Biological Studies of Muramyl Dipeptide Analogues as potential ligands for NOD2 receptor

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

The human body's survival against various foreign disease-causing organisms solely depends on the protection provided by our body's immune system. Our body's complex yet effective immune system comprises cells, molecules and sensory receptors that can activate various immune response pathways to eliminate pathogens. The innate immune system provides the first line of defence through different types of sensor cells with pattern recognition receptors (PRRs) with cytoplasmic proteins like NOD-like receptors (NLRs) that can activate the innate immune system by recognizing the bacterial cell wall component peptidoglycan. The smallest known fragment of peptidoglycan is Muramyl Dipeptide (MDP) which is recognized by the Nucleotide-binding oligomerization domain (NOD2) receptor of the NLR family as a pathogen-associated molecular pattern and can immediately activate the human body's innate immune system to release effective mediators that dominate the destruction of invading pathogens and with adverse side-effects. The parent MDP molecule exhibits high toxicity, hydrophilicity, and rapid elimination from the biological system. Modifications to the MDP structure without losing their immunomodulatory properties can be evaluated to separate desirable biological activities from unwanted side effects that can enhance and modulate the innate immune response and can further be evaluated for their adjuvant potency in vaccines and drugs.

Six novel MDP analogues were synthesized by replacing the D-iso-glutamine residue with artificial aromatic amino acid residue within the dipeptide moiety of the parent MDP structure, followed by adding lipid chains to increase their lipophilicity and among these six, three of the MDP analogues were synthesized with an additional benzyl group. The present study aims to evaluate the biological properties of these MDP analogues as potential immunostimulatory or immunomodulatory agents. This evaluation is conducted by stimulating THP-1 macrophages

with 20 μM of these MDP analogues individually and in combination with lipopolysaccharide (LPS).

Flow cytometry analysis of differentiated THP-1 cells stimulated with these compounds is depicted through the Mean Fluorescence Intensity (MFI) readings for the surface glycoprotein (Intercellular adhesion molecule 1 (ICAM-1)) expression. Furthermore, the concentration of pro-inflammatory cytokines TNF-α and IL-1β is measured in the supernatants obtained from these experiments using ELISA. The MFIs for compounds without the benzyl group and compounds with the benzyl group and a lipid chain of moderate length are relatively low compared to the MFI of the well-known NOD2 agonist Murabutide. In contrast, compound **6** with the benzyl group and a longer lipid chain (C_{16}) shows the highest MFI reading and an elevated concentration of pro-inflammatory cytokines Tumor necrosis factor-alpha (TNF-α) and Interleukin-1 beta (IL-1β) secreted by THP1 macrophages compared to the rest of the compounds.

Further, for MDP analogue **6** with the highest MFI, a concentration-response study was done to find out the optimum dose with the maximum response. Preliminary data indicated that **6** alone at 20 μM induced the highest level of ICAM-1 expression while in the presence of LPS, compound **6** at 20 or 25 μM induced the same and the highest response.

Mechanistic studies were conducted with the commercially available NOD2 inhibitor GSK717 to study the role of the NOD2 receptor in releasing pro-inflammatory cytokines and if NOD2 readily recognized MDP analogues described in the present study as ligands that activated the innate immune response. It was observed that inhibition of the NOD2 receptor showed diminished concentrations of pro-inflammatory cytokines TNF-α and IL-1β induced by **6**, suggesting that MDP analogues **6** activated the NOD2 receptor.

The findings of this study highlight that MDP analogue **6** with a benzyl group and a C_{16}-lipid chain as a lead compound that can be further evaluated as a potential adjuvant for vaccines.

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ABBREVIATIONS

AIM2	Absent in melanoma 2
AMP	Adenosine 5′-monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BIR	Baculoviral inhibition of apoptosis repeat
CARD	Caspase activation and recruitment domain
CMP	Common myeloid progenitor
CpG	Cytosine–guanine dinucleotide
DAMP	Damage associated molecular pattern
DAP	Diaminopimelic acid
DMP	Desmuramylpeptide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
ICAM-1	Intercellular adhesion molecule 1
IL-1β	Interleukin-1 beta

IκB	Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells
IκK	IκB kinase
IRF4	Interferon regulatory factor 4
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
PAMP	Pattern associated molecular pattern
PGN	Peptidoglycan
PI	Propidium iodide
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
PYD	Pyrin domain

RIG-1	Retinoid acid-inducible gene–1
RIPK2	Receptor-interacting protein kinase 2
RLR	Retinoic acid like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAR	Structure-activity relationship
TAK1	Transforming activated kinase 1
TGF-β	Transforming growth factor-β
TIR	Toll-interleukin receptor
TLC	Thin layer chromatography
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF-α	Tumor necrosis factor-alpha
TNFR	TNFα receptor

1. INTRODUCTION

1.1 Importance of the immune system

The human body defends against infections and the damage they cause through a robust immune system consisting of cells and molecules with specific roles that attack invading microbes with two different types of response: the innate or natural immune response and the acquired or adaptive response, based on their speed and specificity (Delves et al., 2000; Parkin et al., 2001). Bone marrow produces white blood cells or leukocytes that regulate the innate and adaptive immune system (Parkin et al., 2001). Innate immunity is essential for survival and provides rapid but less specific physical, chemical, and microbiological barriers. Innate immune responses use natural killer cells, phagocytic cells like neutrophils, monocytes and macrophages, and cells that release inflammatory mediators like basophils, mast cells and eosinophils, while the molecular components of innate immunity include complement, cytokines, and acute-phase proteins. Acquired immunity works on the memory of the attack and is precise, but it takes a while to respond. Acquired response activates antigen-specific reactions using T lymphocytes and B lymphocytes. The surface receptors on cells bind to the attacking agent's antigen to present it to the lymphocytes (Delves et al., 2000; Parkin et al., 2001). Recognizing antigens by T and B cells leads to cell priming, activation, and differentiation within the lymphoid tissue to produce antigen-specific antibodies to eliminate infected cells from the body (Delves et al., 2000; Parkin et al., 2001). The innate and adaptive immune system protects the body against pathogens. The immune system plays an important role in autoimmune diseases and cancers, while immunotherapy could potentially help in the treatment of these diseases.

1.2 The Innate immune system

1.2.1 Cells of the immune system

The common myeloid progenitor (CMP) of the myeloid lineage consists of all immune cells and includes the three types of phagocytic cells of the immune system; macrophages and monocytes, granulocytes, and dendritic cells of the innate immune system (Parkin et al., 2001).

- *Role of Macrophages in the immune system*

Macrophages are the foremost differentiated cell type of the phagocytic system that form the human body's first line of defence and can be found all over the body tissues and are formed from the circulating monocytes in the blood that are formed from progenitor cells (Parameswaran et al., 2010). Macrophages perform various functions for the innate immune system that primarily involves recognition, phagocytosis, and destruction of invading microorganisms (Parkin et al., 2001). The primary functions of macrophages include acute inflammatory responses, killing microbial pathogens using nitric oxide and producing cytokines (Chaplin et al., 2010). Macrophages are also the master regulator of the immune response as they induce inflammation in the human body by releasing small proteins called cytokines and chemokines along with various chemical mediators that effectively recruit and activate immune-system cells for immune response (Parkin et al., 2001). Inflammation in tissues also activates the lining of endothelial cells in blood vessels to express cell-adhesion molecules like intercellular adhesion molecule 1 (ICAM-1) which is a cell surface glycoprotein, and an adhesion receptor that best regulates the recruitment of leukocytes from the circulation to inflammatory sites,

ICAM1 expression is strongly induced in epithelial cells and immune cells in response to inflammatory stimulation (Kenneth et al., 2017).

Macrophages, neutrophils, and dendritic cells are essential sensor cells as they detect pathogens and the damage induced by them through innate recognition receptors that can discriminate and eliminate pathogens or foreign bodies using major protein families with receptors (Parkin et al., 2001). These receptors identify different ligands and initiate the human body's immune response by activating ligand-specific signalling pathways; thus, the importance of acquiring advanced knowledge of the innate immune system for its first line of defence against the invasion of microbes has increased over the past few years (Pan et al., 2007; Saxena et al., 2014).

- *Function and family of innate immune receptors*

Germline-encoded innate immune receptors, also known as pattern recognition receptors (PRRs), initiate innate immune responses by recognizing pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), respectively (Bourhis et al., 2007). These immune receptors sense pathogens or endogenous nonmicrobial danger and activate various inflammatory signalling pathways such as nuclear factor–kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) based on ligand recognition (Chen et al., 2009). Dysregulation of these complex effector pathways is the primary cause of inflammatory diseases and highlights the importance of PRRs in the human body's defence against various microbes and pathogens (Chen et al., 2009). The PRRs family consists of 1) Toll-like receptors (TLRs) that are composed of membrane-anchored proteins, 2) nucleotide-binding and oligomerization domain-

containing receptors; NOD-like receptors (NLRs), which are intracellular, cytoplasmic sensors; and 3) retinoid acid-inducible gene–1 (RIG-1)-like receptors. (RLRs), which are cytosolic helicases that primarily sense viruses (Bourhis et al., 2007; Chen et al., 2009) 4) Absent in Melanoma 2 (AIM2) -like receptors and 5) C-type lectins (Nabergoj et al., 2019; Kim et al., 2016). Over the years, the clinical relevance of TLRs and NLRs has emerged rapidly due to their importance as innate immune sensors in responding to pathogens and carcinogenesis (Ma et al., 2020; Turvey et al., 2010).

1.2.2 Toll-like receptors (TLRs)

- *Family of TLRs*

The human TLRs are transmembrane innate sensors (Kim et al., 2016) that can detect PAMPs and provide effector adaptive responses that allow the innate immune system to distinguish and respond to various microbial epitopes on pathogens (Turvey et al., 2010). Dendritic cells, macrophages and non-immune cells like fibroblast and epithelial cells host the TLRs (Kawasaki et al., 2014). The TLR family consists of 10 receptors (TLR1-TLR10) that exist as dimeric proteins of heterodimers or homodimers (Kenneth et al., 2017). Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 that recognize microbial membrane components like lipids, lipoproteins, and proteins while intracellular TLRs located in the endosome include TLR3, TLR7, TLR8, TLR9 that recognize nucleic acids in bacteria and viruses (Celhar et al., 2012; Kawai et al., 2010; Kawasaki et al., 2014). These TLRs have 18-25 copies (Kenneth et al., 2017) of leucine-rich repeat (LRR) motifs found in the ectodomains that detect pathogens through

receptor-ligand pairs followed by activating the downstream signalling pathways; for example, receptors TLR4 pair with LPS in gram-negative bacteria and lipoteichoic acids in gram-positive bacteria while TLR5 pair with bacterial Flagellin (Turvey et al., 2010; Akira et al., 2004). Ligands like lipoproteins and lipomannans usually pair with several receptors, TLR1/TLR2 /TLR6 and recognize gram-positive organisms (Turvey et al., 2010). Intracellular TLRs like TLR3 pair with ligands of Double-stranded RNA and TLR7/TLR8 with single-stranded RNA. TLR9 ligands include DNA with unmethylated cytosine–guanine dinucleotide (CpG), and TLR10 do not have ligands reported (Kenneth et al., 2017; Takeda et al., 2003).

