonawane, 2015).

CAA specifically describes the deposition of Aβ in the cerebral arteries and veins. Abnormal perivascular drainage of Aβ is an important common trait of both of these diseases and is considered to have a major pathogenic role (Greenberg et al., 2020). CAA appears in 80-90% of all AD cases however it must be recognized that CAA and AD are mutually exclusive. CAA can be present in other forms of dementia making it a precursor for dementia but not specifically for AD (Greenberg et al., 2020). Aβ deposition initiates in the neocortex as CAA and then spreads to the olfactory tubercle, olfactory bulb, preform cortex, and cerebellum (Greenberg et al., 2020). Furthermore, Aβ deposition is five times more likely to occur around arteries than veins and begins at periphery arterioles in presumed interstitial fluid drainage

pathways (Greenberg et al., 2020). There are two main types of CAA: type I and type II. Type I is characterized by Aβ deposition within the brain capillaries and type II is characterized by Aβ deposition in arteries and veins but not in capillaries (Greenberg et al., 2020). In CAA type 1-associated AD cases there are often more severe Aβ plaques but more concerted neurofibrillary tangle (NFT) pathology (Hecht et al., 2018). In type 2 CAA, Aβ deposition is in large blood vessels and in close proximity to smooth muscle cells (Thal et al., 2002). Increased expression of APOE alleles ε4 and ε2 are associated with increases in type 1 CAA and type 2 CAA respectively (Hecht et al., 2018; Thal et al., 2002). CAA and AD are defined by impaired Aβ clearance and creating a positive feedback loop of increased vascular Aβ reduced clearance and subsequent CAA and AD progression. Because there are many similarities between AD and CAA, and clinical trial for either disease is a clinical trial for both. However, it is important to note that the mechanisms that lead to brain injury between these diseases are very different. In AD, the precise mechanism is currently unclear but does center around Aβ-triggered loss of synapses and neurons which is measured as a loss of cortical tissue and the development of hyperphosphorylated tau-containing neurofibrillary lesions (Greenberg et al., 2020). Cortical tissues loss of tau-containing neurofibrillary lesions can be sed as a measure of cognitive function in Aβ positive individuals. CAA on the other hand is more strictly derived from blood vessel dysfunction. This occurs through either the loss of vessel integrity and hemorrhaging or through the loss of normal blood supply and ischemia. Ischemic brain injury in particular loss of structural connectivity in CAA is more commonly correlated with cognitive impairment and slower gait speed (Greenberg et al., 2020). Furthermore, tau deposition is usually not seen in CAA pathology but is occasionally observed around Aβ-laden vessels in sporadic and hereditary CAA (Greenberg et al., 2020).

Vascular changes are predominant throughout AD. Prominent cerebrovascular alterations in AD include smooth muscle loss, vasculitis, CAA with or without microhemorrhaging, blood-brain barrier (BBB) leakage, altered vessel wall collagen contact, and alterations that affect neurovascular coupling (Merlini et al., 2016). It is

difficult to determine if these vascular alterations are AD-related or if they have earlier unrelated origins. Vessel wall pathology is typically observed in normal ageing but may also play a role in primary age-related taupathy (PART). It is believed that tau and amyloid are both capable to negatively affecting cerebral vasculature. In PART, hyperphosphorylation on tau created neurofibrillary tangles (NFT) and this occurs in the absence of Aβ accumulation. NFTs are present in the early stages that could develop into AD or AD with Aβ pathology. This has led scientists to pose that PART could be a pre-AD stage and not a separate disorder (Merlini et al., 2016). Braak tau pathology whether as a subdivision of PART or directly linked to AD or underlying both is accompanied by decreased cerebral blood flow. Decreased cerebral blood flow accompanied by increased vascular resistance and impaired oxygen uptake and arterial stiffness is observed in patients with mild cognitive impairment. These results suggest that early vessel wall remodeling due to changes in vessel anatomy affects blood flow (Merlini et al., 2016). Arterial wall remodeling is important because it cushions pulsatile blood flow waves so that the pulsations experienced by the fragile arteriolar and capillary walls are proportional to the wall's distension capacities. This mechanism prevents microvascular wall damage (Merlini et al., 2016). Cerebral arterial wall integrity is essential for efficient perivascular drainage and it is one of the main cerebral clearance routes. If these mechanisms are not maintained, it leads to vasculitis, BBB leakage, CAA and microhemorrhaging. Vessel specific therapeutics that protect and improve cerebral vessel wall dynamics in addition to improving cerebral clearance and therapeutic targeted against neuronal or aggregated protein targets may hold more promise than AD monotherapy (Merlini et al., 2016).

AD is not only a neurodegenerative disease but also an immune disorder. Neuroinflammation is a widely accepted hallmark of AD. As AD progresses, patients' bodies have a reduced capacity to clear the toxic $A\beta_{40}$ and $A\beta_{42}$ that their bodies are making. This results in neurotoxicity, neuroinflammation, cell death, and blood brain barrier degeneration. Aβ on its own is not inherently toxic. Although the exact functions of Aβ are not known, it is theorized to be involved in modulating synaptic

activity, cell survival, and regulation of potassium and calcium-voltage gated channels (Pearson & Peers, 2006). Excess production and impaired clearance of Aβ affects many cellular pathways including but not limited to lipid metabolism, intracellular signaling cascades, autophagy, neurotransmitter release, and synaptic function. The deregulation of these processes results in neuronal death (Jucker & Walker, 2013).

Aβ is not only toxic to microglia, but also to monocytes. This compound is toxic because phagocytosis of fibrils is restricted with leads to inflammatory responses in monocytes and microglia. This inflammatory response is a call to recruit cells from the periphery and is known as frustrated phagocytosis (Sokolowski & Mandell, 2011). When healthy monocytes are compared with AD age-matched controls, there is more unprocessed Aβ molecules, demonstrating lysosome dysfunction (Saresella et al., 2014). However, in AD peripheral monocytes and macrophages are used to compensate for microglial deficiencies in Aβ processing. This renders them as an important therapeutic target which may be able to reduce Aβ load within the parenchyma. Furthermore, rare variants of CD33 and TREM2 in monocytes causes deficiencies in phagocytosis and Aβ clearance that predisposes individuals to developing AD because they have difficulties processing $A\beta_{42}$ (Zuroff et al., 2017).

Aβ only becomes toxic as oligomeric and fibrillar species according to the amyloid cascade hypothesis. This has led to research to focusing on the fibrillogenesis of Aβ, pathways that lead to increased autophagy, and activation of neuronal signaling to help better maintain neuronal homeostasis and preventing these processes from being blocked by toxic Aβ aggregates (Karran et al., 2011). The formation of Aβ aggregates is not well understood; however, Wang et al. (2012) hypothesize that once a certain concentration of fibrils is reached, they can then catalyze the formation of toxic oligomeric species from the non-toxic and commonly found Aβ monomers. This process has been termed as secondary nucleation. Therapeutics that target this process may be effective in preventing Aβ accumulation. Furthermore, metal ions (Cu^{2+}, Sn^{2+}, and Fe^{2+}) are responsible for stabilizing toxic

Aβ oligomers. Targeting metal ions is another promising target to reduce the toxicity of Aβ oligomers (Savelieff et al., 2013). In addition, targeting proteolytic enzymes like β and γ-secretase that are involved in Aβ processing is another therapeutic that can be used to decrease Aβ load within the brain developing Aβ-specific antibodies. This was recently tested and was used in mice that had an implant containing cells that produced anti-Aβ antibodies and it was effective in decreasing Aβ load (Lathuilière et al., 2016). Finally, upregulating Aβ clearance pathways and increasing autophagy are other potential therapeutics in AD (Wisiewski & Goni, 2015).

1.4.2 Potential Therapeutics

There are only four drugs approved to treat the symptoms of AD: donepezil in 1997, rivastigmine in 2000, galantamine in 2001 and memantine in 2003. Over 100 compounds that were tested as potential therapeutics were either abandoned in development or failed in clinical trials (Mehta et al., 2017). The three main classes of drugs that have potential as therapeutics are monoclonal antibodies, gamma secretase inhibitors, and drugs targeting tau.

Bapineuzumab was the first monoclonal antibody to reach phase III of clinical trials and targeted Ab oligomers and plaque (Vandenberghe et al., 2016). Cerebrospinal fluid (CSF) tau protein was reduced but not to a clinically significant level and did not improve cognitive function (Mehta et al., 2017). Furthermore, this clinical trial was further limited when carriers of APOE ε4 allele were more likely to develop vasogenic edema (Mohandas et al., 2009).

Avagacestat, a gamma secretase inhibitor, was developed because it is implicated in reducing its activity can decrease amyloid production and specifically $A\beta_{42}$ (Coric et al., 2012). During phase II trials, there was a dose dependent decline in Aβ isoforms but T-tau did not show a significant decline (Mehta et al., 2017). Larger doses had increased side effects such as increasing the rate of skin cancer, diarrhea, nausea, and rashes. This drug did not advance to phase III clinical trials

due to its narrow therapeutic window between efficacy and toxicity (Mehta et al., 2017).

Treatments targeting tau (tau aggregation inhibitors (TAIs)) has also become a promising target in AD. According to the tau hypothesis, abnormal phosphorylation of tau results in paired helical filaments tau (PHF-tau) or neurofibrillary tangles. Once these abnormal forms stabilize microtubule assembly when subsequently inhibits axonal transport which causes cell death (Mullane & Williams, 2013). Trials with TAIs have been the most promising but have yet to be published in peer revied journals.

MDP analogs are different from the previous classes of drugs because it aims to work from the outside in. MDP analogs may help improve vascular Aβ clearance through an increased level of patrolling monocytes, with increased levels of phagocytosis in the early stages of CAA and AD. In the early stages of AD or CAA, an increased level of patrolling monocyte mediated phagocytosis would result in prolonged blood-brain Aβ homeostasis and prevent disease progression. This allows for the peripheral-sink effect to occur. This relies of monocytes phagocytosing Aβ in the blood which lowers the level of free Aβ this then causes the brain to release its store of the peptide. This storage and then release of circulating Aβ creates a shift in the concentration gradient of Aβ between the brain and blood, leading to an efflux of Aβ out of the brain. It has been proposed that reducing Aβ peptides in the blood could lead to diminished Aβ load in the blood (Xiang et al., 2015).

This treatment would allow Aβ transporters to continue to be expressed. Receptors like lipoprotein receptor-related protein 1(LRP-1), ABC transporter subfamily member A family 1 (ABCA1), and ABC transporter subfamily member B family 1 (ABCB1) are dysregulated and downregulated as the severity of AD increases (Johanson et al., 2006; El Ali & Rivest, 2013). These transporters are found in the BBB, a specialized endothelial cell membrane that lines cerebral microvessels (Zenaro et al., 2016). The BBB is essential to the generation and maintenance of chronic inflammation in AD and operates within the neurovascular unit (NVU) which

includes glial cells, neurons, and pericytes. As the NVU degrades in AD, so does the BBB, leading to functional changes that advance neuronal injury and cognitive decline (Zenaro et al., 2016). Leukocytes are able to enter the CNS through three routes: from the blood to parenchyma using the walls of parenchymal post-capillary venules, from the blood to the subarachnoid space through the walls of meningeal vessels, and from the blood to CSF across the venule wall and then through the stroma and epithelium of the choroid plexus (Ransohoff et al., 2003). During inflammation, the first two sites are used by leukocytes to invade the CNS while the third is primarily involved in immunosurveillance (Ransohoff et al., 2003). Patrolling monocytes have been seen surveying the walls of $A\beta^{+}$ veins, implying that they can naturally depose of Aβ in the lumen of the veins (Michaud et al., 2013). Maintaining the integrity of the BBB in AD may prevent the progression of the disease (El Ali & Rivest, 2013). LRP1 is involved in Aβ transport in the BBB but its exact role in uncertain. Selective deletion of LRP1 in brain endothelial cells in 5XFAD mice reduced plasma Aβ levels and increased soluble brain Aβ which led to increased spatial learning and memory deficits (Storck et al., 2016). ABCA1 is a potential AD therapeutic target because its increased activity can suppress cholesterol accumulation and can prevent Aβ accumulation, slowing the progression of the disease (Matsuo, 2022). ABCA1 prevents Aβ aggregation in an APOE dependent manner and promotes its elimination from the brain (El Ali & Rivest, 2013). As ABCA1 transporters are exposed to Aβ oligomers, their expression is significantly reduced in vitro (Sierri et al., 2022). ABCB1 is able to directly transport Aβ from the brain into the blood and is induced after orphan nuclear receptor (ONR) and pregnane X receptor (PXR) are activated human brain microvessels (El Ali & Rivest, 2013). The stimulation of these receptors provides a mechanism for increased Aβ clearance from the brain.