- *Function of TLRs*

TLRs are located on the cell surface and in the membranes of endosomes and recognize most microbial pathogens and activate multiple host defence signalling pathways. TLRs contain LRRs required for the recognition of pathogen-associated molecular patterns (PAMPs) (Martinon et al., 2004; Martinon et al., 2005). For TLRs, binding of PAMPs to the LRRs of TLRs promotes conformational changes that lead to receptor activation such that the intracellular Toll-interleukin receptor (TIR) domains of these transmembrane receptors dimerize and recruit TIR-containing adapter proteins that use signalling proteins to initiate downstream signalling pathways of TLRs (Medzhitov, 2001) like the NF-κB pathway to induce pro-inflammatory cytokines like TNF-α and IL-1β (Kenneth et al., 2017)

1.2.3 NOD-like receptors (NLRs)

NLRs are cytoplasmic receptors that recognize PAMPs and DAMPs essential for innate immune response and regulate apoptosis and early development (Kim et al., 2016). NLRs can be divided into four subfamilies based on their function; "inflammasome assembly, signalling transduction, transcription activation, and autophagy" (Kim et al., 2016). Interestingly, polymorphism of genes encoding NLR can shift the balance between pro- and anti-inflammatory cytokines, potentially increasing the risk of infection and several cancers in the human body. Inflammatory stimulus or genetic mutation that causes a shift in either gain or loss of function of the NLR gene can elicit a significant disturbance in the immune system, leading to chronic inflammation, which is the main cause of cancer (Chen et al., 2009; Akira et al., 2004; Kutikhin et al., 2011).

- *Structure of NLRs*

The structural features of NLRs include 1) central nucleotide-binding oligomerization domain, 2) variable N-terminal protein-protein interaction domain defined by the caspase recruitment domain (CARD) containing proteins, 3) C-terminal LRR that senses pathogen-associated molecular patterns and ligands (Saxena et al., 2014).

- *Family of NLRs*

The N-terminal domain of the NLR divides the 22 human NLRs into five categories with unique functional characteristics for each NLR. The sub-families include 1) NLRA (CIITA), which contains an N-terminal acidic transactivation domain, 2)

NLRB (NAIP) contains an N-terminal baculoviral inhibition of apoptosis repeat (BIR), 3) NLRC (CARD) contains an N-terminal caspase activation and recruitment domain, 4) NLRP with pyrin domain (PYD), and 5) NLRX that has an N-terminal mitochondria targeting sequence (Ting et al., 2008; Saxena et al., 2014).

- *Functions of NLRP1 and NLRP3*

Another subfamily of NLR proteins has a pyrin domain instead of a CARD domain at their amino terminus and is known as the NLRP family (Kenneth et al., 2017). NLRP1 and NLRP3 are two of the most studied members of the NLR protein family and possess a nucleotide-binding fold known as the NACHT domain (Koonin et al., 2000), and a variable number of leucine-rich repeat (LRR) domains. NLRP1 plays a critical role in the innate immune response by recognizing specific pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that are released during infection or cellular stress. The NLRP1 protein contains both PRYIN and CARD domains located at opposite ends of the protein. The only PAMP identified so far for NLRP1 is muramyl dipeptide (MDP) which can induce a conformational change in NLRP1 that makes the protein competent to bind Adenosine triphosphate (ATP) (Kenneth et al., 2017). When activated, NLRP1 forms a large multiprotein complex called the inflammasome, which leads to the activation of caspases, particularly caspase 1, which initiates the inflammatory response by binding to apoptosis-associated speck-like protein (ASC) through the N-terminal PYD. ASC, in turn, binds pro-caspase 1, forming a multi-protein complex called the inflammasome (Martinon et al., 2002).

NLRP3 resides in an inactive form in the cytoplasm, where its LRR domains are thought to bind the heat shock chaperone protein HSP90 and the cochaperone SGT1, which keeps NLRP3 in an inactive state instead of activating NF-κB, as in NOD1 and NOD2 signalling, NLRP3 signalling leads to the production of pro-inflammatory cytokines and cell death by forming a multiprotein complex called the inflammasome (Kenneth et al., 2017). Activation of inflammation occurs in several stages. The first is the aggregation of the LRR domains of multiple NLRP3 molecules or other NLRP molecules by a specific triggering or recognition event. This aggregation causes the pyrin domains of NLRP3 to interact with the pyrin domains of the ASC which is an adapter protein consisting of an amino terminal pyrin domain and a carboxy-terminal CARD domain. The pyrin and CARD domains are both capable of forming polymeric filamentous structures. The interaction of NLRP3 with ASC further promotes the formation of a polymeric ASC filament with the pyrin domains in the center and the CARD domains facing outward (Kenneth et al., 2017). These CARD domains then interact with the CARD domains of inactive procaspase 1, triggering its CARD-dependent polymerization into discrete caspase 1 filaments. This aggregation triggers pro-caspase 1 autocleavage, releasing the active caspase fragment from its autoinhibitory domains. These members of the caspase family mainly process pro-inflammatory cytokines, particularly IL-1β and IL-18, into their active forms (Kenneth et al., 2017). Caspase 1 activation also induces cell death through an unknown inflammation-related mechanism called pyroptosis ("fiery death") because these pro-inflammatory cytokines are released upon cell rupture (Kenneth et al., 2017). For inflammasome activation to produce inflammatory cytokines, there must first be a preparatory step in which cells induce and translate mRNAs encoding precursors of IL1β, IL-18, or other cytokines. The cytokine IL-1β activates NF-κB to produce more IL1β; additionally, IL-1β

induces the transcription of many other pro-inflammatory genes that is important in inflammation (Moltke et al., 2013). Another essential function of NOD2 is autophagy which destroys damaged proteins and organelles that recycle biomolecules (Akira et al., 2004; Boyle et al., 2014).

- *Sub-family and role of NLRC in innate immunity*

The NLRC is further divided into subfamily of NLRC 1-5 depending on CARD and facilitates the interaction between the NLRC family and other CARD-carrying adaptor proteins. NOD1/NLRC1, previously known as CARD4 and NOD2/NLRC2, previously known as CARD15 (Saxena et al., 2014), are intracellular proteins located in the cytosol (Nabergoj et al., 2019) that are key sensors of peptidoglycan (PGN) (Philpott et al., 2014; Saxena et al., 2014). PGN also known as murein is an important component of bacterial cell wall and functions to keep the cell structure intact, preserve cell integrity and also anchor components like lipoproteins (Rogers et al., 1980; Park, 1996; Nanninga, 1998; Mengin-Lecreulx & Lemaitre, 2005; Vollmer et al., 2008). NOD1 and NOD2 play an essential role in various biological processes like stimulating innate immune responses, apoptosis, autophagy, and reactive oxygen species (ROS) generation (Ma et al., 2020; Soyocak et al., 2020; Kutikhin et al., 2011).

NOD1 and NOD2 members of the NLRC family have unique roles in activating various pathways to induce pro- or anti-inflammatory responses (Chen et al., 2009). NOD1 and NOD2 display variable tandem C-terminal LRRs, responsible for ligand

recognition of PGN fragments found in bacterial cell walls. The NOD1 receptor recognizes meso-diaminopimelic acid (DAP)-containing fragments of PGN, such as the D-glutamyl-meso-diaminopimelic acid (iE-DAP) and L-alanyl-γ-D-glutamylmeso-DAP (Tri-DAP) (Nabergoj et al., 2019; Udden et al., 2017). The NOD2 receptor recognizes N-(acetylmuramyl) -L-alanyl-D-isoglutamine; muramyl dipeptide (MDP) (Udden et al., 2017).

NOD1 and NOD2 interact with the actin cytoskeleton in the cytoplasm to remain inactive. On activation by their respective ligands, NOD1 and NOD2 undergo complex conformational changes that result in self-oligomerization to recruit serine/threonine receptor-interacting protein kinase 2 (RIPK2) through homotypic CARD–CARD interaction (Correa et al., 2012). The serine/threonine RIPK2 then recruits and activates the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) Ser-Thr kinase that is necessary for the activation of IKK complex made of two kinases (IKKα and IKKβ) (Caruso et al., 2014) and the mitogen-activated protein kinase (MAPK) cascades (Velloso et al., 2004). The NF-κB cascade is essentially controlled by the IKK kinase complex that phosphorylates the NF-κB inhibitor IκBα to polyubiquitination (pUb) followed by degradation that allows NF-κB to translocate to the nucleus and activate downstream signalling pathway, transcription of immune response genes and production of inflammatory cytokines (Akira et al., 2004; Israël, 2012). It is important to note that both TLRs and NLRs activate the downstream signalling pathways that initiate innate immune responses (Akira et al., 2004).

1.2.4 Summary

The review above of the innate immune system cells explain the complex biological interactions between cells, receptors and molecules that play a vital role in protecting the human body against diseases. Macrophages exhibit multiple receptor systems that recognize various pathogens and elicit a rapid cellular response in defence, along with inducing inflammation that triggers an increase of ICAM-1 expression on the cell surface. TLRs on the cell surface and in the membranes of endosomes, along with NLRs, detect invading pathogens and activate multiple host defence signalling pathways, including the NF-κB pathway, that leads to the release of cytokines like TNF-α and IL-1β, which are important mediators of immunologic and inflammatory reactions. NLRs also signal through the inflammasome, which produces pro-inflammatory cytokines and induces pyroptosis, a type of cell death. Thus, measuring the ICAM-1 expression and concentration of TNF-α and IL-1β secreted by macrophages with cell viability over 90% can help us understand and enhance the activities of the innate immune system.

1.3 Nucleotide-binding oligomerization domain 2 (NOD2)

1.3.1 NOD2 receptor and associated ligands

The most researched receptor of the NLR family belonging to the PRR family is the NOD2 receptor, expressed in macrophages, dendritic cells, lymphocytes, and epithelial cells. NOD2 recognizes bacterial components released during the synthesis or degradation of peptidoglycan (Watanabe et al., 2006). NOD2 receptor comprises two N-terminal CARDs, a central nucleotide-binding domain, and a C-terminal LRR (Watanabe et al., 2006). NOD2 is an MDP sensor, a peptidoglycan component found in Gram-

positive and Gram-negative bacteria (Udden et al., 2017). NOD2 has also been shown to recognize other bacterial ligands, including mycobacterial cell wall components and fungal β-glucans. This broad recognition ability suggests that NOD2 plays a major role in host defense against a wide range of microbial infections (Girardin et al., 2003).