1.5 Hypothesis and Objectives

The conversion of $Ly6C^{high}$ to $Ly6C^{low}$ monocytes by NOD2 agonists reduces the amount of vascular Aβ invoked by AD pathophysiology. Based on this hypothesis, my work was to evaluate if MDP analogs are capable of binding specifically to NOD2,

do not elicit a proinflammatory response, are not toxic to peripheral blood mononuclear cells (PBMCs) or HepG2 cells, increase rates of Aβ phagocytosis, and are able to elicit phenotypic changes in monocytes *in vivo*.

To answer the aims of our hypothesis, we used many models to characterize the effects of NOD2 agonists *in vitro* and *in vivo*. We used HEK-Blue NOD2 and HEK-Blue TLR2 cell lines to determine which agonists specifically and optimally activated NOD2. We used PBMCs taken from AD patients to ascertain if their stimulation by NOD2 agonists increased rates of phagocytosis. In parallel, we conducted *in vivo* experiments to determine if the switch in monocyte phenotype after agonist administration is NOD2 dependent. After selecting the most effective NOD2 agonists we then tested if they negatively affected cell viability in PBMCs and the HepG2 cell line, derived from the liver. We also tested to see if they induced a proinflammatory response in human serum using the Cytometric Bead Array (CBA).

Chapter 2 MDP analogs bind to NOD2 and not TLR2

2.1 Background

In order to ensure that our MDP analogs selectively bind to NOD2, we used the HEK-Blue NOD2 and HEK-Blue TLR2 cell lines. These cell lines both use the HEK 293 engineered cell line as the base cell line where the receptor is expressed. NLRs are broadly expressed throughout body and recognize bacteria. NOD2 is specifically expressed in immune and epithelial cells. The HEK-Blue NOD2 cell line is used to study the stimulation of NOD2 and uses the activation of NF-κB/AP-1 to study now strongly NOD2 is stimulated. NOD2 is able to recognize peptidoglycan motifs associated with gram-positive and gram-negative bacteria. The HEK-Blue TLR2 cell line is used to study simulation of TLR2 and uses the activation of NF-κB/AP-1 to study how strongly TLR2 is stimulated. TLRs are able to recognize many classes of microbial molecules. TLR2 is able to recognize peptidoglycans, lipoteichoic acid, lipoarabinomannan from mycobacteria, and zymosan from the yeast cell wall (Girard et al., 2003). TLR2 is able to cooperate with TLR6 and associates with TLR1 to recognize and respond to mycoplasmal lipopeptide and triacylated lipopeptides respectively. Extracellular and intracellular domains are important for ligand recognition in TLR1 and TLR2 and signal activation (Sandor et al., 2003). HEK-Blue NOD2 and HEK-Blue TLR2 both express human or murine NOD2/TLR2 genes and in NF-kB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene that is monitored using HEK-Blue Detection media. Previous research has indicated that unmodified MDP does not bind to TLR2 (Uehori et al., 2005). The analogs that we are using are based off of MDP, but due to our contract with Amorchem, the exact modifications cannot be described here.

2.2 Methods

HEK-Blue NOD2 and HEK-Blue TLR2 cell lines were used to determine if varying analogs bound to the NOD2 or TLR2 receptor. Both cell lines use a SEAP reporter gene transfected into HEK293 cells. In the HEK-Blue NOD2 cell line, the SEAP reporter gene is placed under the IL-12 p40 promoter. This promoter is fused to five NF-κB and AP-1 binding sites. Stimulation of NOD2 by an agonist activates NF-κB

and AP-1 which induces the production of SEAP. In the HEK-Blue TLR2 cell line the SEAP reporter gene is under the control of the IFN-β promoter which is fused to AP-1 and NF-κB binding sites. In addition, the CD14 co-receptor gene was transfected into these cells in order to increase the TLR2 response. Stimulation of TLR2 with a TLR2 ligand activates NF-κB and AP-1 which then induces the production of SEAP. Agonists were tested at 0, 0.1, 0.5, 1, 3, 5, 8, 10 µg/mL and positive controls were tested at 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 µg/mL. Our positive controls are MDP for the HEK-Blue NOD2 cell line and Pam3CSK4 for the HEK-Blue TLR2 cell line. Cells from both cell lines were incubated for 16 hours at a concentration of 50,000 cells/well and reached a 70% confluency. Flat-bottomed plates from Sarstedt were used and wells measured 0.29 cm^2. Cells were maintained in an incubator at 37°C, 5% CO_2. SEAP levels were determined using HEK-Blue Detection media and a SpectraMax i3 spectrophotometer.

2.3 Results

MDP and its analogs showed varying affinity to NOD2. Using the HEK-Blue NOD2 cell line we showed that MDP, Agonist 1 and Agonist 2 all activated NOD2 in a concentration-dependent manner although Agonist 2 does activate NOD2 but not as strongly as MDP and Agonist 1 (Figure 1). Some MDP analogs did not activate NOD2 at all whereas some activated NOD2 to some degree and few activated NOD2 very well. In the HEK-Blue TLR2 cell line we used Pam3CSK4 as the positive control and strongly activated TLR2 in the HEK-Blue TLR2 cell line whereas Agonist 1 and 2 did not activate TLR2 at all (Figure 2). It is important that our agonists are specific to NOD2 as triggering TLR2 will cause further NF-kB and AP-1 production. These results demonstrate that the agonists that we are using are specific to the NOD2.

2.4 Figures

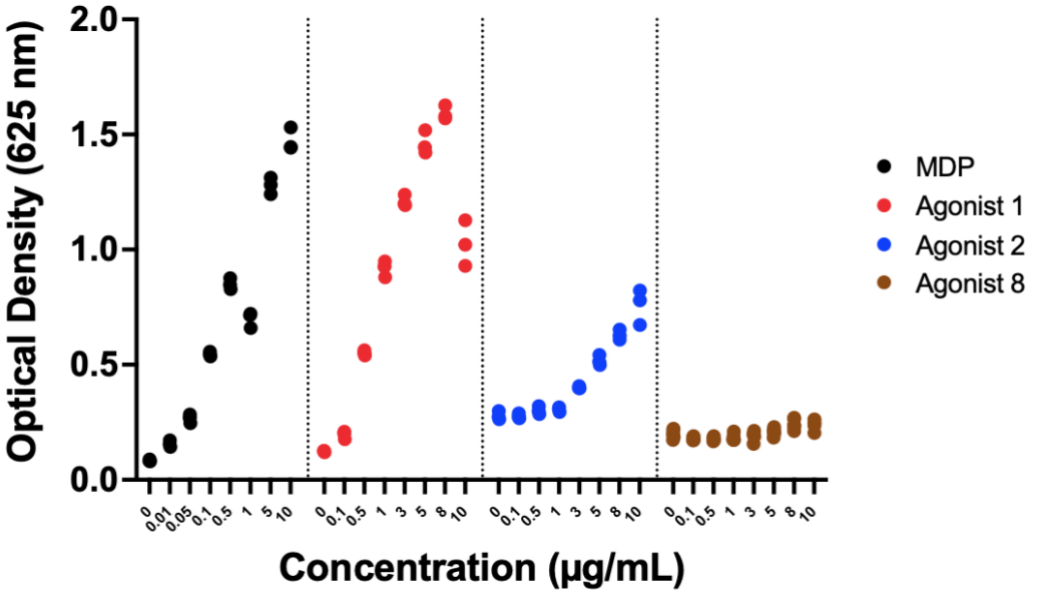


Figure 2. 1 Agonist binding in the HEK-Blue NOD2 cell line.

Agonist 1 activated to NOD2 in a concentration-dependent manner. Agonist 2 activated NOD2 but less effectively than Agonist 1 at 0, 0.1, 0.5, 1, 3, 5, 8 and 10 µg/mL (n=3, 50,000 cells/well, 70% confluency). MDP is our positive control for NOD2 (n=3, 50,000 cells/well, 70% confluency).

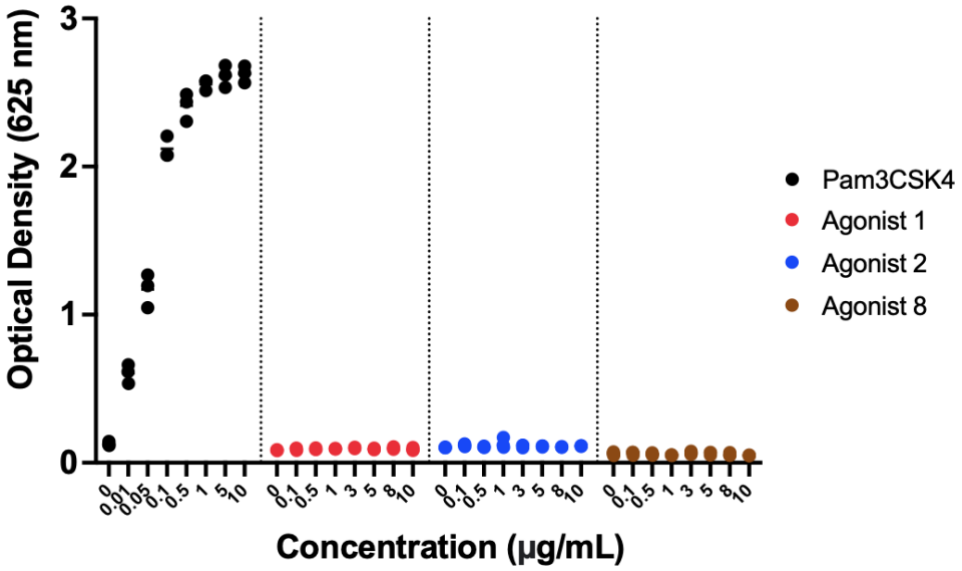


Figure 2. 2 Agonist binding in the HEK-Blue TLR2 cell line.

Pam3CSK4 activated TLR2 in a concentration-dependent manner (n=3, 50,000 cells/well, 70% confluency). Agonist 1 and Agonist 2 do not activate TLR2 (n=3, 50,000 cells/well, 70% confluency).

Agonist	Binds to NOD2	Lowest effective concentration (µg/mL)	Efficacy as compared to MDP (ie. Agonist X > MDP)	Maximal effective concentration (ug/mL)	Binds to TLR2
MDP	Yes	0.05	N/A	10	No
Agonist 1	Yes	0.5	>	10	No
Agonist 2	Yes	3.0	<	10	No
Agonist 3	Yes	0.1	<	10	No
Agonist 4	Yes	1.0	=	8	No
Agonist 5	No	N/A	<	10	No
Agonist 6	Yes	0.5	=	10	No
Agonist 7	Yes	0.5	>	10	No
Agonist 8	No	N/A	<	10	No
Agonist 9	No	N/A	<	10	No
Agonist 10	Yes	0.1	<	10	No
Agonist 11	Yes	0.5	<	10	No
Agonist 12	Yes	3.0	<	10	No
Agonist 13	Yes	0.5	<	10	No
Agonist 14	Yes	0.5	=	10	No
Agonist 15	Yes	0.1	>	10	No
Agonist 16	Yes	0.1	=	10	No
Agonist 17	No	8.0	<	10	No
Agonist 18	No	N/A	<	10	No
Agonist 19	Yes	0.5	=	10	No
Agonist 20	Yes	0.1	<	10	No
Agonist 21	Yes	0.5	<	10	No
Agonist 22	No	N/A	<	10	No
Agonist 23	Yes	5.0	<	10	No
Agonist 24	No	N/A	<	10	No
Agonist 25	Yes	3.0	<	10	No

Table 2. 1 Summary of agonist activation in HEK-Blue NOD2 and HEK-Blue TLR2 assays.

Activity was determined based off of the HEK-Blue NOD2 and HEK-Blue TLR2 assays. Lowest effective concentration is the first significantly different concentration above untreated. We then determined if the agonists were more, less, or as effective as MDP. We determined equal efficacy to MDP by comparing 5ug/mL, 8 ug/mL and 10 ug/mL of a given analog to MDP, the symbols >, <, and = signify respectively more, less, and equal to MDP. This was done using a one-way ANOVA ($n=3$, $\alpha = 0.05$) The maximal effective concentration was the concentration that OD was the highest. None of the NOD2 agonists bind to TLR2.

Chapter 3 MDP analogs do not elicit a proinflammatory response

3.1 Background

Neuroinflammation is a common trait in AD. It is important to evaluate proinflammatory cytokine production in order to ensure that the treatment does not cause further inflammation. Our MDP analogs have the potential to bind to other NLRs and TLRs, activating these receptors would cause further neuroinflammation. We performed cytometric bead arrays in order to ensure that NOD2-activated cells do not produce proinflammatory molecules.

3.2 Methods

The Cytometric bead array (CBA) Human Inflammatory Cytokines kit from BD Bioscience were used to determine if proinflammatory cytokines were produced after exposure to MDP or its analogs. Five bead populations with distinct fluorescence intensities were coated with capture antibodies specific for IL-8, IL-1β, IL-10, TNF, and Interleukin-12p70 (IL-12p70). These five bead populations are mixed together to create a bead array that can be read in a red channel of a flow cytometer. These bead populations aka Capture Beads are then mixed with PE-conjugated detection antibodies and incubated with recombinant standards or test tables to create sandwich complexes. Human Chemokine Standards are provided with the BD CBA kit and are reconstituted and then serially diluted before mixing with Capture Beads and PE Detection Reagent. Serial dilutions were performed between Human Chemokine Standards and Assay Diluent at concentrations of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. One tube contained only Assay Diluent to serve as our negative control. This panel of dilutions served as our standard for the assay. These standards can detect chemokine concentrations in a range of 10-2500 pg/mL. Human blood was taken from AD patients and then treated with LPS, MDP, DMSO or agonists for three hours. MDP, DMSO, and agonists were added at a concentration of 10 µg/mL whereas LPS, our positive control, was added at a

concentration of 1 μg/mL. We evaluated the production of TNF, IL-8, IL-12p70, IL-10, IL-6 and IL-1β, according to the CBA assay. Serum was then taken to be used as the sample which was centrifuged from the whole blood and then collected. A 1:1 ratio of Capture Beads and standard/sample was used, indicating that the number of chemokines present in 50μL of serum was within the 10-2500 pg/mL range. A flow cytometer acquired data from the samples (n=2). FlowJo and GraphPad Prism was used to process this data. A logarithmic standard curve was created to determine the pg/mL of IL-8. Then a one-way ANOVA was used to compare IL-8 production between MDP and its analogs.

3.3 Results

MDP and its analogs did not trigger the production of many proinflammatory cytokines except for IL-8 (Figure 3). MDP produces much larger amounts of IL-8 when compared to agonists, LPS, and DMSO. IL-8 is involved in the recruitment of neutrophils to the site of injury and those neutrophils cause subsequent inflammation. IL-8 is also secreted in order to increase the adhesion of cells to the walls of cerebral blood vessels. The effects of IL-8 are context dependent These results demonstrate that the MDP analogs synthesized by Amorchem do not cause the production of proinflammatory cytokines when present in blood. Some of the agonists were not tested in the CBA assay due to their elimination from the screening based off of the data from the previous HEK-Blue NOD2 and HEK-Blue TLR2 screenings.

3.4 Figures

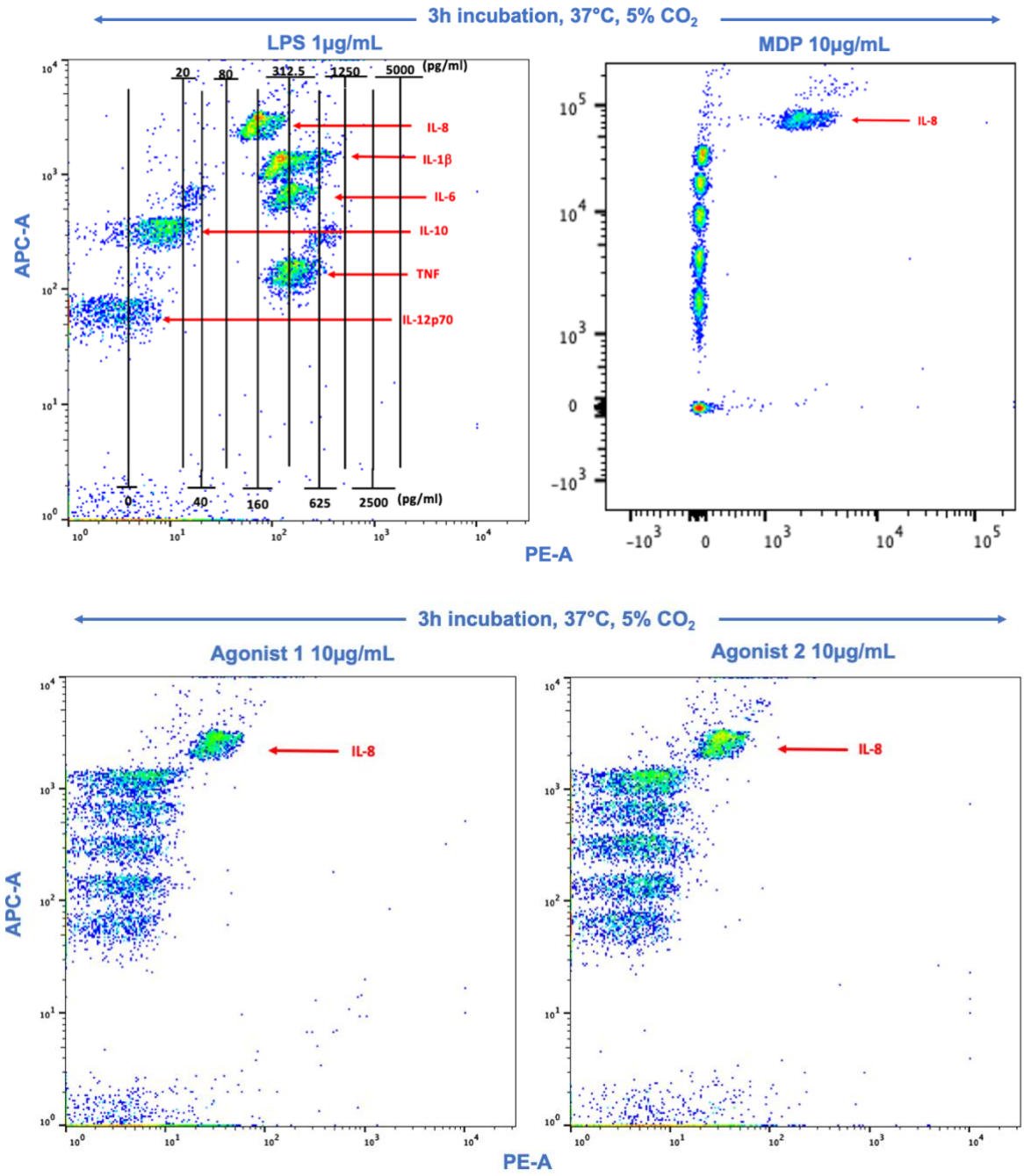


Figure 3. 1 MDP, Agonist 1, and Agonist 2 produce IL-8 after exposure in whole blood.

LPS at 1µg/mL elicited a strong proinflammatory response causing the release of Il-1β, IL-6, and TNF whereas MDP at 10 µg/mL did not elicit a proinflammatory response but did release IL-8. Agonists 1 and 2 at 10 µg/mL released IL-8 as well (n=2).

Agonist	Produces IL-8	IL-8 concentration (pg/mL)	Significance level (X : MDP)
LPS	Yes	160.00	****
DMSO	Yes	35.141	****
MDP	Yes	5002.221	N/A
Agonist 1	Yes	40.420	****
Agonist 2	Yes	43.764	****
Agonist 3	Yes	43.764	****
Agonist 4	Yes	43.764	****
Agonist 5	Yes	35.141	****
Agonist 6	Yes	40.420	****
Agonist 7	Yes	37.570	****
Agonist 8	Yes	43.764	****
Agonist 9	Yes	23.559	****
Agonist 10	Yes	40.420	****
Agonist 11	Yes	43.764	****
Agonist 12	Yes	37.570	****
Agonist 13	Yes	43.764	****
Agonist 14	Yes	35.141	****
Agonist 15	Yes	43.764	****
Agonist 16	Yes	43.764	****
Agonist 17	Yes	40.420	****
Agonist 18	Yes	43.764	****
Agonist 19	Yes	40.420	****
Agonist 20	Yes	43.764	****
Agonist 21	Yes	40.420	****
Agonist 22	N/A	N/A	N/A
Agonist 23	Yes	33.072	****
Agonist 24	N/A	N/A	N/A
Agonist 25	N/A	N/A	N/A

Table 3. 1 Summary of agonist cytokine production in CBA assay.
Agonists were tested in the CBA assay in order to determine how much of selected proinflammatory cytokines are produced after exposing agonists to whole blood. IL-8 production of the agonists was then compared to IL-8 production from MDP. n=2, $\alpha = 0.05$, ****$p \leq 0.0001$.

Chapter 4 MDP analogs are not toxic to PBMCs or HepG2 cells

4.1 Background

In order to ensure that MDP or the NOD2 agonists did not affect cell viability, we used the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay in peripheral blood mononuclear cells from AD patients and in the HepG2 cell line. The HepG2 cell line was isolated and immortalized from a human liver, an organ responsible for the degradation of many molecules and metabolites.

4.2 Methods

The MTS cell viability assay was used to determine if analogs reduced cell viability in peripheral blood mononuclear cells (PBMCs) isolated from AD patients and the HepG2 cell line. PBMCs were isolated from AD patients using the and then resuspended in Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS), and pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). HepG2 cells were kept in Eagle's minimum essential medium (EMEM), 10% FBS, and pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). Cells were plated at 100,000 cells/well for PBMCs and 50,000 cells/well for HepG2 cells. Flat-bottomed plates from Sarstedt were used and wells measured 0.29 cm^2. Cells were maintained in an incubator at 37°C, 5% CO_2. Saline, MDP, and Agonists were all added at a concentration of 10 µg/mL when the cells were initially plated and then again at 24 hours. MTS was added to wells at a concentration of 10µL MTS/100µL media at 72 hours. The agonists provided by Amorchem are suspended in dimethyl sulfoxide (DMSO) and the 1/100 control concentration of DMSO was used in order to replicate the actual concentration of DMSO in the wells with the agonist. MDP is suspended in endotoxin-free water and saline is used as a control for MDP. Our negative controls for PBMCs and HepG2 cells were frozen PBMCs that were plated at a concentration of 100,000 cells/well and frozen HepG2 cells that were plated at a concentration of 50,000 cells/well. These cells serve as our control because they were frozen only in DMEM/EMEM and lysed upon freezing, rendering them

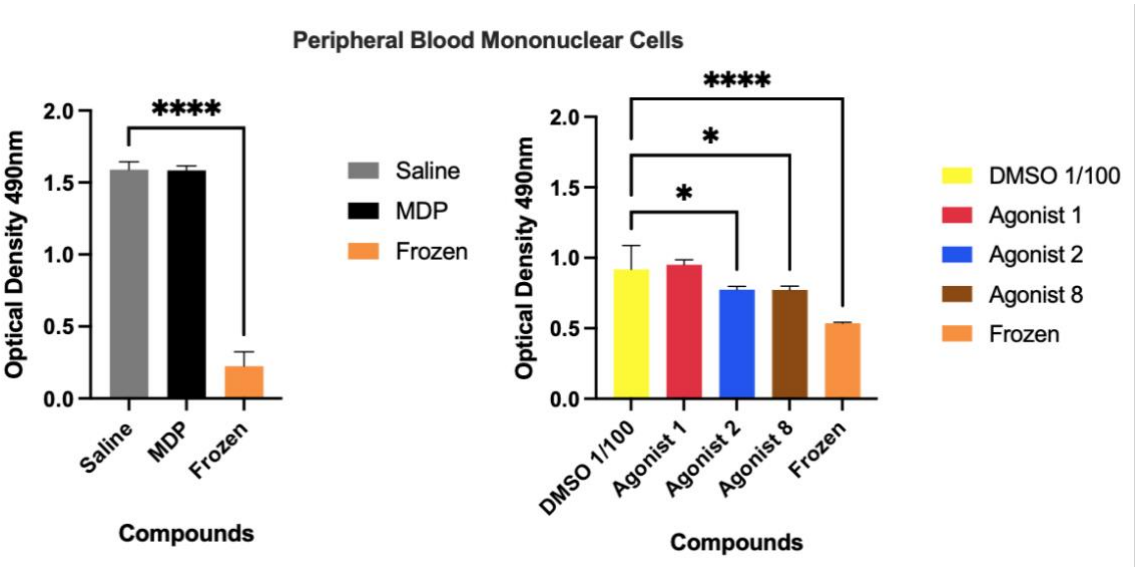
completely unviable. Once the cells were plated they were left to incubate for 72 hours. One round of analogs is added upon seeding and 24 hours later. MTS is added at 72 hours and cells are left to incubate with the MTS for 3-4 hours and read the plate in the SpectraMax i3 spectrophotometer. A one-way ANOVA was used to determine if agonists were toxic to PBMCs or HepG2 cells ($n=6$, $\alpha = 0.05$).