1.3.2 NOD2 agonists

The primary role of NOD2 agonists is to enhance innate and adaptive immune responses to antigens with lower immunostimulatory capabilities determined by the capacity of NOD2 agonists to stimulate monocytes and macrophages (Nabergoj et al., 2019). One of the most potent and extensively studied NOD2 agonists is MDP, the smallest peptidoglycan fragment that can elicit NOD2 activation. MDP can synergize with various cytokines , (IL-8, IL-1β, and TNF-α) to increase immune response (Nabergoj et al., 2019).

1.3.3 Muramyl dipeptide

MDP is the smallest biologically potent component of PGN. The structure of PGN has an N-acetylyglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide chain along with four to five amino acid chains that have L-Ala as the first chain and D-Glu as the second amino acid chain which are linked from the lactyl group of one of the N-acetylmuramic acid to the other (Ogawa et al., 2011). MDP is a low molecular weight molecule composed of an N-acetylmuramic acid linked to a dipeptide consisting of L-alanine (L-Ala) and D-isoglutamine (D-isoGln) (Figure 1). MDP-induced pyrogenicity is a protective response to bacterial infection, as fever can help kill bacteria

and activate other components of the immune system. However, excessive, or prolonged fever can also be harmful and cause complications such as dehydration and metabolic disorders thus MDP needs to be modified to reduce its pyrogenicity before administering it to the human body and increase the molecule's in-vivo activity by reducing water solubility and rapid bodily elimination (Nabergoj et al., 2019). Over the years, numerous MDP analogs have been synthesized in laboratories and distinguished into four different groups named lipophilic derivatives (Mifamurtide, Romurtide), hydrophilic derivatives (GMPD, Murabutide), conjugates (biomolecules, small molecule drugs), and desmuramylpeptides (LK-409, LK-410) (Nabergoj et al., 2019). The structure-activity relationship (SAR) of MDP with its adjuvant activity is extensively studied to enhance its properties for treating cancer and various other diseases (Nabergoj et al., 2019) as the NOD2 receptor recognizes MDP and undergoes self-oligomerization to activate NF-κB and MAPK signalling pathways for pro-inflammatory and antimicrobial response (Caruso et al., 2014; Mo et al., 2012; Jakopin et al., 2014).

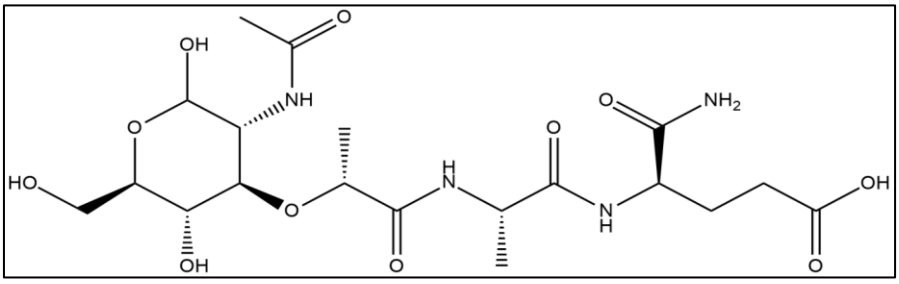

Figure 1: Structure of parent MDP. Chemdraw (RRID:SCR_016768)

1.3.4 Mifamurtide

Mifamurtide is a synthetic lipophilic derivative of MDP (addition of lipophilic moieties into MDP) also known as Muramyl tripeptide phosphatidylethanolamine or MTP-PE. Compared to the parent MDP, Mifamurtide has an improved half-life and less toxicity (Nabergoj et al., 2019). Mifamurtide is an important NOD2 agonist that can modulate the innate immune system to activate macrophages and monocytes to secrete chemicals that act as an antitumor and control microscopic metastatic cancer (Nabergoj et al., 2019). The European Medicine Agency has approved the treatment of osteosarcoma with Mifamurtide in combination with standard adjuvant chemotherapy and surgery to improve overall survival in patients with osteosarcoma, especially those with a poor prognosis or metastatic disease (Kager et al., 2010), but it has side effects like fever, chill, and nausea therefore, mifamurtide needs to be explored further with potential combination therapies to improve outcomes in patients with osteosarcoma (Effenberg et al., 2017).

1.3.5 Murabutide

Murabutide has a similar adjuvant activity as parent MDP but without its pyrogenic and toxic properties (Nabergoj et al., 2019). Murabutide is a hydrophilic MDP derivative that can balance lipophilicity and hydrophilicity to enhance the biological activity of innate immune responses (Danklmaier et al., 1990; Khan et al., 2017). Murabutide has a lipophilic chain D-Gln-n-butyl-ester residue introduced in place of D-isoGln at the peptide end of MDP (Figure 2). Studies have shown that Murabutide is a safe

immunomodulator that can alter the immune response by enhancing the anticancer activities of cytokines compared to traditional immunomodulatory agents exhibiting limited efficacy (Jakopin et al., 2014; Danklmaier et al., 1990; Sollner et al., 1993; Khan et al., 2017) and can enhance resistance to infection by interacting with the human body's immune system (Nabergoj et al., 2019).

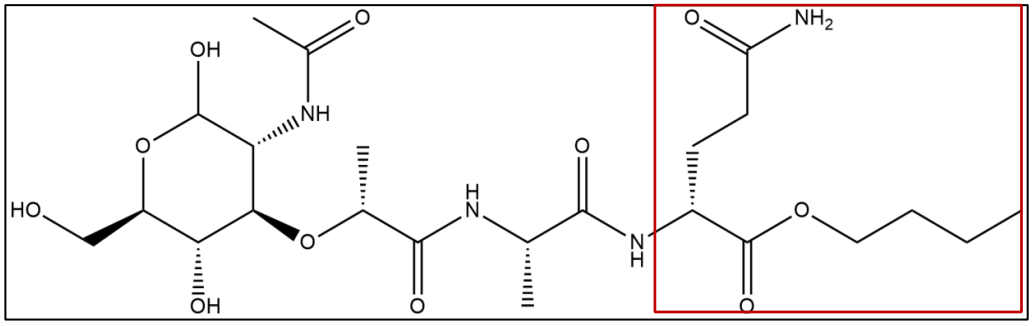

Figure 2: Structure of the hydrophilic derivative of MDP; Murabutide Chemdraw (RRID:SCR_016768) with the red box indicating structural modification to parent MDP.

1.3.6 Desmuramylpeptides

Desmuramylpeptides (DMPs) are MDP analogues without the parent MDP's Nacetylmuramyl (carbohydrate) moiety. Studies show increased adjuvant activity by MDP analogues with lipophilic substituents as desmuramylpeptides enter cells through passive absorption (Bourhis et al., 2007). Desmuramylpeptides can increase host defence against microbial infections and show notable antitumor potency (Sersa et al., 1992). The desmuramylpeptide LK-409 has its N-acetylmuramyl moiety replaced by the N-(7-

oxooctanoyl) acetyl group (Figure 3) and was found to delay tumour growth and decrease pyrogenicity, while LK-410 has N-trans-2-((2′-(acetylamino)cyclohexyl)oxy) acetyl group (Kikelj et al., 1998; Kotnik et al., 2020). The importance of desmuramylpeptides has increased over the years due to their ability to restore the immune cell function of cancer patients impaired by chemotherapeutics (Nabergoj et al., 2019)

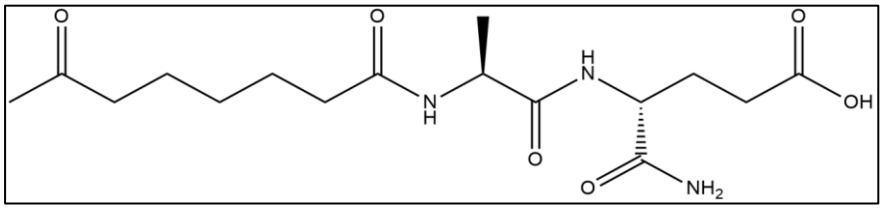

Figure 3: Modification done to N-acetylmuramyl moiety of parent MDP molecule; desmuramylpeptide in present-day clinical trials; LK-409. Chemdraw (RRID:SCR_016768)

1.4 NOD2 antagonists

The dysregulation of NOD2 signalling has been associated with various inflammatory diseases, autoimmune diseases, and certain types of cancer (Guzelj et al., 2020), suggesting that small molecule inhibitors of this signalling complex can be therapeutically valuable (Rickard et al., 2013). NOD2 antagonists have recently been identified as potential anticancer agents with a unique mechanism of action. Unlike NOD2 agonists, which generally produce pro-inflammatory tumour microenvironment (TME) and enhance the ability of immune cells to fight cancer cells, NOD2 antagonists have been proposed to primarily mediate their antitumor effects by suppressing the

generation of inflammatory TME (Nabergoj et al., 2019). Regulated suppression of NOD2 activity has thus been highlighted as a new approach to treating these diseases while keeping other innate immune mechanisms intact (Guzelj et al., 2020).

Studies done by Rickard et al. identified selective inhibitors of NOD2 signaling by screening approximately 1.9 million compounds for their ability to inhibit MDP-stimulated IL-8 secretion in human NOD2-expressing HEK293 cells. Active compounds were further tested to determine their inhibitory effects on NOD1, TNFR1, and TLR2-mediated responses to confirm the selectivity of NOD2 signaling and led to the discovery of Benzimidazole diamides are the only known class of NOD2-selective antagonists. Further investigation on the selective inhibition of NOD2 vs NOD1 mediated signalling pathways showed that GSK669 demonstrated complete selectivity towards NOD2 mediated IL-8 secretion but had no effect on NOD1 mediated IL-8 secretion in HCT116 colon carcinoma cells that express both NOD1 and NOD2 receptors. A more active analogue of GSK669 that is commercially available is a cell-permeable benzimidazole diamide compound GSK717 that selectively blocks MDP-stimulated cytokine release and downstream NF-κB and MAPK signalling pathways. Cell lines treated with MDP significantly upregulated NOD2 expression and increased their respective NF-κB transcriptional activities (Alyami et al., 2019), while these activities were suppressed by the NOD2 antagonist (Guzelj et al., 2020). Studies showed that THP-1 cells treated with the NOD2 antagonists prior to the addition of MDP significantly reduced the release of TNF-α and IL-8. Cell viability results also confirmed that the NOD2 antagonists were not cytotoxic at the maximum concentration tested (20 μM) (Guzelj et al., 2020). Though most research has focused on creating MDP derivatives with higher adjuvant activity,

developing MDP analogues that suppress rather than enhance immune responses is also a promising area of research.