4.3 Results

Our lab demonstrated that MDP and Agonist 1 are not toxic to PBMCs from AD patients in the early stages of AD. Agonists 2 and 8 demonstrated decreased cell viability in PBMCs (Figure 4.4A). MDP and Agonists 1, 2, and 8 were not toxic to HepG2 cells (Figure 4.4B). These results could be explained by the differing functions and properties of innate immune system cells and hepatic cells. Hepatic cells can metabolize a wide range of toxic and non-toxic molecules whereas innate immune cells not as readily equipped to do this. It is important that these agonists do not induce cell death in PBMCs because it would further reduce the number of cells that are able to phagocytose Aβ in AD patients. Ensuring that synthesized NOD2 agonists do not induce a proinflammatory response is key to the development of this project because prevalent cytotoxicity to the liver or PBMCs would have stopped this project. Some of the agonists were not tested in HepG2 cells due to their elimination from the screening based on the data from the previous HEK-Blue NOD2 and HEK-Blue TLR2 screenings.

4.4 Figures

A.



B.

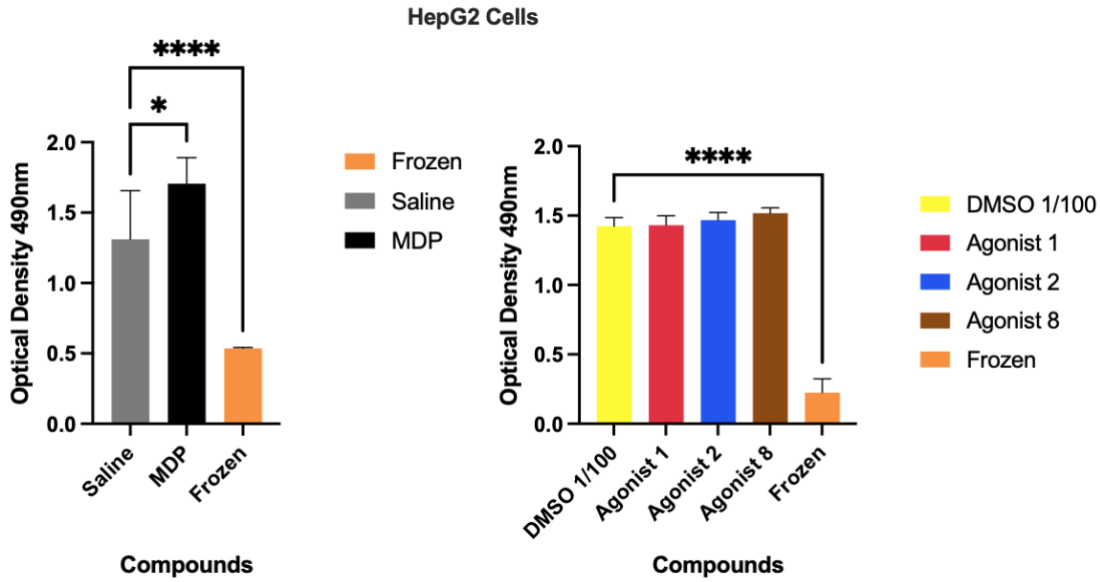


Figure 4. 1 Agonists are not toxic to PBMCs from AD patients or HepG2 cells. (A) Cells were plated at a concentration of 100,000 cells/well in DMEM 10% FBS pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). Saline, MDP, and Agonists were all added at a concentration of 10 µg/mL upon cell plating and again

at 24 hours. MTS was added to wells at a concentration of 10µL MTS/100µL media at 72 hours. Toxicity was not seen in Agonist 1 when compared to DMSO 1/100 in PBMCs. Toxicity was demonstrated in Agonist 2 in PBMCs when compared to DMSO 1/100 using a one-way ANOVA. Frozen PBMCs/frozen HepG2 cells were used as our negative control. Increases in toxicity are demonstrated by decreases in optical density. Agonists are suspended in DMSO while MDP is suspended in endotoxin-free water. A one-way ANOVA was used n=6, $\alpha = 0.05$, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 ****p ≤ 0.0001 (B) Cells were plated at a concentration of 50,000 cells/well EMEM 10% FBS pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). Saline MDP, and Agonists were all added at a concentration of 10 µg/mL upon cell plating and again at 24 hours. MTS was added to wells at a concentration of 10µL MTS/100µL media at 72 hours. Toxicity was not seen in Agonist 1 or Agonist 2 when compared to DMSO 1/100 in HepG2 cells. Increases in toxicity are demonstrated by decreases in optical density. Agonists are suspended in DMSO while MDP is suspended in endotoxin-free water. A one-way ANOVA was used n=6, $\alpha = 0.05$, *p ≤ 0.05, ****p ≤ 0.00005.

Agonist	PBMCs toxic when compared to 1/100 DMSO	Significance level (α = 0.05)	HepG2 cells toxic when compared to 1/100 DMSO
Agonist 1	No		No
Agonist 2	Yes	***	No
Agonist 3	No		No
Agonist 4	No		No
Agonist 5	No		N/A
Agonist 6	No		No
Agonist 7	No		N/A
Agonist 8	No		No
Agonist 9	No		N/A
Agonist 10	No		N/A
Agonist 11	No		No
Agonist 12	Yes	****	No
Agonist 13	No		No
Agonist 14	No		No
Agonist 15	No		No
Agonist 16	Yes	***	No
Agonist 17	No		No
Agonist 18	No		No
Agonist 19	No		No
Agonist 20	No		No
Agonist 21	No		No
Agonist 22	No		N/A
Agonist 23	No		No
Agonist 24	No		N/A
Agonist 25	No		No

Table 4. 2 Summary of agonist toxicity in PBMCs and HepG2 cells in the MTS assay.

Agonists were tested in PBMCs isolated from AD patients in the early stages of the disease in addition to HepG2 cells. PBMCs were isolated from AD patients using the and then resuspended in DMEM 10% FBS pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). HepG2 cells were kept in EMEM 10% FBS pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). Cells were plated at 100,000 cells/well for PBMCs and 50,000 cells/well for HepG2 cells (n=6). Toxicity was tested for in PBMCs and HepG2 cells when they were compared to their respective DMSO 1/100 conditions using a one-way ANOVA. n=6, $\alpha = 0.05$, ***$p \leq 0.001$ ****$p \leq 0.0001$

Chapter 5 MDP analogs increase rates of Aβ phagocytosis

5.1 Background

Increasing phagocytosis is an important aspect of our hypothesis, and in order to determine if analogs can enhance amyloid uptake compared to MDP we conducted a phagocytosis assay using HiLyte Fluor 488 $Aβ_{1-40}$. This kind of amyloid is the main form found in blood vessels. Phagocytosis of Aβ allows for the sink effect to occur which involves monocytes phagocytosing Aβ in the blood which lowers the level of free Aβ this then causes the brain to release its store of the peptide. This creates a shift in the concentration gradient of Aβ between the brain and blood, leading to an efflux of Aβ out of the brain. It has been proposed that reducing Aβ peptides in the blood could lead to diminished Aβ load in the blood (Xiang et al., 2015).

5.2 Methods

Phagocytosis assays were performed to determine if analogs could induce increased phagocytosis of soluble $Aβ_{40}$ by patrolling monocytes at a higher rate than the positive control. Blood was taken from AD patients with a Mini-Mental State Examination (MMSE) score of 19 to 24 (early-stage AD). During the first day of treatment, 30 mL of human blood was collected from participants and then 400 µL was transferred to EDTA tubes. 10 µg/mL of MDP or agonists were added to the blood. 10 µg/mL of DMSO was used to account for the effect of DMSO on blood. Our agonists are solubilized in DMSO and our positive control is 10 µg/mL DMSO and 1 µg/mL of HiLyte Fluor 488 $Aβ_{1-40}$. The blood is then kept at 37°C, 5% CO_2 for 24 hours. During the second day of treatment, 10 µg/mL of MDP, agonists, and DMSO are added to their respective EDTA tubes and incubated for another 24 hours on a rotator at 37°C, 5% CO_2. During the third day of treatment, 1 µg/mL of HiLyte Fluor 488 $Aβ_{1-40}$ is added to all tubes except the negative control which only contains blood and has not been treated. The tubes are then left to incubate for four hours on a rotator. Then a fluorescence activated single-cell sorting (FACS) is performed and the blood is stained with CD14 and CD16 to separate them as classical

(CD14^{++}CD16^{-}) and non-classical (CD14^{+}CD16^{++}) human monocytes. Cells were then prepared for the FACS using the following antibodies: CD14/PE and CD16/AF700. Aβ is already marked with HiLyte Fluor 488 $Aβ_{1-40}$. The cells that had the CD14CD16 staining and HiLyte Fluor 488 $Aβ_{1-40}$ were counted as cells that had phagocytosed Aβ. GraphPad Prism was used to conduct the one-way ANOVAs $α = 0.05$.

5.3 Results

The results showed that MDP was able to match the rate of the positive control. Further testing is needed to see if statistical significance can be achieved in MDP or other agonists. Agonist 1 was also able to match the rate of the positive control. Many of the agonists were tested once in this assay and further testing will be needed if they are chosen for *in vivo* work. Further specific studies on phagocytic capacities of monocyte subsets in needed in order to determine if a certain monocyte subtype has the best phagocytic capacity.

5.4 Figures

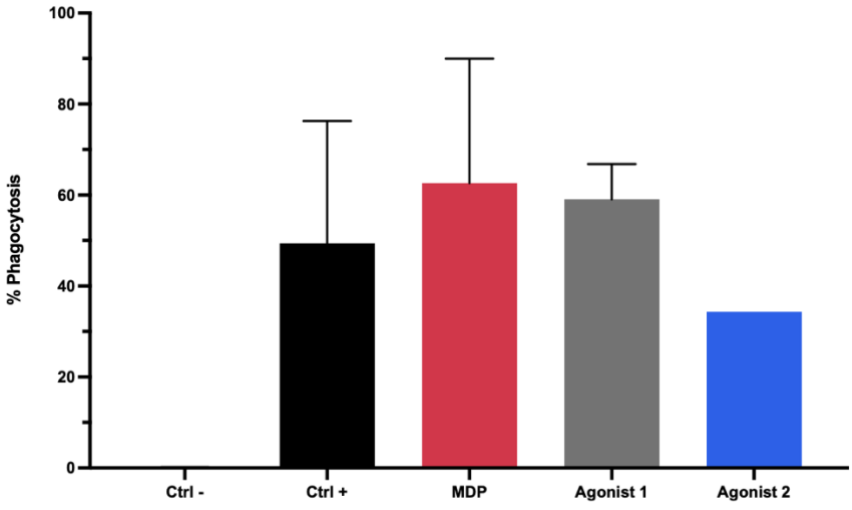


Figure 5. 1 Agonist 1 has increased phagocytosis in whole blood compared to the positive control.
Early data suggests that Agonist 1 may phagocytose $Aβ_{40}$ at an equal rate to MDP but a larger sample size is needed to reach statistical significance. Whole blood was incubated for 48 hours with Agonists at a concentration of 10 μg/mL and then four-

hour incubation was conducted with HiLyte Fluor 488 $A\beta_{1-40}$ at a concentration of 1 µg/mL. Statistics were performed using a one-way ANOVA $\alpha = 0.05$.

Agonist	% Phagocytosis Aβ+ monocytes: total monocytes	N	Significance level
Negative control	0	9	N/A
Positive control	58.34	9	N/A
MDP	62.61	5	ns
Agonist 1	59.04	8	ns
Agonist 2	34.32	1	N/A
Agonist 3	36.12	1	N/A
Agonist 4	N/A	1	N/A
Agonist 5	41.77	1	N/A
Agonist 6	33.22	1	N/A
Agonist 7	N/A	1	N/A
Agonist 8	N/A	1	N/A
Agonist 9	N/A	1	N/A
Agonist 10	50.09	1	N/A
Agonist 11	55.80	7	ns
Agonist 12	24.78	1	N/A
Agonist 13	28.81	1	N/A
Agonist 14	54.66	7	ns
Agonist 15	23.23	1	N/A
Agonist 16	18.95	1	N/A
Agonist 17	34.73	1	N/A
Agonist 18	54.76	1	N/A
Agonist 19	29.90	1	N/A
Agonist 20	29.07	1	N/A
Agonist 21	18.85	1	N/A
Agonist 22	N/A	1	N/A
Agonist 23	N/A	1	N/A
Agonist 24	N/A	1	N/A
Agonist 25	N/A	1	N/A

Table 5. 1 Summary of agonists in phagocytosis assay.
Whole blood was incubated for 48 hours with Agonists at a concentration of 10 µg/mL and then four-hour incubation was conducted with HiLyte Fluor 488 $A\beta_{1-40}$. at a concentration of 1 µg/mL. Statistics were performed using a one-way ANOVA; $\alpha = 0.05$.

Chapter 6 MDP analogs elicit phenotypic changes in monocytes *in vivo*

6.1 Background

Previous research has demonstrated that MDP is capable of changing monocyte phenotype from inflammatory to patrolling but we reconducted these experiments to support our previous findings (Lessard et al., 2017). The exact mechanisms of inflammatory to patrolling monocytes are not known

6.2 Methods

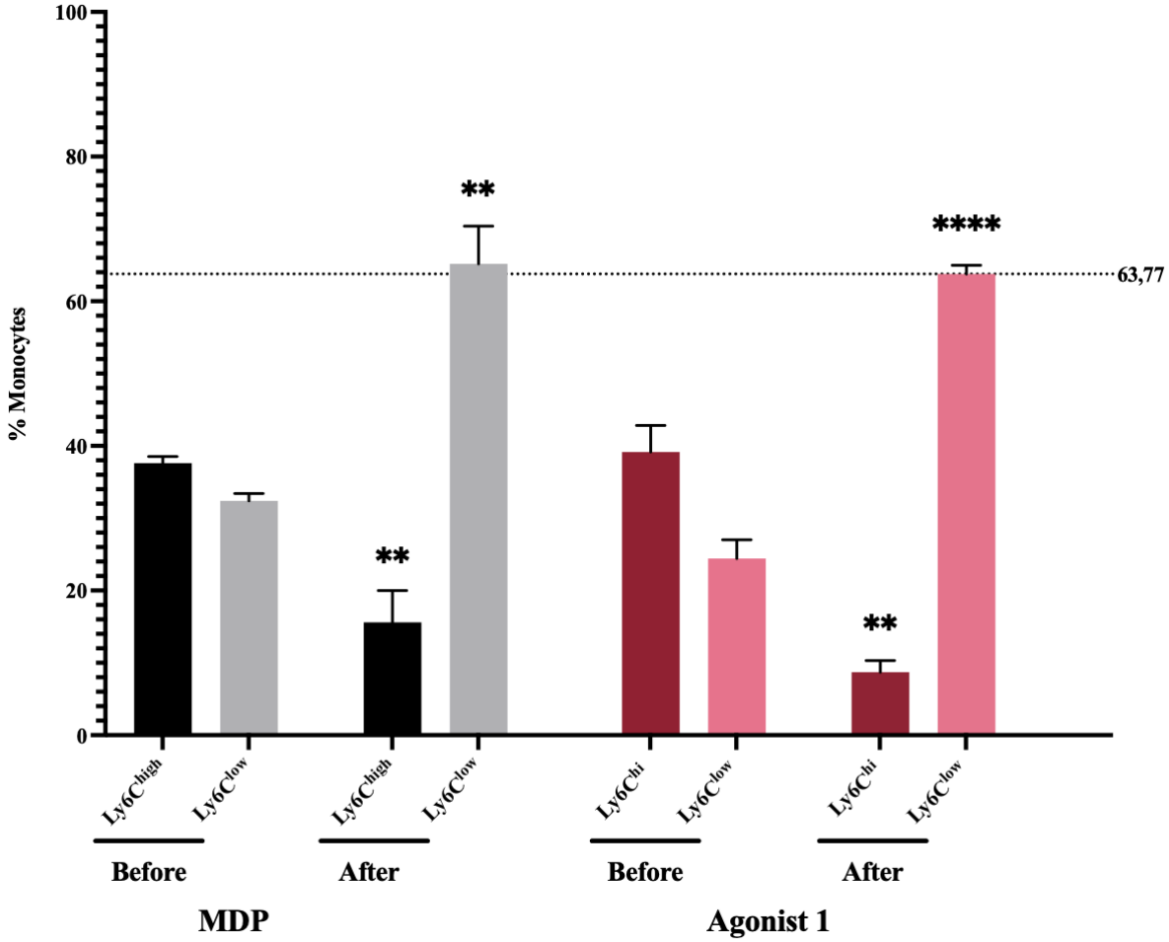
Tests were performed *in vivo* to determine if monocyte phenotype could be changed from the inflammatory to the patrolling phenotype after MDP or analog injections. Injections were done 1x daily for 3 days at 10mg/kg in WT mice and NOD2 KO mice. A FACS was performed the 4th day using blood from the submandibular vein. The antibodies used to differentiate between different phenotypes of monocytes are CD11b, Ly6C, CD45, and Live-Dead staining. A two-way t-test was used between the before and after of each Ly6C type, high and low ($\alpha = 0.05$, **p=0.01, ****p=0.0001).

6.3 Results

Only a few agonists from our *in vitro* assessments were chosen for the *in vivo* assessments. We assessed if Agonist 1 could cause a change in phenotype. As shown in Figure 5A, MDP and Agonist 1 were capable of changing monocyte phenotype from inflammatory to patrolling *in vivo*. To confirm that the switch is NOD2 specific we used NOD2 KO mice which demonstrated no change in monocyte phenotype after MDP and Agonist 1 administration (Figure 5B), implying that NOD2 activation is necessary for monocyte conversion.

6.4 Figures

A.


100
80
60
40
20
0
% Monocytes
**
**
****
63,77
Ly6C^high
Ly6C^low
Ly6C^high
Ly6C^low
Ly6C^hi
Ly6C^low
Ly6C^hi
Ly6C^low
Before
After
Before
After
MDP
Agonist 1

B.

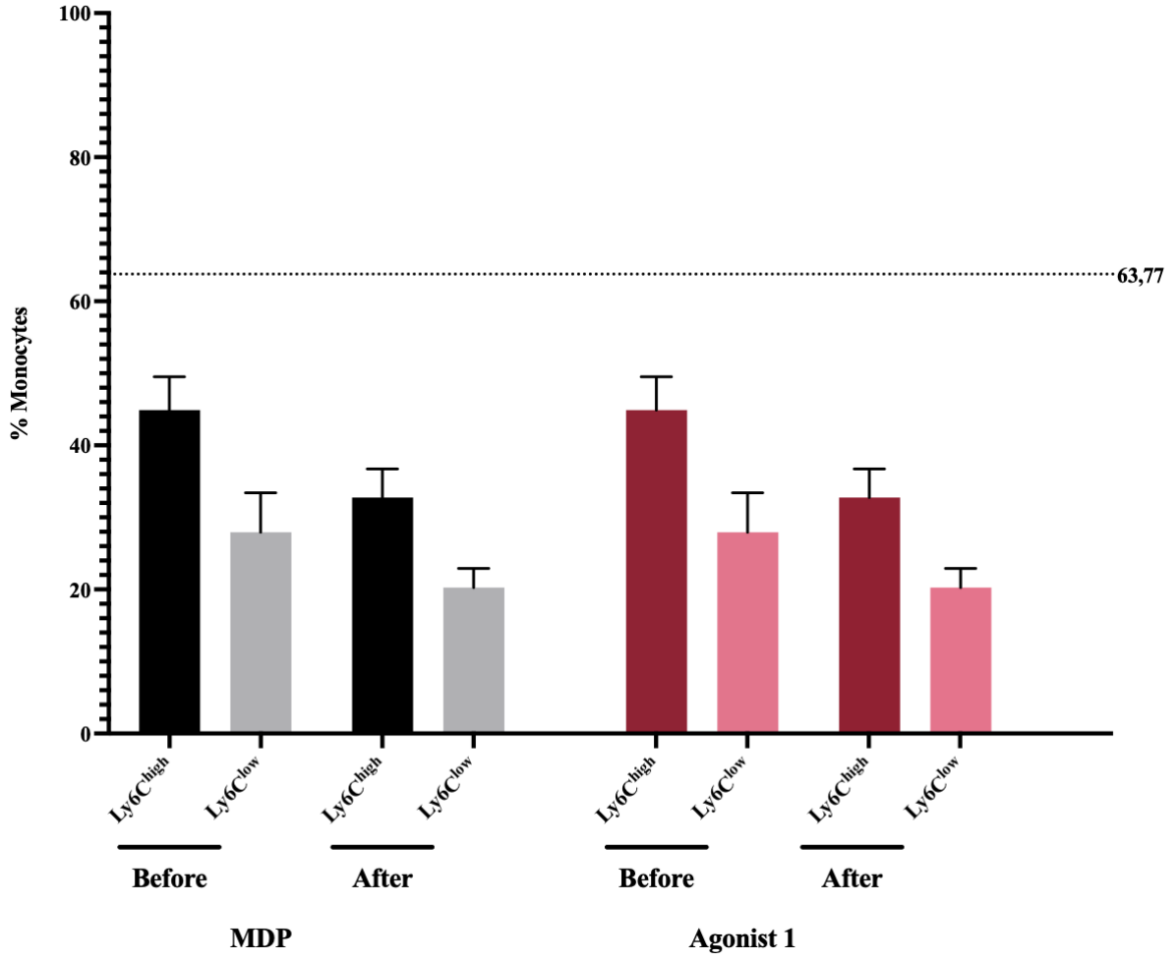


Figure 6. 1 Ly6Clow monocytes are significantly increased after MDP or Agonist 1 injections in mice and NOD2 KO prevents monocyte conversion after MDP and Agonist 1 injections.

(A) Injections were done 1x daily for 3 days at 10mg/kg in WT mice (n=4). A FACs was performed the 4th day using blood from the submandibular vein. The antibodies used to differentiate between different phenotypes of monocytes are CD11b, Ly6C, CD45, and Live-Dead staining. A two-way t-test was used to determine if there was statistical significance between the before and after of each Ly6C type (high and low), $\alpha = 0.05$, $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. **(B)** Injections were done daily for 3 days at 10mg/kg in NOD2 KO mice (n=4). A two-way t-test was used, $\alpha = 0.05$. A FACs was performed the 4th day using blood from the submandibular vein. The antibodies used to differentiate between different phenotypes of monocytes are CD11b, Ly6C, CD45, and Live-Dead. A two-way t-test was used to determine if there was statistical significance between the before and after of each Ly6C cell type (high and low) and none was found.

Chapter 7 Discussion

AD is the most prominent form of dementia but it is also a neuroinflammatory disorder. AD frequently overlaps with vascular dementia due to the high prominence of CAA in AD patients. With increased understanding of the inflammatory components of AD it leaves scientists wondering if these mechanisms are the primary cause of damage or if they are the responses to the root pathogenic processes. Basic research studies and clinical research have given direct and tangential evidence for the neurodegenerative role of AD inflammatory processes. Central nervous system (CNS) inflammation in AD is multifaceted from vascular to changes in the kind and accumulation of Aβ. This inflammation is likely to start decades before clinical symptoms appear. Once a certain threshold of Aβ or tau is reached, surveying microglia within the brain are activated, eliminating Aβ laden neurons but amplifying toxic molecular responses (Cuello, 2017). This response constitutes a proinflammatory process whose mechanisms could be controlled with anti-inflammatory agents like MDP and its analogs (Cuello, 2017).

Chapter 2

Our findings support previous research that MDP and its analogs are NOD2 specific. Our results from HEK-Blue NOD2 and HEK-Blue TLR2 cell culture lines demonstrate that the synthesized MDP analogs are specific to NOD2 and bind in a concentration dependent manner. TLR2 is one of the main receptors that $A\beta_{42}$ binds to in order to trigger neuroinflammation in AD (Liu et al., 2012). TLRs are expressed on innate immune cells such as DCs, microglia and macrophages in addition to non-immune cells like fibroblasts and epithelial cells (Kawasaki & Kawai, 2014). Liu et al. found that deficiencies in TLR2 in myeloid cells causes them to shift from M1 into M2 inflammatory activation in AD mouse brains. Myeloid cells include granulocytes, monocytes, macrophages, and dendritic cells (De Kleer et al., 2014). Stimulating myeloid cells may cause them to differentiate into M1 macrophages, increasing neuroinflammation. Microglial TLR2 mediates pathological processes in AD by identifying Aβ and causing subsequent microglial activation and blocking microglial TLR2 results in Aβ accumulation (Lax et al., 2020). TLR2 stimulation may be

involved in the progressive loss of cortical neurons when they bind to external TLR2 agonists (Lax et al., 2020). It is important that our analogs do not activate TLR2 leading to the previously stated consequences.