1.5 *NOD2 signalling pathways*

1.5.1 NOD2 and TLR pathway

Numerous studies have demonstrated the importance of NOD2 receptors in decreasing tumorigenesis and inflammatory disorders in the gut. NOD2 and TLRs have similar downstream targets; for example, upon recognition of their respective PAMPs, the primarily membrane-bound TLRs recruit adaptor proteins such as Myeloid differentiation primary response 88 (MyD88), which activate the MAPK and NF-κB signalling pathways, resulting in the induction of pro-inflammatory and antimicrobial mediators such as interleukin IL-6, tumour necrosis factor-alpha (TNF-α), and IL-1β. Activation of NLRs and TLRs leads to activation of the NF-κB pathway that induces pro-inflammatory cytokines crucial for an effective immune response against invading pathogens and in most studies have shown to be a synergistic response. Activation of NLRs can upregulate TLRs expression and these pathways work together to enhance immune responses while separately a weaker response is reported like when only NOD2 receptor is activated by MDP a weaker immune response is observed and reported compared to the immune responses by the activation of NF-κB pathway by both lipopolysaccharide (LPS) through TLR4 and MDP through NOD2 receptor (Kobayashi et al., 2005; Boyle et al., 2014). Gobec et al. describe this interaction as a powerful amplification effect of MDP on TLR4-induced cytokine production (Gobec et al., 2018).

Few independent biochemical studies support the hypothesis that NOD2 can inhibit Toll-like receptor (TLR)-mediated activation, another important immune response pathway. The exact mechanism by which NOD2 inhibits TLR-mediated activation is not fully understood, but it is believed that NOD2 may affect signaling pathways downstream of TLRs, such as the NF-κB and MAPK pathways. This inhibition helps regulate the immune response and prevent excessive inflammation. Further studies are needed to fully explain the molecular mechanisms involved in NOD2 inhibiting the TLR-mediated activation of NF-κB and MAPK pathways (Gobec et al., 2018). NF-κB is the central pathway for inflammatory responses, and studies have proven that it plays an essential role in carcinogenesis and cell protection against apoptosis and promotes resistance to various cancer drugs (Pikarsky et al., 2004).

The regulation of the NF-κB and MAPK pathways is critical for producing surface cellular adhesion molecules, antimicrobial peptides and cytokines involved in the host response against infection. NOD2 receptors in the intestine in homeostatic conditions activate the NF-κB and MAPK pathways and suppress these pathways during acute and chronic inflammation. Thus MDP-dependent activation of NOD2 plays a vital role in immune responses even though bacterial TLR ligands more potently activate the NF-κB pathway than MDP (Udden et al., 2017; Kaser et al., 2010).

The ability of NOD2 to suppress TLR signaling pathways highlights the intricate and complex regulatory networks that exist in the immune system. This also indicates that dysregulation of these networks may contribute to the pathogenesis of inflammatory and autoimmune diseases (Udden et al., 2017; Kaser et al., 2010. The crosstalk between NOD2 and TLR signaling pathways is essential for adaptive immunity and immune

homeostasis. NOD2 agonists can enhance the adjuvant capacity of TLR ligands, resulting in stronger immune responses. This interaction between NOD2 and TLRs highlights the importance of NLRs in shaping immune responses and offers new opportunities for the development of new vaccines (Gobec et al., 2018).

1.5.2 NOD2 and AMPK pathway

Studies show that NOD2 present in hepatocellular carcinoma (HCC) cells can activate the adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) signalling pathway. NOD2 directly binds with serine/threonine-protein kinase STK11 (liver kinase B1; LKB1)–AMPK complex and regulates the LKB1/AMPK pathway inducing autophagy-mediated apoptosis of HCC cells. NOD2 is an efficient regulator of the AMPK pathway, and its deficiency leads to cancer progression and increases cancer cell resistance to various chemotherapeutic drugs. Resistance to chemotherapeutic drugs has always been a challenging obstacle. Studies also show that NOD2 can exert an antitumour effect that can enhance the sensitivity of cancer cells to induce apoptosis by activating the AMPK pathway (Ma et al., 2020). Reports confirm that NOD2 agonists can activate the cytotoxic potential of immune cells in a tumour microenvironment (TME) and actively enable their interactions with tumour cells (Ma et al., 2020).

1.6 Mutations in NOD2

NOD2 loss-of-function mutations in humans have been linked to an inflammatory bowel disease called Crohn's disease. Some patients with this disease have mutations in

the LRR domain of NOD2 that impair their ability to sense MDP and activate NF-κB. It is believed that it reduces the production of defensins and other antimicrobial peptides, which weaken the natural barrier function of the intestinal epithelium and cause inflammation characteristic of this disease. Functional mutations in human NOD2 are associated with inflammatory diseases, early-onset sarcoidosis and Blau syndrome, characterized by spontaneous inflammation in tissues such as the liver or joints, eyes and skin. Activating mutations in the NOD domain appear to promote a signalling cascade in the absence of a ligand, leading to an inappropriate inflammatory response in the absence of pathogens (Kenneth et al., 2017).

1.7 Conclusion and applications of MDP analogues

Major infectious diseases can be controlled with vaccinations, and studies show several MDP derivatives can be used in place of mycobacteria in Freund's Complete Adjuvant (FCA) since MDP is the smallest fragment in PGN that can mimic the immunostimulatory activities of FCA and can be used in immunological studies to enhance the immune response to an antigen. The adjuvant activity shown by MDP makes it an important compound that can be synthetically modified to improve chemical and biological properties. MDP derivatives have several clinical uses and therapeutic potential. MDP derivatives synthesized over the years as NOD2 ligands show antitumor and antimetastatic properties beneficial in clinical trials. For example, Murabutide has been used to boost the immune response as a cancer treatment. As mentioned, the European Medicine Agency approved one NOD2 agonist, Mifamurtide, for treating osteosarcoma. Though NOD2 ligands might not be able to eliminate or treat cancer

completely, existing cancer immunotherapies, chemotherapy, or radiation can use these ligands as adjuvants or in recombinant vaccines. The discovery of NOD2 antagonist and their potential to regulate pathways responsible for inflammation and tumorigenesis have expanded the study area for NOD2 ligands as they can recognize and act on the infiltration of immune cells like macrophages, lymphocytes, and natural killer (NK) cells in the early stages of tumour development which is crucial for an appropriate anticancer immune response. NOD2 agonists can act as (i) immunotherapeutic or (ii) adjuvants in cancer vaccines. NOD2 antagonists mediate their antitumor activity by preventing the formation of inflammatory TME.

2. RATIONALE AND OBJECTIVES

MDP is a popular adjuvant derived from PGN that can mimic the immunostimulatory effect of FCA (Effenberg et al., 2017). However, the parent MDP molecule and its derivatives show multiple drawbacks like high pyrogenicity and toxicity in clinical trials that need to be eliminated for MDP analogues to be a potential adjuvant. Only a few MDP analogues like Romurtide and Mifamurtide have been introduced into medical practices which means there is a serious need for new MDP analogues with no significant side effects. Another significant issue that needs to be addressed is the cost of production of these MDP analogues. Therefore, a new series of MDP analogues with modifications were designed and synthesized to further increase the parent MDP's therapeutic potential (Almzene, 2018). Six new MDP analogues were designed and synthesized by N. Almzene (Figure 4) by (a) replacing the D-isoglutamine (D-iso-Gln) in the MDP molecule with an aromatic amino acid, 3,4-di-aminobenzoic acid (DABA) and, (b) attaching a lipophilic chain of different length at the C-terminal of the dipeptide moiety to potentially increase the lipophilicity of the molecule and their transfer through biological membrane (Almzene, 2018).

Replacing the expensive D-isoglutamine (D-iso-Gln) in the MDP molecule with an aromatic amino acid, 3,4-di-aminobenzoic acid (DABA) is more cost effective, replacing D-iso-Gln with DABA on the NOD2 receptor may have different effects, but in general it can affect the ability of the receptor to recognize and respond to the modified MDP analogues and could potentially lead to a weakened or altered immune response to bacterial infections and also, depending on the specific substitution and its location in the receptor sequence, it can increase or decrease the overall functionality of the receptor

which means this replacement can either enhance or impair the NOD2-mediated immune response (Kenneth et al., 2017) therefore these novel MDP analogues **1-6** (Figure 4) must be biologically evaluated for their NOD2 receptor sensitivity, immunomodulatory activities and toxicity to potentially use them in low doses as an immunotherapeutic or adjuvant in cancer vaccines and drugs.

ICAM-1, TNF-α and IL-1β were chosen as markers in this present immunological and inflammatory studies as they play a key role in the inflammatory response, a key process of the immune system. Immune cells and other cell types produce and release them in response to infection, tissue damage, or various other immune problems (Bui et al., 2020). Studying these markers helps researchers understand the molecular mechanisms behind inflammation as these biomarkers are useful indicators of immune system activity and potential disease states. The use of established markers such as ICAM-1, TNF-α, and IL-1β allows for consistent comparisons between different studies and research groups. Researchers can design experiments based on existing knowledge about these markers and interpret results effectively. Reliable and validated assays are available for measuring ICAM-1, TNF-α, and IL-1β levels in biological samples, making them a practical choice for experimental analysis.

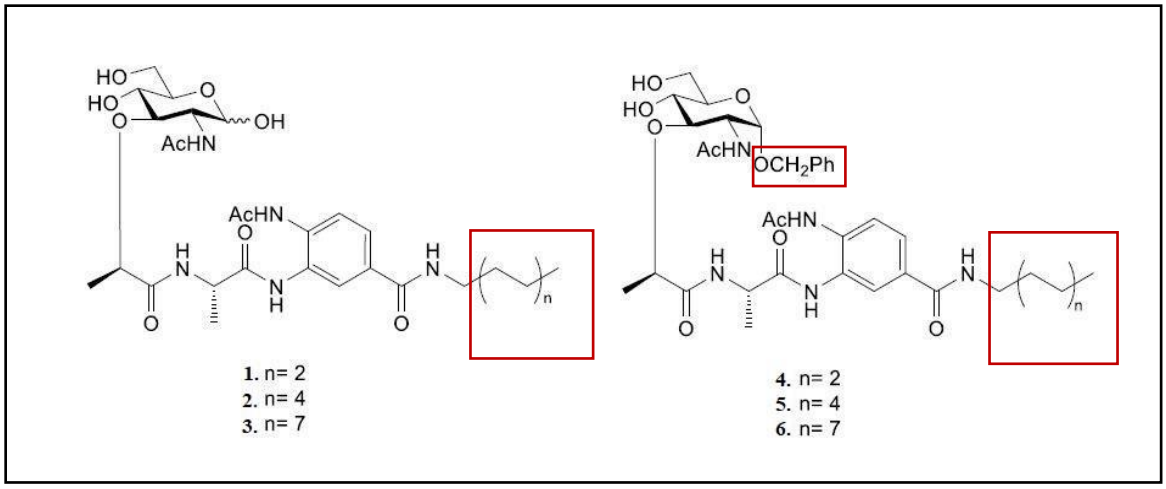

Figure 4: Novel MDP analogues synthesized by Almzene in 2018 at Lakehead University, Thunder Bay, Ontario, Canada. (Almzene, 2018)

The objectives of this thesis are:

1. Optimize an in-vitro experimental model through protocols in literatures to validate positive and negative controls and further use the designed experiment to document innate immune responses by THP-1 macrophages stimulated with the novel MDP analogues.
2. Evaluate the immunomodulatory properties of MDP analogues **1-6** alone and in combination with LPS for their potential to induce ICAM-1 expression and production of pro-inflammatory cytokines such as TNF-α and IL-1β by THP-1 macrophages.
3. Evaluate and test if the NOD2 receptor mediates these MDP analogues' biological activities (immunostimulatory/immunomodulatory) by employing known NOD2 inhibitor GSK717.