It is not expected that MDP analogs would bind to TLR2 in the manner that we have modified them. Research has demonstrated that specific alterations of MDP such as acetylation allowing for the addition of a long fatty acid chain, give it the capacity of binding to TLR2, but these modifications have not been performed in our MDP analogs. The addition of the fatty acid chain allows the molecule to insert itself into the TLR2 pocket and activate the receptor (Uehori et al., 2005; Jin et al., 2007). Furthermore, NOD2 and TLR2 are distinctly different from each other. It is predicted that MDP binds on the concave surface of the leucine rich region (LRR) in NOD2 (Figure 1.2). TLR2 has distinct pockets that hold one long hydrocarbon chain per pocket and is fixed in place by a polar group at the head of the chain. TLR2 responds to lipoteichoic acid (LTA) and the synthetic bacterial lipopeptide Pam3-Cys-Ser-Lys4 (Pam3CSK4) (Brandt et al., 2013).

Chapter 3

The CBA assay revealed that IL-8 is produced when whole blood is exposed to MDP and MDP analogs. It is important to note that MDP analogs produce less than one tenth of IL-8 when compared to MDP. IL-8 is typically secreted from leukocytes and endothelial cells after they are exposed to IL-1 and TNF-α. IL-8 is able to bind to two heterotrimeric G protein-coupled receptors CXCR1 and CXCR2. These receptors are found in neutrophils, monocytes, and endothelial cells (Long et al., 2016). IL-8 a potent angiogenic factor implicated in tumor growth and metastasis in addition to being a biomarker in chronic inflammatory conditions (Warner et al., 2015). IL-8 is produced by macrophages and is likely to be involved in the recruitment of inflammatory cells (Apostolopoulos et al., 1996). IL-8 is responsible for attracting and activating neutrophils in inflammatory regions. Neutrophils make up between 40-70% of all white blood cells, are able to kill and digest bacteria and fungi, and are also implicated in found healing and infection fighting. Neutrophils in response to IL-

8 stimulation migrate to the inflamed region, release granule enzymes, among other intra- and extracellular changes (Bickel, 1993). It is important to note that the biological effects of IL-8 are context-dependent (Warner at al., 2015). IL-8 regulated by NF-κB at the transcriptional level, but IL-8 secretion can occur independently of NF-κB. These IL-8 specific regulators are likely to influence IL-8 secretion downstream of NF-κB or in parallel. More research is needed to understand the steps of IL-8 gene regulation in addition to individual genetic variation, local vs systemic production, type of stimulus, and tissue specificity. More work is needed to better understand the role of IL-8 in the context of AD.

Vascular cell adhesion molecule 1 (VCAM-1) is a member of the immunoglobin super family and is usually expressed by endothelial cells and is specifically involved in adhesion of only lymphocytes and monocytes to the endothelium (Zulliani et al., 2008). IL-8 has been previously shown to trigger adhesion of monocytes to the vascular endothelium because it is able to increase expression of molecules like VCAM-1 and E-selectin (Gerszten et al., 1999). VCAM-1 is expressed in large and small blood vessels and is more common in atherosclerotic plaques than in healthy segments (Zullianai et al., 2008). Because none of the other classically pro-inflammatory chemokines were produced in this assay, we propose that IL-8 may be involved in increasing expression of VCAM-1 which allows for monocyte adhesion and recruitment to endothelial cell walls in cerebral blood vessels. Further testing is needed to ensure that IL-8 does not cause neutrophil recruitment and subsequent inflammation.

Chapter 4

The assay that we primarily used to detect toxicity in PBMCs and HepG2 cells was the MTS assay. This assay measures NADPH-dependent dehydrogenase enzymes to reduce the tetrazolium dye into formazan in metabolically active cells. The cells' ability to reduce the dye and quantity of formazan produced is thought to reflect their metabolic capacity. The optic density is used as an estimation of the number of active mitochondria and therefore the number of living cells within a sample. There are

certain limitations to be observed in this assay. A study by Lü et al. (2012) found that the insoluble form of formazan from the MTT assay could exacerbate cell injury. In the MTS assay, the formazan is soluble and does not pose a physical threat to the cell membrane. However, the MTT metabolism and exocytosis of MTT has the potential to damage cells (Lu et al., 2012). The exact biological effects of MTS remain elusive and there is potential that the MTS assay did not negatively affect the viability of PBMCs or HepG2 cells.

When using PBMCs they were isolated from whole blood from AD patients and placed into DMEM supplemented with 10% FBS and pen strep (100 I.U./mL penicillin and 100 ug/mL streptomycin). It was important to test PBMCs in AD patients in order to see if there was outright toxicity to PBMCs from the MDP analogs. Because the PBMCs were tested outside of their natural environment and incubated for 72 hours, results from these cells were less consistent than the HepG2 cell line. The HepG2 cell line provided much more stability from an assay standpoint because it is an immortalized cell culture line from cancerous liver cells and are able to provide some insights to if MDP analogs are toxic to liver cells. Due to their proliferative abilities, these cells were easier to measure and provided clearer results. HepG2 cells have retained some features of hepatocytes such as albumin secretion, insulin-stimulated glycogen synthesis, and glutathione-based detoxification (Krammerer & Küpper, 2018). However, this cell line does not reflect the biology and physiology of healthy hepatocytes. Because HepG2 cells are derived from a hepatoblastoma, there is very little to no expression of human liver phase I (except CYP2B6) and phase II enzymes. These enzymes are involved in these phases are responsible for breaking down fat-soluble toxins and eliminating water-soluble waste (Muhammad et al., 2018). Another limitation of the MTS method is that metabolite buildup is observed at around 4 hours, and it becomes more difficult to distinguish over time if there are changes in cell viability due to the high background.

Chapter 5

Using soluble $A\beta_{1-40}$ (HiLexi Fluor 488) does not account for the oligomers and plaques that form in AD pathology. More research is needed to see if oligomers and plaques can be phagocytosed by patrolling monocytes. Agonist 1 is one of our more promising analogs whereas Agonist 2 activates to NOD2 but not particularly strongly. In this assay, Agonist 2 was only tested once because it was not a strong candidate from the HEK-Blue NOD2 assay (Figure 2.1). Furthermore, many of the agonists were only tested once in this assay, more testing will be required in order to determine if there is a significant increase in phagocytosis (Table 5.1).

Chapter 6

Not only does our lab see NOD2 specific activation *in vitro*, but also clearly see activation of NOD2 resulting in phenotypic conversion *in vivo* (Figure 2.1; Figure 6.1 A&B). When we administered MDP and its analogs *in vivo* in NOD2 KO mice we do not see phenotypic conversion of monocytes. However, this does not allow us to conclude that MDP and its analogs could bind to other NLRs. Further research is needed to verify that MDP analogs are not capable of binding to other NLRs. This can be achieved through the use of various NLR cell culture lines. Further research is needed to better understand how long this phenotypic conversion is maintained in addition to what kind of Aβ the patrolling monocytes phagocytose *in vivo*.

General Discussion

Our findings demonstrate that MDP analogs are NOD2 specific, do not induce a strong proinflammatory response, are generally not toxic to PBMCs or HepG2 cells, may increase the rate of phagocytosis and can change monocyte phenotype *in vivo*.

The mechanisms that cause $Ly6C^{high}$ monocyte conversion remains to be elucidated. Our lab's previous research has demonstrated that after triggering NOD2 with MDP, a significant proportion of $Ly6C^{high}$ monocytes express the surface markers typically associated with patrolling monocytes. In $NOD2^{-/-}$ mice, we do not see an increase in the number of patrolling monocytes, indicating that this conversion mechanism is NOD2 mediated. When our lab treated Nr4a1-/- mice with

MDP, we found that a significant proportion of blood monocytes had the typical surface marker expression and functions of patrolling monocytes. Furthermore, our lab's mRNA analysis demonstrated that Nr4a1 was significantly increased in Ly6C^{high} monocytes and only moderately affected Ly6C^{low} monocytes after *in vivo* treatment with MDP. It is likely that Nr4a1 contributes to the NOD2 cascade in Ly6C^{high} monocytes but because conversion from Ly6C^{high} to Ly6C^{low} monocytes was observed in Nr4a1-deficient mice there may be another signaling cascade at play. It is theorized that C/EBPβ acts upstream of Nr4a1. MDP treatment increases C/EBPβ expression in monocyte subsets. Our lab theorizes activating NOD2 may also trigger C/EBPβ downstream leading it to bind to other promoters that participate in differentiation of Ly6C^{high} monocytes when Nr4a1 is absent. Further research of the mechanism is needed to support this theory.

The specific role of NOD2 in the regulation of inflammatory responses is ambiguous. It is not clear if NOD2 variant-related inflammation is caused by an impaired ability to control bacterial clearance or to regulate excessive inflammatory responses. Our lab's previous research has demonstrated that treatment with MDP reduces inflammatory responses in a murine model of systemic inflammation and in an arthritis mouse model of chronic inflammation (Lessard et al., 2017). Both models had increased levels of circulating Ly6C^{low} patrolling monocytes which suggests that the conversion of Ly6C^{high} monocytes to Ly6C^{low} monocytes after NOD2 stimulation could control excessive inflammation. We suspect that Ly6C^{low} monocytes could differentiate into M2-like macrophages after their recruitment to inflamed tissue after MDP treatment. This is supported by the activation of M2 associated factor IRF4 and chemokine CCL22 after MDP treatment in Ly6C^{low} monocytes (Lessard et al. 2017). This hypothesis coincides with our genomic analyses which indicate that after MDP treatment Ly6C^{high} monocytes have an upregulation to genes that are associated with an anti-inflammatory signature (Lessard et al., 2017). NOD2 has roles in host defense against microorganisms but this receptor also appears to regulate distinct cellular functions. It may regulate these processes through transcriptional regulation of key innate immune genes. We propose two distinct responses that are enacted

via NOD2 activation. The first is an “immediate” response following the recognition of bacterial fragments or viral RNAs which leads to the subsequent production of inflammatory factors that counteract infection. The second is a “late” response that leads to the emergence of patrolling monocytes which could be used to regulate homeostasis in inflamed tissues (Lessard et al., 2017). These responses necessitate further study in order to better understand the role of NOD2 in inflammatory diseases.

Although NOD2 is specifically activated by MDP and its derivatives, it is not specific to a pathogen per se and can be described as a “boost of natural immunity” (Coulombe et al., 2019). Further research is needed to determine the key cellular and molecular events that are induced by MDP treatment. This has led to efforts to generate immunomodulatory compounds that keep protective “boosting” activity while having minimal toxicity. Compounds such as murabutide, romurtide and mifamurtide are three MDP derivatives that have low toxicity and are promising new molecules for the treatment of certain infections and cancers such as HIV and osteosarcoma (Coulombe et al. 2012). Determining essential cellular and molecular mechanisms enacted by MDP treatment will lead to increased understanding of the mechanism of action of muramyl peptide immunomodulators. Clinically approved and novel MDP analogs should be considered to treat infections and cancers.

We plan to continue collaborating with Amorchem to conduct further tests in cell culture and in mice. We are looking to treat APP mice with the most promising MDP analogs over an extended period of time to see if the amount of Aβ in cerebral blood vessels is reduced and to see if there is any rescue in memory using the Novel Object Recognition test. We will continue to work with Amorchem to further characterize NOD2 agonists *in vivo* and *in vitro*. Furthermore, if these agonists are able to reduce Aβ in cerebral blood vessels in APP mice and leads to improvements in the novel object recognition test then testing will be done to further elucidate specific processes in humans such as the specifics of agonist binding, how long the agonists stay in the body, and which organ(s) metabolize the agonists.

Currently we are testing three promising NOD2 agonists using the NOR test, Western blot analysis, and cortical plaque quantification in hopes that these agonists may show signs of rescuing cognitive deficits in addition to allowing for the clearance of Aβ from the cortex into cerebral blood vessels and reducing the number of cortical Aβ plaques. In partnership with Amorchem, we are currently treating APP mice once per week at dosage of 10mg/kg from 3 to 6 months of age. APP mice develop a familial form of AD and are characterized by aberrant amyloid deposition within the brain parenchyma and vasculature.

Our hope is that if these agonists demonstrate to be effective in Aβ phagocytosis in AD, that it can be paired with other monoclonal antibody treatments like aducanumab which was recently approved in the United States by the Food and Drug Administration (FDA). Aducanumab is a monoclonal antibody treatment that is able to mark Aβ. Pairing this drug with NOD2 agonists would allow for better phagocytosis of Aβ in cerebral blood vessels and would signal to patrolling monocytes where the Aβ primarily resides. If NOD2 agonists become viable treatment options in AD, then they could be paired with future monoclonal antibody treatments as a preventative treatment option in the early stages of the disease.

Conclusions and Perspectives

The immunomodulatory effects of MDP and its analogs could be beneficial in neuroinflammatory diseases like AD. However, the exact mechanism of conversion of inflammatory to patrolling monocytes is not well understood. In addition, it will be important to determine that these NOD2 agonists do not bind to other NLRs. The modifications of MDP need further studying to determine how they affect MDP-mediated immune modulation. As CAA progresses in humans, the BBB is degraded it will be important to see if MDP analogs can aid in maintaining BBB integrity. Further investigations are needed to demonstrate if treatment by MDP leads to a reduced number of cortical plaques in male and female APP mice in addition to if these results are generated with MDP analogs like Agonist 1. If these experiments demonstrate that cortical plaques are reduced, it supports the theory of the sink effect. The kinds of Aβ that can be phagocytosed by monocytes needs to be explored further. Given that MDP and its analogs are effective in the early stages of the disease, future studies are needed to determine to what are the extents of its applications and if it is possible to use MDP analogs in later stages of AD. Our primary focus is to continue studying these analogs *in vitro* and *in vivo* to further characterize their effects.

Bibliography

Abreu, M. T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature Reviews Immunology, 10(2), 131-144.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., ... & Wyss–Coray, T. (2000). Inflammation and Alzheimer's disease. *Neurobiology of aging*, *21*(3), 383-421.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Innate immunity. In *Molecular Biology of the Cell. 4th edition*. Garland Science.

Anbazhagan, K., Duroux-Richard, I., Jorgensen, C., & Apparailly, F. (2014). Transcriptomic network support distinct roles of classical and non-classical monocytes in human. *International reviews of immunology*, *33*(6), 470-489.

Anderson, R. M., Hadjichrysanthou, C., Evans, S., & Wong, M. M. (2017). Why do so many clinical trials of therapies for Alzheimer's disease fail?. *The Lancet*, *390*(10110), 2327-2329.

Apostolopoulos, J., Davenport, P., & Tipping, P. G. (1996). Interleukin-8 production by macrophages from atheromatous plaques. *Arteriosclerosis, thrombosis, and vascular biology*, *16*(8), 1007-1012.

Baumgart, D. C., & Sandborn, W. J. (2012). Crohn's disease. The Lancet, 380(9853), 1590-1605.

Barage, S. H., & Sonawane, K. D. (2015). Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. *Neuropeptides*, *52*, 1-18.

Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R., & Podolsky, D. K. (2005). Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor–κB activation in muramyl dipeptide recognition. Journal of Cell Biology, 170(1), 21-26.

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology*, *81*(1), 1-5.

Bickel, M. (1993). The role of interleukin-8 in inflammation and mechanisms of regulation. *Journal of periodontology*, *64*(5 Suppl), 456-460.

Blander, J. M., & Medzhitov, R. (2006). Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature, 440(7085), 808-812.

Bloksma, N., Hofhuis, F. M., & Willers, J. M. (1985). Muramyl dipeptide analogues as potentiators of the antitumor action of endotoxin. Cancer Immunology, Immunotherapy, 19(3), 205-210.

Boyette, L. B., Macedo, C., Hadi, K., Elinoff, B. D., Walters, J. T., Ramaswami, B., ... & Metes, D. M. (2017). Phenotype, function, and differentiation potential of human monocyte subsets. PloS one, 12(4), e0176460.

Brandt, K. J., Fickentscher, C., Kruithof, E. K., & De Moerloose, P. (2013). TLR2 ligands induce NF-κB activation from endosomal compartments of human monocytes. *PloS one*, *8*(12), e80743.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., ... & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. science, 303(5663), 1532-1535.

Bumet, F. M. (1959). The clonal selection theory of acquired immunity. *Cambridge Amer. Press, New York*.

Burnet, F. M. (1960). *Sir Frank Macfarlane Burnet – Nobel Lecture*. Nobelprize.Org. Retrieved February 11, 2022, from https://www.nobelprize.org/prizes/medicine/1960/burnet/lecture/

Chen, G. Y., & Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nature Reviews Immunology*, *10*(12), 826-837.

Cheng, W. C., You, T. Y., Teo, Z. Z., Sayyad, A. A., Maharana, J., Guo, C. W., ... & Meng, F. C. (2020). Further Insights on Structural Modifications of Muramyl Dipeptides to Study the Human NOD2 Stimulating Activity. Chemistry–An Asian Journal, 15(22), 3836-3844.

Chi, H., Cao, W., Zhang, M., Su, D., Yang, H., Li, Z., ... & Cui, B. (2021). Environmental noise stress disturbs commensal microbiota homeostasis and induces oxi-inflammmation and AD-like neuropathology through epithelial barrier disruption in the EOAD mouse model. *Journal of Neuroinflammation*, *18*(1), 1-16.

Coric, V., van Dyck, C. H., Salloway, S., Andreasen, N., Brody, M., Richter, R. W., ... & Berman, R. M. (2012). Safety and tolerability of the ɣ-secretase inhibitor avagacestat in a phase 2 study of mild to moderate Alzheimer disease. *Archives of neurology*, *69*(11), 1430-1440.

Coulombe, F., Fiola, S., Akira, S., Cormier, Y., & Gosselin, J. (2012). Muramyl dipeptide induces NOD2-dependent Ly6Chigh monocyte recruitment to the lungs and protects against influenza virus infection. *PLoS One*, *7*(5), e36734.

Cuello, A. C. (2017). Early and late CNS inflammation in Alzheimer's disease: two extremes of a continuum?. *Trends in pharmacological sciences*, *38*(11), 956-966.

Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H., & Simon, M. C. (2003). Regulation of macrophage and neutrophil cell fates by the PU. 1: C/EBPα ratio and granulocyte colony-stimulating factor. *Nature immunology*, *4*(10), 1029-1036.

De Kleer, I., Willems, F., Lambrecht, B., & Goriely, S. (2014). Ontogeny of myeloid cells. *Frontiers in immunology*, *5*, 423.

Dempsey, P. W., Allison, M. E., Akkaraju, S., Goodnow, C. C., & Fearon, D. T. (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science, 271(5247), 348-350.

Egarnes, B., Blanchet, M. R., & Gosselin, J. (2017). Treatment with the NR4A1 agonist cytosporone B controls influenza virus infection and improves pulmonary function in infected mice. *PLoS One*, *12*(10), e0186639.

ElAli, A., & Rivest, S. (2013). The role of ABCB1 and ABCA1 in beta-amyloid clearance at the neurovascular unit in Alzheimer's disease. Frontiers in physiology, 4, 45.

Friedman, A. D. (2007). Transcriptional control of granulocyte and monocyte development. *Oncogene*, *26*(47), 6816-6828.

Gabay, C., & Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. New England journal of medicine, 340(6), 448-454.

Gatz, M., Pedersen, N. L., Berg, S., Johansson, B., Johansson, K., Mortimer, J. A., ... & Ahlbom, A. (1997). Heritability for Alzheimer's disease: the study of dementia in Swedish twins. *The*

Journals of Gerontology Series A: Biological Sciences and Medical Sciences, *52*(2), M117-M125.

Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone, M. A., ... & Rosenzweig, A. (1999). MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature*, *398*(6729), 718-723.

Girard, R., Pedron, T., Uematsu, S., Balloy, V., Chignard, M., Akira, S., & Chaby, R. (2003). Lipopolysaccharides from Legionella and Rhizobium stimulate mouse bone marrow granulocytes via Toll-like receptor 2. *Journal of cell science*, *116*(2), 293-302.

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., ... & Sansonetti, P. J. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. Journal of Biological Chemistry, 278(11), 8869-8872.

Gong, T., Liu, L., Jiang, W., & Zhou, R. (2020). DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nature Reviews Immunology, 20(2), 95-112.

Greenberg, S. M., Bacskai, B. J., Hernandez-Guillamon, M., Pruzin, J., Sperling, R., & van Veluw, S. J. (2020). Cerebral amyloid angiopathy and Alzheimer disease—One peptide, two pathways. Nature Reviews Neurology, 16(1), 30-42.

Grimes, C. L., Ariyananda, L. D. Z., Melnyk, J. E., & O'Shea, E. K. (2012). The innate immune protein Nod2 binds directly to MDP, a bacterial cell wall fragment. Journal of the American Chemical Society, 134(33), 13535-13537.

Hanna, R. N., Carlin, L. M., Hubbeling, H. G., Nackiewicz, D., Green, A. M., Punt, J. A., ... & Hedrick, C. C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C− monocytes. Nature immunology, 12(8), 778-785.

Hansen JD, Vojtech LN, Laing KJ. Sensing disease and danger: a survey of vertebrate PRRs and their origins. *Dev Comp Immunol* (2011) **35**:886–97. doi:10.1016/j.dci.2011.01.008

Hecht, M., Krämer, L. M., von Arnim, C. A., Otto, M., & Thal, D. R. (2018). Capillary cerebral amyloid angiopathy in Alzheimer's disease: association with allocortical/hippocampal microinfarcts and cognitive decline. *Acta Neuropathologica*, *135*(5), 681-694.

Heijenoort, J. V. (2001). Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology, 11(3), 25R-36R.

Heil, M., & Land, W. G. (2014). Danger signals–damaged-self recognition across the tree of life. *Frontiers in Plant Science*, *5*, 578.

Hoeffel, G., & Ginhoux, F. (2018). Fetal monocytes and the origins of tissue-resident macrophages. *Cellular immunology*, *330*, 5-15.

Ishii, K. J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., ... & Akira, S. (2008). TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature, 451(7179), 725-729.

Iwasaki, A., & Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. Nature immunology, 16(4), 343-353.

Iwasaki, A., & Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. science, 327(5963), 291-295.

Iwasaki, A., & Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nature immunology, 5(10), 987-995.

Janeway, C. (1989). Immunogenecity signals 1, 2, 3... and 0. *Immunology today*, *10*(9), 283-286.

Janeway, C. (1989, January). Approaching the asymptote? Evolution and revolution in immunology. In *Cold Spring Harbor symposia on quantitative biology* (Vol. 54, pp. 1-13). Cold Spring Harbor Laboratory Press.

Jha, P., & Das, H. (2017). KLF2 in regulation of NF-κB-mediated immune cell function and inflammation. *International Journal of Molecular Sciences*, *18*(11), 2383.

Jin, M. S., Kim, S. E., Heo, J. Y., Lee, M. E., Kim, H. M., Paik, S. G., ... & Lee, J. O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell*, *130*(6), 1071-1082.

Johanson, C., Flaherty, S., Messier, A., Duncan, J., & Silverberg, G. (2006). Expression of the beta-amyloid transporter, LRP-1, in aging choroid plexus: implications for the CSF-brain system in NPH and Alzheimer's disease. Cerebrospinal Fluid Research, 3(1), 1-2.

Jucker, M., & Walker, L. C. (2013). Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. Nature, 501(7465), 45-51.

Kamboh, M. I. (2004). Molecular genetics of late-onset Alzheimer's disease. *Annals of human genetics*, *68*(4), 381-404.

Kammerer, S., & Küpper, J. H. (2018). Human hepatocyte systems for in vitro toxicology analysis. *Journal of Cellular Biotechnology*, *3*(2), 85-93.

Kapellos, T. S., Bonaguro, L., Gemünd, I., Reusch, N., Saglam, A., Hinkley, E. R., & Schultze, J. L. (2019). Human monocyte subsets and phenotypes in major chronic inflammatory diseases. Frontiers in immunology, 10, 2035.

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. *Frontiers in immunology*, 461.

Kitaura, H., Ishida, M., Kimura, K., Sugisawa, H., Kishikawa, A., Shima, K., ... & Shen, W. R. (2018). Role of muramyl dipeptide in lipopolysaccharide-mediated biological activity and osteoclast activity. Analytical Cellular Pathology, 2018.

Land, W. (2003). Allograft injury mediated by reactive oxygen species: from conserved proteins of Drosophila to acute and chronic rejection of human transplants. Part III: interaction of (oxidative) stress-induced heat shock proteins with toll-like receptor-bearing cells of innate immunity and its consequences for the development of acute and chronic allograft rejection. *Transplantation Reviews*, *17*(2), 67-86.

Lathuiliere, A., Laversenne, V., Astolfo, A., Kopetzki, E., Jacobsen, H., Stampanoni, M., ... & Aebischer, P. (2016). A subcutaneous cellular implant for passive immunization against amyloid-β reduces brain amyloid and tau pathologies. Brain, 139(5), 1587-1604.

Lausen, M., Pedersen, M. S., Rahman, N. S. K., Holm-Nielsen, L. T., Farah, F. Y. M., Christiansen, G., & Birkelund, S. (2020). Opsonophagocytosis of Chlamydia pneumoniae by human monocytes and neutrophils. *Infection and immunity*, *88*(7), e00087-20.