4. Analyze the structure-activity relationship of these novel MDP analogues and the usefulness of 3,4-di-aminobenzoic acid (DABA) as a replacement of D-iso-glutamine (D-iso-Gln) in the generation of novel MDP analogues as NOD2 agonists or antagonists.
5. Select one lead compound as a potential immunomodulatory/immunostimulatory agent for further studies.

3. RESULTS AND DISCUSSION

3.1 Synthesis summary of MDP analogues 1-6

Compounds **1-6** (Figure 4) were modified within the dipeptide component of parent MDP by replacing the D-iso-glutamine residue with an artificial aromatic amino acid residue. The synthesis of these compounds was described by N. Almzene (Almzene, 2018) and is herein summarized. The first step of the synthesis was to replace the D-isoglutamine (D-iso-Gln) of parent MDP with the structurally analogous aromatic amino acid, 3,4-di-aminobenzoic acid (DABA) (Figure 5), to enhance the stability of MDP analogues.

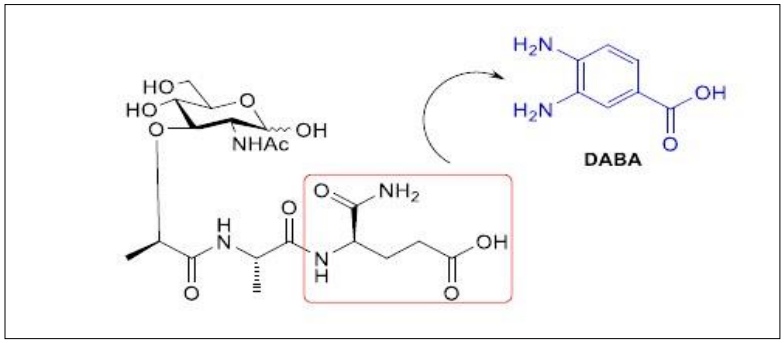

Figure 5: Replacement of D-iso-glutamine of parent MDP with the structurally analogous aromatic amino acid, 3,4-di-aminobenzoic acid. (Almzene, 2018)

In the process of synthesis, DABA was found to be quite unstable when exposed to air, therefore 4-acetamido-3-aminobenzoic acid was used as the surrogate of DABA. 4Acetamido-3-nitro-benzoic acid was the starting material, which, following the

reduction of the nitro group, was coupled with L-alanine. Finally, a lipophilic chain of different lengths was attached to the C-terminal of the dipeptide moiety to obtain molecules with higher lipophilicity as they tend to cross biological membranes and stick more easily to lipid-based cellular structures. Various lipid chain lengths were introduced at the C-terminal to acquire lipophilic dipeptide analogues, which were then coupled with an *N*-acetylmuramic acid derivative to get protected MDP analogues. Compounds **1-3** were fully deprotected after global deprotection, while compounds **4-6** were obtained as α-benzyl glycosides (Almzene, 2018) (Figure 6).

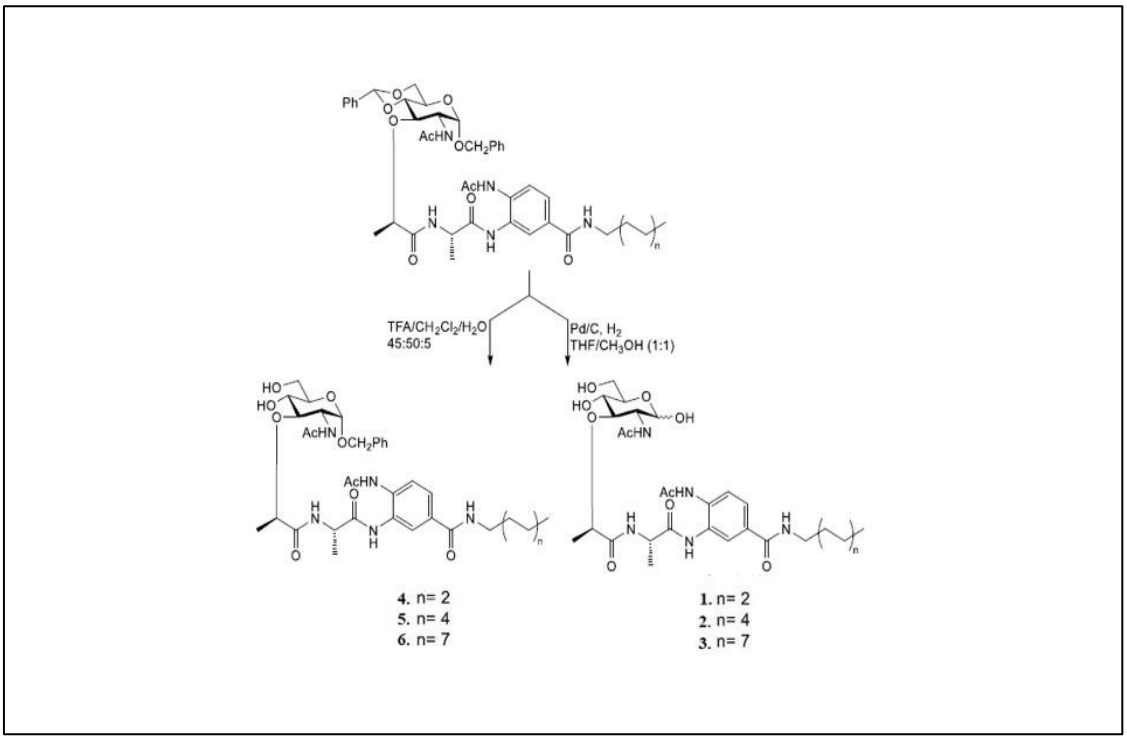

Figure 6: Deprotected MDP analogues 1-6. (Almzene, 2018).

3.2 Optimization of an *in-vitro* experimental model with THP-1 macrophages

- Recognition of MDP analogue by THP-1 macrophages was initially tested through ICAM-1 expression via immunostaining and Fluorescence activated cell sorting (FACS) analysis. To develop the in-vitro model for this study, experiments were conducted primarily to validate the positive controls (Murabutide and LPS) and negative control (PBS), and the protocols for the experiments were derived from studies on THP-1 macrophages (Khan et al., 2017; Chahal, 2021). The first positive control tested is LPS at 1 ng/ml, 10 ng/ml and 100 ng/ml, with reference to the protocols mentioned in literature, from the results it was determined that LPS at 100 ng/ml showed most consistent data and depicted high MFI reading 951±127 ($p<0.0001$) compared to non-stimulated THP-1 macrophages with PBS (Figure 7). Next the positive control Murabutide is tested at 20 μM following the protocol mentioned in literature and consistent MFI reading 750±36 was recorded with $p<0.0001$ compared to non-stimulated THP-1 macrophages. Finally, THP-1 macrophages were stimulated with Murabutide (20 μM) in co-stimulation with LPS (100 ng/ml) to study the potential synergistic effect. Though the MFI reading were high 1512±88 ($p<0.0001$) compared to non-stimulated macrophages, synergy by Murabutide and LPS was not detected (Figure 7). Minimal changes were made to cell culture incubation time in terms of differentiating THP-1 cells to macrophages using Phorbol myristate acetate (PMA) between 24 – 48 hours and there was no difference in the MFI measured for the positive controls, similarly the incubation time for LPS was tested between 18 – 20 hours and no difference in MFI was detected. The variation in nutrient supply (FBS, RPMI, PBS) for the cell cultures was studied and the results showed no significant difference. The optimized method is explained in detail in the

experimental sections below and was used in this study to further evaluate the new MDP analogues.

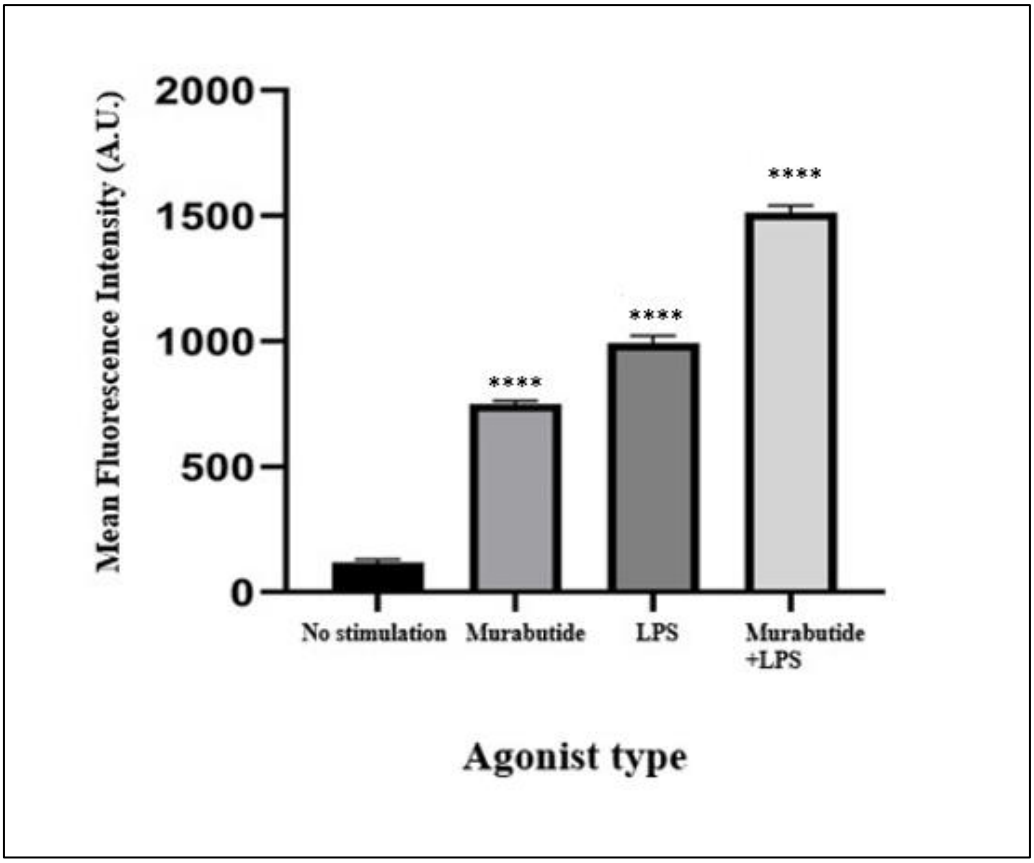

Figure 7: ICAM-1 expression in differentiated THP-1 cells stimulated by Murabutide (20 µM), LPS (100 ng/ml), and Murabutide (20µM) plus LPS (100ng/ml). ICAM-1 expression was measured via immunostaining and flow cytometry, and the results were expressed as the average of three separate experiments ± (SD) with three replicates. Statistical significance comparison was determined by one-way ANOVA, where **** $p<0.0001$ vs non-stimulated THP-1 macrophages.

3.3 Biological evaluation of MDP analogues 1-6

3.3.1 Induction of ICAM-1 expression

ICAM-1 is a membrane-bound glycoprotein and adhesion receptor that regulates the recruitment of leukocytes from the bloodstream to sites of inflammation and is expressed in epithelial cells and immune cells in response to inflammatory stimuli and functions as a biosensor by transmitting signals from outside the cell through its cytoplasmic domain, which associates with the actin cytoskeleton when its extracellular domain interacts with ligands (Bui et al., 2020). As a result, ICAM-1 plays an important role in various cellular functions in the initiation and resolution of pathological conditions (Bui et al., 2020). ICAM-1 is the most extensively studied among the other ICAM molecules because of its wide distribution and specific regulation and plays a critical role in immune and inflammatory responses; though ICAM-1 is constitutively expressed at low levels in endothelial cells and some lymphocytes and monocytes, its expression can be significantly increased in the presence of cytokines (TNF-α, IL-1, IFN-γ) and reactive oxygen species. ICAM-1 is involved in inflammatory cell trafficking, cell-cell interactions during antigen presentation, microbial pathogenesis, and through extrinsic signalling events. ICAM-1 binding has been documented to activate specific kinases through phosphorylation, leading to transcription factor activation and increased cytokine production, cell membrane protein expression, reactive oxygen species production, and cell proliferation (Hubbard & Rothlein, 2000). The clinical value of ICAM-1 could be re-examined to improve therapeutic strategies as its diverse roles in health and disease are better understood and new mechanistic insights into its function are gained (Bui et al. 2020).

ICAM-1 is an adhesion molecule and an important regulator for innate immune responses as NF-κB in response to TNF-α or IL-1β induces up-regulation of ICAM-1 expression (Bui et al., 2020). Only a few studies show the relationship between MDP and ICAM-1 expression nor evidence of ICAM-1 expression by macrophages from co-stimulation with LPS and MDP (Khan et al., 2017; Pashenkov et al. 2019). THP-1 cells are known for their high concentration of NOD2 receptors that can bind to MDP and induce the NF-κB pathway, and THP-1 macrophages induce a more robust immunomodulatory response than the THP-1 cell line (Khan et al., 2017). Therefore, the present study evaluated the novel MDP analogues (**1-6**) and their potential synergy with LPS in their capacity to induce ICAM-1 expression by THP-1 cells differentiated to THP-1 macrophages using phorbol 12-myristate 13-acetate (PMA). Earlier studies showed that 20 ng/ml of PMA can sufficiently differentiate THP-1 monocytes from macrophages by showing more than 90% adherence after incubating the cells for 24-48 hours (Khan et al., 2017; Chahal, 2021). THP-1 macrophages were then stimulated with the novel MDP analogues **1-6** at a concentration of 20 μM and incubated for 20 hours. The test concentration of MDP analogues at 20 μM was determined based on our previous studies that support the maximum capacity of MDP-stimulated activation of NOD2 at 20 μM (Khan et al., 2017; Khan et al., 2021). To better understand the potential synergism between MDP and LPS, THP-1 macrophages were incubated with the newly synthesized MDP analogues for one hour at 20 μM prior to stimulation by LPS (100 ng/ml) for 20 hours. ICAM-1 expression on cell surfaces of treated THP-1 macrophages was studied using flow cytometry analysis.

- *ICAM-1 expression induced by **1**–**6***

As observed in Figure 8, the positive controls Murabutide and LPS that have been studied over time in various studies (Khan et al., 2017) show a significant difference with MFI of 754±36 ($p<0.0001$) and 926±128 ($p<0.0001$), respectively, in comparison to no stimulation. Compound **1** (**1**, n=2, Figure 4) alone shows an MFI of 203±12 ($p=0.1$) and the statistical analysis depicts not statistically significant compared to untreated macrophages. which indicates low to no activity by compound **1**. Similarly, increasing the chain length from n-hexyl to n-decyl for compound **2** (**2**, n=4, Figure 4) gives us the same results of not statistically significant compared to untreated macrophages with MFI of 199±12 ($p=0.2$) while compound **3** has an n-hexadecyl (**3**, n=7; Figure 4) at the C-terminal and shows MFI of 257±17 ($p<0.002$) indicating some activity by compound **3** in inducing ICAM-1 expression in THP-1 macrophages. Compound **4** with an additional benzyl group (**4**, n=2, Figure 4) shows an MFI of 179±14 ($p=0.4$) and compound **5** with a benzyl group and an n-decyl chain (**5**, n=4, Figure 4) shows the lowest MFI 160±16 ($p=0.8$) among compounds **1**-**6**. Compounds **4** and **5** are not statistically significant compared to untreated THP-1 macrophages. Therefore, adding the benzyl group and an alkyl chain with moderate chain length (C_6 or C_{10}) does not increase the surface ICAM-1 expression and is statistically not significant (compounds **1-3** vs. compounds **4** and **5**) and between compounds **1**-**5**. Interestingly, a much higher ICAM-1 expression was induced by compound **6** of 1098±72 ($p<0.001$) compared to the non-stimulated THP-1 macrophages. Compound **6** also shows a high MFI compared to the positive controls with a value of $p<0.0001$ statistical difference compared to Murabutide and $p=0.0001$ compared to LPS. Compound **6** also has a higher MFI compared to the rest of compounds

1-5 with a statistical significance of $p<0.0001$ (Figure 8). Compound **6** at 20 μM shows 46% higher agonist activity than Murabutide (20 μM) and 15% higher than LPS in inducing ICAM-1 expression in THP-1 macrophages. The surface ICAM-1 expression in THP-1 macrophages depends on the NF-κB pathway and the increase in ICAM-1 regulation exerted by **6** might be due to the combined presence of the benzyl group instead of the reducing sugar and the increased lipophilicity resulting from a longer chain length (C_{16}), relative to compounds **1**-**5**.

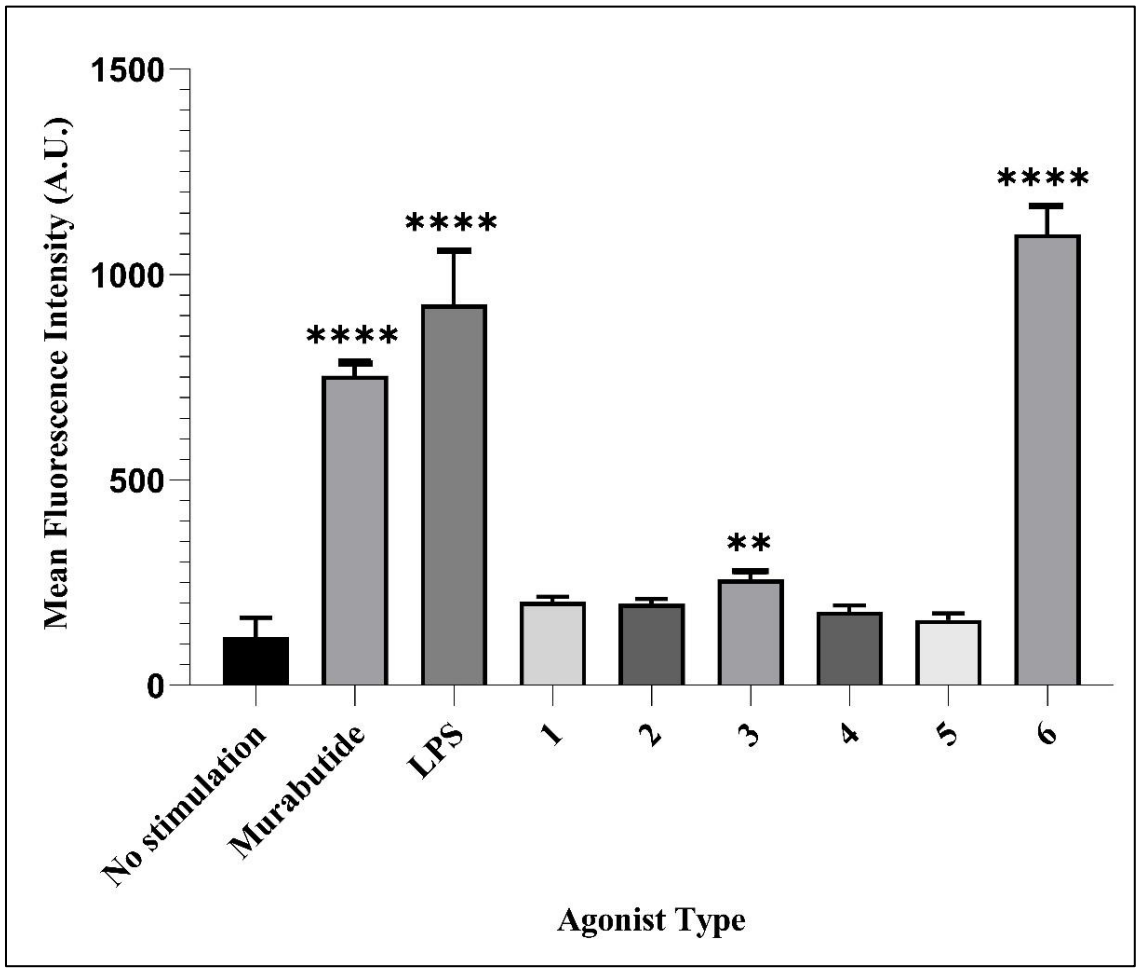

Figure 8: Surface ICAM-1 expression in differentiated THP-1 cells treated with 20 μM of MDP analogues 1-6. FACS analysis of THP-1 macrophages treated with MDP analogues **1-6** (20 μM), Murabutide (20 μM), and LPS (100 ng/ml) compared to unstimulated macrophages, measured via immunostaining and flow cytometry. The results are expressed as the means of three separate experiments ± (SD) with three replicates. Cell viability was determined by incubating unstained THP-1 macrophages with 1 μg/mL (8 μL) Propidium Iodide (PI) for one minute and then subjected to flow cytometry; cells with viability over 90% are used in this study. The ICAM-1 expression results are presented as the mean fluorescence intensity (MFI) on the FL2 channel acquiring 10,000 events. One-way ANOVA with multiple comparisons determined statistical significance, where **** $p<0.0001$, ** $p<0.002$ difference compared to untreated THP-1 macrophages.