Lax, N., Fainstein, N., Nishri, Y., Ben-Zvi, A., & Ben-Hur, T. (2020). Systemic microbial TLR2 agonists induce neurodegeneration in Alzheimer's disease mice. *Journal of Neuroinflammation*, *17*(1), 1-12.

Lee, J. M., Lee, K. H., Weidner, M., Osborne, B. A., & Hayward, S. D. (2002). Epstein–Barr virus EBNA2 blocks Nur77-mediated apoptosis. Proceedings of the National Academy of Sciences, 99(18), 11878-11883.

Lessard, A. J., LeBel, M., Egarnes, B., Préfontaine, P., Thériault, P., Droit, A., ... & Gosselin, J. (2017). Triggering of NOD2 receptor converts inflammatory Ly6Chigh into Ly6Clow monocytes with patrolling properties. *Cell reports*, *20*(8), 1830-1843.

Liu, S., Liu, Y., Hao, W., Wolf, L., Kiliaan, A. J., Penke, B., ... & Fassbender, K. (2012). TLR2 is a primary receptor for Alzheimer's amyloid β peptide to trigger neuroinflammatory activation. *The Journal of Immunology*, *188*(3), 1098-1107.

Lü, L., Zhang, L., Wai, M. S. M., Yew, D. T. W., & Xu, J. (2012). Exocytosis of MTT formazan could exacerbate cell injury. *Toxicology in Vitro*, *26*(4), 636-644.

Long, X., Ye, Y., Zhang, L., Liu, P., Yu, W., Wei, F., ... & Yu, J. (2016). IL-8, a novel messenger to cross-link inflammation and tumor EMT via autocrine and paracrine pathways. *International journal of oncology*, *48*(1), 5-12.

Maccioni, R. B., Farías, G., Morales, I., & Navarrete, L. (2010). The revitalized tau hypothesis on Alzheimer's disease. *Archives of medical research*, *41*(3), 226-231.

Martinon, F., Mayor, A., & Tschopp, J. (2009). The inflammasomes: guardians of the body. Annual review of immunology, 27, 229-265.

Matsuo, M. (2022). ABCA1 and ABCG1 as potential therapeutic targets for the prevention of atherosclerosis. *Journal of Pharmacological Sciences*, *148*(2), 197-203.

Matzinger, P. (2007). Friendly and dangerous signals: is the tissue in control?. *Nature immunology*, *8*(1), 11-13.

McMorrow, J. P., & Murphy, E. P. (2011). Inflammation: a role for NR4A orphan nuclear receptors?. *Biochemical Society Transactions*, *39*(2), 688-693.

Medzhitov, R., & Janeway Jr, C. (2000). Innate immune recognition: mechanisms and pathways. Immunological reviews, 173, 89-97.

Mehta, D., Jackson, R., Paul, G., Shi, J., & Sabbagh, M. (2017). Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. *Expert opinion on investigational drugs*, *26*(6), 735-739.

Mendez, M. F. (2012). Early-onset Alzheimer's disease: nonamnestic subtypes and type 2 AD. *Archives of medical research*, *43*(8), 677-685.

Merlini, M., Wanner, D., & Nitsch, R. M. (2016). Tau pathology-dependent remodelling of cerebral arteries precedes Alzheimer's disease-related microvascular cerebral amyloid angiopathy. *Acta neuropathologica*, *131*(5), 737-752.

Mildner, A., Schönheit, J., Giladi, A., David, E., Lara-Astiaso, D., Lorenzo-Vivas, E., ... & Jung, S. (2017). Genomic characterization of murine monocytes reveals C/EBPβ transcription factor dependence of Ly6C− cells. Immunity, 46(5), 849-862.

Mildner, A., Yona, S., & Jung, S. Chapter Three—A Close Encounter of the Third Kind: Monocyte-Derived Cells. *Advances in Immunology; Murphy, KM, Merad, M., Eds*, 69-103.

Mitchell, R. N. (2013). Innate and adaptive immunity: The immune response to foreign materials. In Biomaterials Science (pp. 512-533). Academic Press.

Mohandas, E., Rajmohan, V., & Raghunath, B. (2009). Neurobiology of Alzheimer's disease. *Indian journal of psychiatry*, *51*(1), 55.

Muhammad, I., Wang, H., Sun, X., Wang, X., Han, M., Lu, Z., ... & Zhang, X. (2018). Dual role of dietary curcumin through attenuating AFB1-induced oxidative stress and liver injury via modulating liver phase-I and phase-II enzymes involved in AFB1 bioactivation and detoxification. *Frontiers in Pharmacology*, *9*, 554.

Mukherjee, T., Hovingh, E. S., Foerster, E. G., Abdel-Nour, M., Philpott, D. J., & Girardin, S. E. (2019). NOD1 and NOD2 in inflammation, immunity and disease. Archives of biochemistry and biophysics, 670, 69-81.

Mullane, K., & Williams, M. (2013). Alzheimer's therapeutics: continued clinical failures question the validity of the amyloid hypothesis—but what lies beyond?. *Biochemical pharmacology*, *85*(3), 289-305.

Negroni A, Pierdomenico M, Cucchiara S, Stronati L. NOD2 and inflammation: current insights. *J Inflamm Res*. 2018; 11:49-60. Published 2018 Feb 12. doi:10.2147/JIR.S137606

Pearson, H. A., & Peers, C. (2006). Physiological roles for amyloid β peptides. The Journal of physiology, 575(1), 5-10.

Pecchi, E., Dallaporta, M., Jean, A., Thirion, S., & Troadec, J. D. (2009). Prostaglandins and sickness behavior: old story, new insights. Physiology & behavior, 97(3-4), 279-292.

Pons, V., & Rivest, S. (2022). Targeting Systemic Innate Immune Cells as a Therapeutic Avenue for Alzheimer Disease. *Pharmacological Reviews*, *74*(1), 1-17.

Rajpal, A., Cho, Y. A., Yelent, B., Koza-Taylor, P. H., Li, D., Chen, E., ... & Winoto, A. (2003). Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. The EMBO journal, 22(24), 6526-6536.

Richardson, R. M., Marjoram, R. J., Barak, L. S., & Snyderman, R. (2003). Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. *The journal of immunology*, *170*(6), 2904-2911.

Sandor, F., Latz, E., Re, F., Mandell, L., Repik, G., Golenbock, D. T., ... & Finberg, R. W. (2003). Importance of extra-and intracellular domains of TLR1 and TLR2 in NFκB signaling. *The Journal of cell biology*, *162*(6), 1099-1110.

Saresella, M., Marventano, I., Calabrese, E., Piancone, F., Rainone, V., Gatti, A., ... & Clerici, M. (2014). A complex proinflammatory role for peripheral monocytes in Alzheimer's disease. *Journal of Alzheimer's Disease*, *38*(2), 403-413.

Savelieff, M. G., Lee, S., Liu, Y., & Lim, M. H. (2013). Untangling amyloid-β, tau, and metals in Alzheimer's disease. ACS chemical biology, 8(5), 856-865.

Saxena, M., & Yeretssian, G. (2014). NOD-like receptors: master regulators of inflammation and cancer. Frontiers in immunology, 5, 327.

Schmid, D., & Münz, C. (2007). Innate and adaptive immunity through autophagy. Immunity, 27(1), 11-21.

Schenk, M., Mahapatra, S., Le, P., Kim, H. J., Choi, A. W., Brennan, P. J., ... & Modlin, R. L. (2016). Human NOD2 recognizes structurally unique muramyl dipeptides from Mycobacterium leprae. Infection and immunity, 84(9), 2429-2438.

Serbina, N. V., Cherny, M., Shi, C., Bleau, S. A., Collins, N. H., Young, J. W., & Pamer, E. G. (2009). Distinct responses of human monocyte subsets to Aspergillus fumigatus conidia. The Journal of Immunology, 183(4), 2678-2687.

Sierri, G., Dal Magro, R., Vergani, B., Leone, B. E., Formicola, B., Taiarol, L., ... & Re, F. (2022). Reduced Levels of ABCA1 Transporter Are Responsible for the Cholesterol Efflux Impairment in β-Amyloid-Induced Reactive Astrocytes: Potential Rescue from Biomimetic HDLs. *International journal of molecular sciences*, *23*(1), 102.

Skrzeczyńska-Moncznik, J., Bzowska, M., Lo˝ seke, S., Grage-Griebenow, E., Zembala, M., & Pryjma, J. (2008). Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. Scandinavian journal of immunology, 67(2), 152-159.

Sokolowski, J. D., & Mandell, J. W. (2011). Phagocytic clearance in neurodegeneration. *The American journal of pathology*, *178*(4), 1416-1428.

Stetson, D. B. (2009). Connections between antiviral defense and autoimmunity. Current opinion in immunology, 21(3), 244-250.

Strober, W., & Watanabe, T. (2011). NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. Mucosal immunology, 4(5), 484-495.

Thal, D. R., Ghebremedhin, E., Rüb, U., Yamaguchi, H., Del Tredici, K., & Braak, H. (2002). Two types of sporadic cerebral amyloid angiopathy. *Journal of Neuropathology & Experimental Neurology*, *61*(3), 282-293.

Thomas, C. J., & Schroder, K. (2013). Pattern recognition receptor function in neutrophils. Trends in immunology, 34(7), 317-328.

Uehori, J., Fukase, K., Akazawa, T., Uematsu, S., Akira, S., Funami, K., ... & Seya, T. (2005). Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. *The Journal of Immunology*, *174*(11), 7096-7103.

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell proliferation, 36(3), 131-149.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., ... & Perlmann, T. (2003). Structure and function of Nurr1
identifies a class of ligand-independent nuclear receptors. Nature, 423(6939), 555-560.

Wang, C., Yang, A., Li, X., Li, D., Zhang, M., Du, H., ... & Wang, C. (2012). Observation of molecular inhibition and binding structures of amyloid peptides. Nanoscale, 4(6), 1895-1909.

Warner, N., Burberry, A., Pliakas, M., McDonald, C., & Núñez, G. (2014). A genome-wide small interfering RNA (siRNA) screen reveals nuclear factor-κB (NF-κB)-independent regulators of NOD2-induced interleukin-8 (IL-8) secretion. *Journal of biological chemistry*, *289*(41), 28213-28224.

Wekerle, H., Linington, C., Lassmann, H., & Meyermann, R. (1986). Cellular immune reactivity within the CNS. *Trends in neurosciences*, *9*, 271-277.

Weller, R. O., Carare, R. O., & Boche, D. (2009). Amyloid: vascular and parenchymal.

Wilson, R. S., Segawa, E., Boyle, P. A., Anagnos, S. E., Hizel, L. P., & Bennett, D. A. (2012). The natural history of cognitive decline in Alzheimer's disease. *Psychology and aging*, *27*(4), 1008.

Wisniewski, T., & Goñi, F. (2015). Immunotherapeutic approaches for Alzheimer's disease. Neuron, 85(6), 1162-1176.

Wolf, A. A., Yáñez, A., Barman, P. K., & Goodridge, H. S. (2019). The ontogeny of monocyte subsets. *Frontiers in immunology*, *10*, 1642.

Wong, K. L., Tai, J. J. Y., Wong, W. C., Han, H., Sem, X., Yeap, W. H., ... & Wong, S. C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood, The Journal of the American Society of Hematology, 118(5), e16-e31.

Yáñez, A., Coetzee, S. G., Olsson, A., Muench, D. E., Berman, B. P., Hazelett, D. J., ... & Goodridge, H. S. (2017). Granulocyte-monocyte progenitors and monocyte-dendritic cell progenitors independently produce functionally distinct monocytes. *Immunity*, *47*(5), 890-902.

Yoneyama, M., & Fujita, T. (2008). Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity, 29(2), 178-181.

Zenaro, E., Piacentino, G., & Constantin, G. (2017). The blood-brain barrier in Alzheimer's disease. *Neurobiology of disease*, *107*, 41-56.

Zuliani, G., Cavalieri, M., Galvani, M., Passaro, A., Munari, M. R., Bosi, C., ... & Fellin, R. (2008). Markers of endothelial dysfunction in older subjects with late onset Alzheimer's disease or vascular dementia. *Journal of the neurological sciences*, *272*(1-2), 164-170.

Zuroff, L., Daley, D., Black, K. L., & Koronyo-Hamaoui, M. (2017). Clearance of cerebral Aβ in Alzheimer's disease: reassessing the role of microglia and monocytes. *Cellular and Molecular Life Sciences*, *74*(12), 2167-2201.