- *ICAM-1 expression induced by **1 – 6** plus LPS*

The next set of experiments is the ICAM-1 expression in THP-1 macrophages induced by compounds **1-6** in combination with LPS (Figure 9), Murabutide in co-stimulation with LPS has MFI of 1512±87 ($p<0.0001$) compared to macrophages treated with LPS alone which means there is a significant increase in agonist activity by MDP compound in co-stimulation with LPS but not enough to conclude a synergistic effect between Murabutide and LPS. Compound **1-4** in co-stimulation with LPS show statistical analysis of ns (**1**, $p=0.8$; **2**, $p=0.5$; **3**, $p=0.4$; **4**, $p=0.9$) in comparison to expression levels in THP-1 macrophages stimulated with LPS alone (Figure 9); this is consistent with the

values from the previous tests (Figure 8) that these compounds (**1** – **4**) do not display any activity.

Compound **5** in co-stimulation with LPS depicts an increase in MFI at 1045±9 ($p=0.001$) in THP-1 macrophages compared to macrophages treated with LPS alone. In comparison to compound **5** alone, there is an increase in agnostic activity in ICAM-1 expression when co-stimulated with LPS more studies need to be done to further conclude their potential in various concentrations to determine if they can induce synergistic effects. In the above section (Figure 8), compound **5** alone shows the lowest MFI, but in co-stimulation with LPS shows a higher MFI compared to the rest of the compounds **1-4** and LPS alone (Figure 9). Statistical analysis shows that compounds **1**-**6** in co-stimulation with LPS are not statistically significant among each of them. Compound **6** in co-stimulation with LPS induces an ICAM-1 expression of 1634±99 ($p<0.0001$) by THP-1 macrophages (Figure 9) compared to macrophages stimulated with LPS alone. Compound **6** does not show synergism with LPS in inducing ICAM-1 expression by THP-1 macrophages (Figure 9).

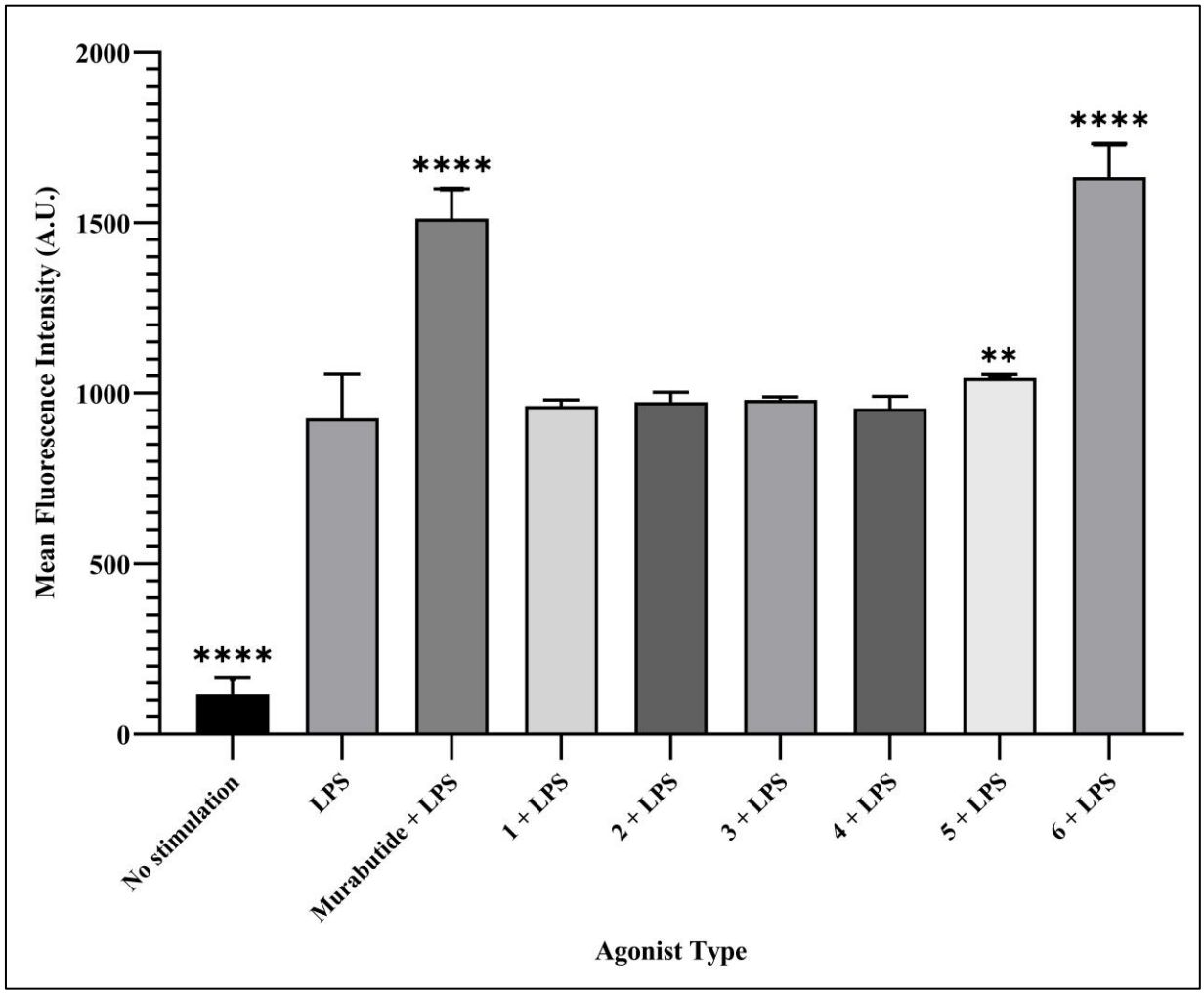

Figure 9: Surface ICAM-1 expression in differentiated THP-1 cells treated with 20 µM of MDP analogues 1-6 in co-stimulation with LPS (100 ng/ml). FACS analysis of THP-1 macrophages treated with MDP analogues **1-6** (20 µM) plus LPS (100 ng/ml), positive controls; Murabutide (20 µM) plus LPS (100 ng/ml), LPS (100 ng/ml) and unstimulated macrophages, measured via immunostaining and flow cytometry, and the results are expressed as the mean of three separate experiments ± (SD), each with three replicates. Cell viability was determined by incubating unstained THP-1 macrophages with 1 µg/mL (8 µL) Propidium Iodide (PI) for one minute and then subjected to flow

cytometry; cells with viability over 90% are used in this study. The ICAM-1 expression results are presented as the Mean Fluorescence Intensity (MFI) on the FL2 channel acquiring 10,000 events. One-way ANOVA determined statistical significance, **** $p<0.0001$, *** $p=0.0005$ and ** $p=0.001$ difference compared to LPS (100 ng/ml) treated THP-1 macrophages.

*- ICAM-1 expression induced by MDP analogue **6** and its dose-response*

Compound **6** in the above section shows the highest activity alone in THP-1 macrophages; therefore, its concentration-dependent response was investigated in a separate single experiment (Figure 10). THP-1 macrophages were stimulated with compound **6** at different concentrations of 5 μM, 10 μM, 15 μM and 25 μM. THP-1 macrophages treated with compound **6** at the mentioned concentrations were also co-stimulated with LPS (100 ng/ml), and the results of their combined induction of ICAM-1 expression is presented below (Figure 10). This set of experiments highlights the maximum ICAM-1 expression induced in THP-1 macrophages by compound **6** at 20 μM. Several studies have reported this characteristic of MDP analogues where there is a drop in the agonist activity after a specific concentration with or without co-stimulation of LPS (Khan et al., 2017; Khan et al., 2021) and further trials need to be attempted with multiple replicates to draw a conclusion regarding the responses to compound **6** at various concentrations and to construct a complete dose-response curve with EC_{50} calculations.

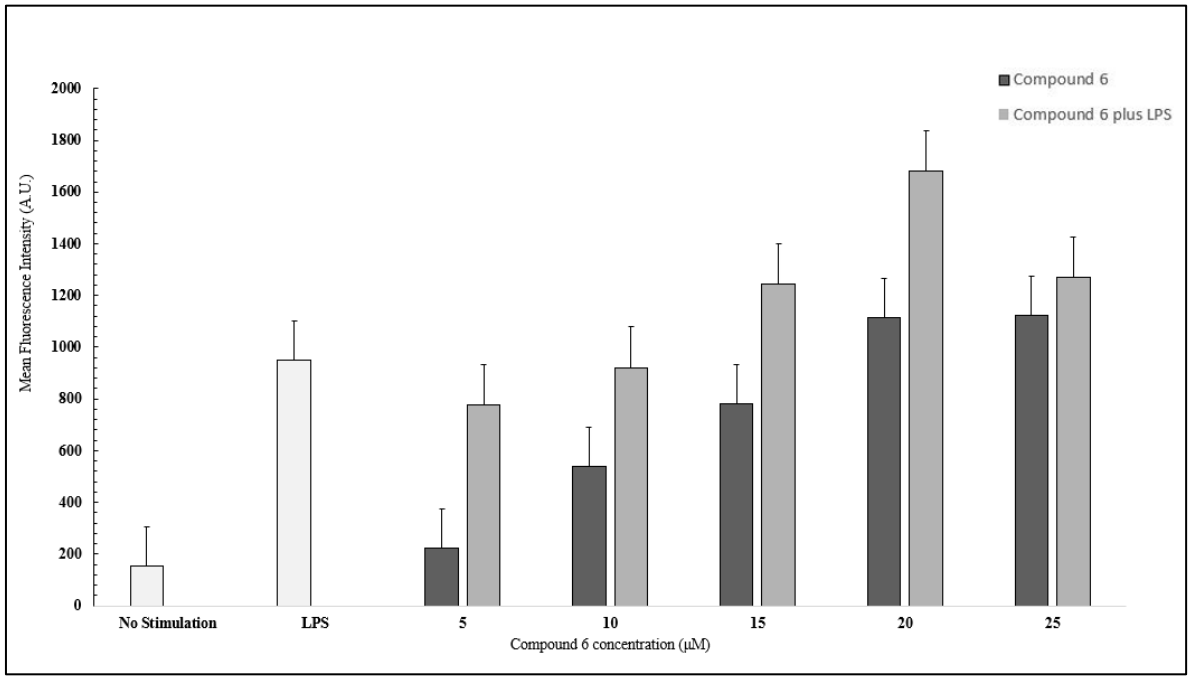

Figure 10: Surface ICAM-1 expression of THP-1 macrophages stimulated by Compound 6 at concentrations 5μM, 10 μM, 20 μM, and 25 μM and in co-stimulation with LPS (100 ng/ml). FACS analysis of THP-1 macrophages treated with MDP analogue **6** at concentrations 5μM, 10 μM, 20 μM, and 25 μM and in co-stimulation, with LPS (100 ng/ml), positive controls; LPS (100 ng/ml) and unstimulated macrophages, measured via immunostaining and flow cytometry, and the results are expressed as the mean of an experiment ± (SD) with three replicates. Cell viability was determined by incubating unstained THP-1 macrophages with 1 μg/mL (8 μL) Propidium Iodide (PI) for one minute and then subjected to flow cytometry; cells with viability over 90% are used in this study. The ICAM-1 expression results are presented as the Mean Fluorescence Intensity (MFI) on the FL2 channel acquiring 10,000 events.

- *Summary*

The developed *in-vitro* experimental model using THP-1 macrophages proved to be successful at stimulating ICAM-1 expression, an innate immune response activated in response to signals from NOD2 receptors that function to signal the presence of infectious agents. In this case, the positive controls Murabutide and LPS showed a high surface glycoprotein ICAM-1 expression by THP-1 macrophages, and the new MDP analogues could be tested using the same model. Though compounds **1-5** showed minimal activity when stimulated on THP-1 macrophages, compound **6** could be one potential immunomodulatory agent that induces high ICAM-1 expression in THP-1 macrophages.

3.3.2 Induction of TNF-α secretion

Activation of innate immune pathways by NOD2 ligands like MDP binding to the NOD2 receptor induces a broad range of pro- and anti-inflammatory signals, including the secretion of TNF-α. Disruption in the balance of these signals leads to chronic inflammatory states that directly affect cellular processes, such as cell cycle progression and apoptosis, creating a background context for various diseases. The primary importance of TNF-α is to contain the infection within the area of pathogen attack which is mainly secreted by macrophages as a pro-inflammatory cytokine through interaction between the MDP analogues and NOD2 receptors (Kenneth et al., 2017). Therefore, studying the induction of TNF-α secretion by THP-1 macrophages stimulated by MDP analogues could increase our knowledge of their immunomodulatory activity.

- *TNF- α secretion induced by **1 – 6***

The concentration of TNF-α secreted by THP-1 macrophages stimulated with compounds **1**-**6** alone at 20 μM was measured using an enzyme-linked immunosorbent assay (ELISA). These results are shown below in Figure 11. The positive controls Murabutide and LPS induce minimal concentrations of TNF-α secretion by THP-1 macrophages in this present study, Murabutide shows a concentration of 1.3±0.2 pg/ml ($p<0.0001$) and LPS shows 19±5 pg/ml ($p<0.0001$) compared to untreated THP-1 macrophages. It can be noted that compound **1** (0.05±0 pg/ml, $p=0.01$) alone does not induce TNF-α secretion by THP-1 macrophages and shows values lower than untreated THP-1 macrophages. In comparison, compound **2** elicits 2±0.7 pg/ml ($p<0.0001$) of TNF-α secretion compared to untreated macrophages and is not statistically significant than the positive control Murabutide but significantly lower than the concentration of TNF-α secreted by THP-1 macrophages stimulated with the positive control LPS (19±5 pg/ml, $p=0.0001$). It can be noted that compound **3** induces 0.6±0.3 pg/ml and compound **4** induces only 0.2±0.05 pg/ml and is statistically not statistically significant to the amount of TNF-α by untreated macrophages. Compound **5** induces 0.5±0.3 pg/ml ($p=0.006$) of TNF-α by THP-1 macrophages which are slightly higher than the untreated macrophages. Though MDP analogues **1-5** and Murabutide alone at 20 μM show minimal to no activity in stimulating TNF-α secretion by THP-1 macrophages, compound **6** shows a drastically high concentration of TNF-α of 1963±16 pg/ml ($p<0.0001$), which is higher than both the positive controls Murabutide and LPS (Figure 11) which is consistent with the much higher ICAM-1 level induced by compound **6** than other tested compounds (Figure 8). The data indicates that compound **6** alone displays potent activity

in inducing the production of pro-inflammatory cytokine TNF-α and can be described as a potent immunostimulatory agent in activating the innate immune response.

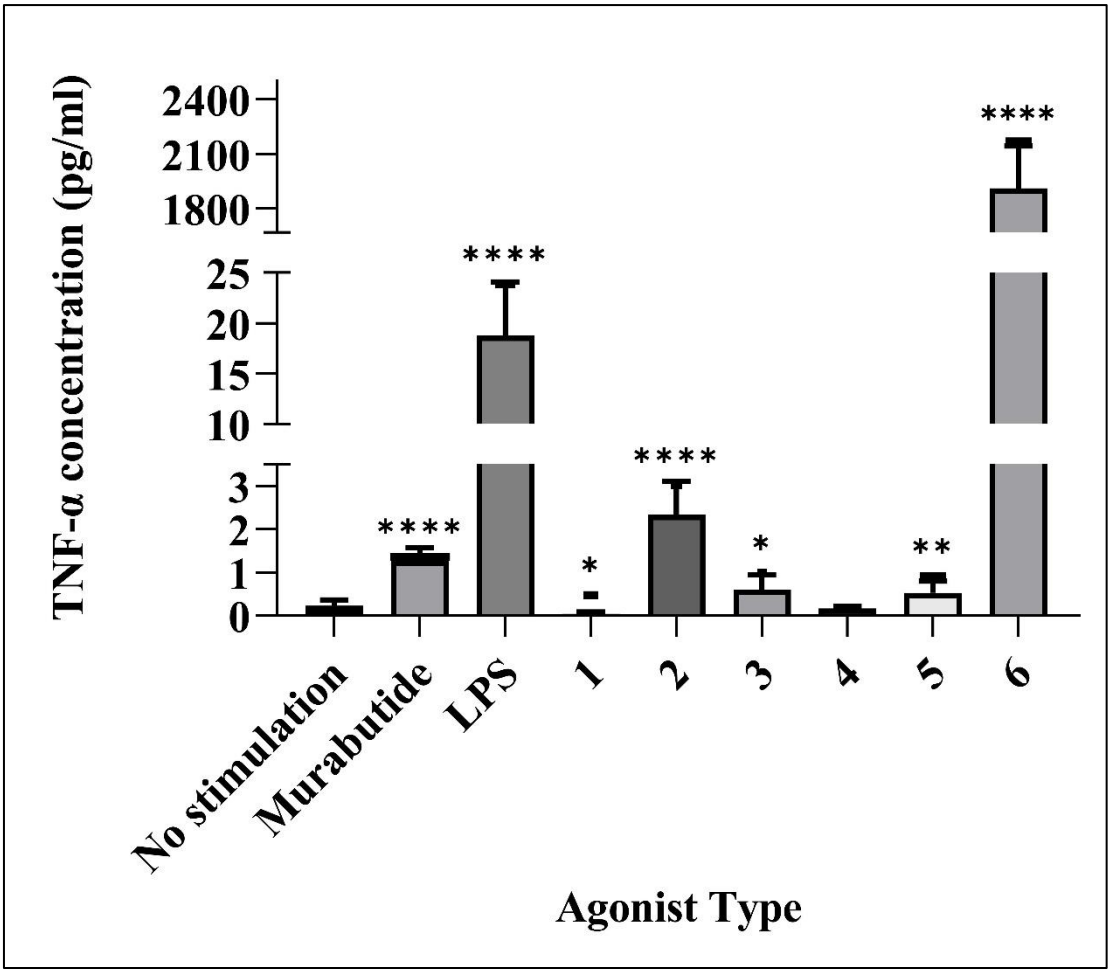

Figure 11: The concentrations of TNF-α secreted by THP-1 macrophages treated with 20 µM MDP analogues. ELISA test of supernatants collected from THP-1 macrophages treated with MDP analogues **1-6** (20 µM), positive controls; Murabutide (20 µM), LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SD), each with three replicates. The

concentrations of TNF-α results are presented in pg/ml. One-way ANOVA determined statistical significance, where ns is the non-significant difference, **** $p<0.0001$, ** $p=0.006$ and * $p=0.02$ difference compared to untreated THP-1 macrophages.

- *TNF-α secretion induced by **1 – 6** plus LPS*

The next set of experiments is to measure the concentration of TNF-α secreted by THP-1 macrophages by compounds **1**-**6** in combination with LPS (Figure 12), Murabutide in co-stimulation with LPS shows concentration of 871±154 pg/ml ($p<0.0001$) compared to macrophages treated with LPS alone at 18±7 pg/ml which means there is a significant increase in agonist activity of MDP compound in co-stimulation with LPS and also depicts a synergistic effect between Murabutide and LPS. Compounds **1** and **2** in co-stimulation with LPS increase the concentration of TNF-α secreted by THP-1 macrophages to 272±77 pg/ml and 244±27 pg/ml respectively with $p<0.0001$ compared to macrophages treated with LPS alone. Compound **3** shows a concentration of 348±49 pg/ml ($p=0.001$) TNF-α secretion by THP-1 macrophages compared to macrophages treated with LPS alone. Compounds **4** and **5** in co-stimulation with LPS show a steady increase in TNF-α secreted by THP-1 macrophages of 406±48 pg/ml and 592±118 pg/ml, respectively, which again is significantly higher ($p<0.0001$) compared to positive control LPS but low compared to TNF-α secreted by THP-1 macrophages stimulated with Murabutide (20 μM) in co-stimulation with LPS (100 ng/ml). The data depicts that compounds **1**-**5** in co-stimulation with LPS show a significant synergistic effect which can be classified as a synergy between the NOD2 ligand and TLR4 ligand (NLR signalling and TLR signalling). The observed synergy in turn suggests that these MDP

analogues are likely mediated by NOD2 receptor in terms of TNF-α secretion by THP-1 macrophages. Compound **6** in co-stimulation with LPS induces 480±79 pg/ml of TNFα, which is significantly higher than the positive control LPS ($p<0.0001$). It can be noted that when combined with LPS, the induced TNF-α level was reduced by 75%, suggesting that TLR4 agonists may regulate the immunostimulatory activity of compound **6** in inducing TNF-α response. The immune system must constantly balance activation and inhibition to avoid harmful and inappropriate inflammatory responses, and timely control of TLR4-mediated desensitization is essential to limit excessive inflammatory responses (Liew et al., 2005; Medvedev et al., 2007) which is highlighted in this study with compound **6** and LPS, and the important role of maintaining immune system balance to prevent harmful and inappropriate inflammatory responses. In the context of tolerance, this means that the immune system must be able to distinguish between harmless and harmful stimuli, respond appropriately to pathogens and threats, and prevent excessive inflammation if necessary. Overstimulation of proinflammatory cytokines is often associated with toxicity and side effects and the next logical step would be conducting immune-based toxicity because if the immune system continually overreacts to the same stimulus, chronic inflammation and tissue damage can occur. This could also be avoided by lowering the concentration of compound **6** and conducting a concentration-response curve to further confirm compound **6** as a potential lead compound.

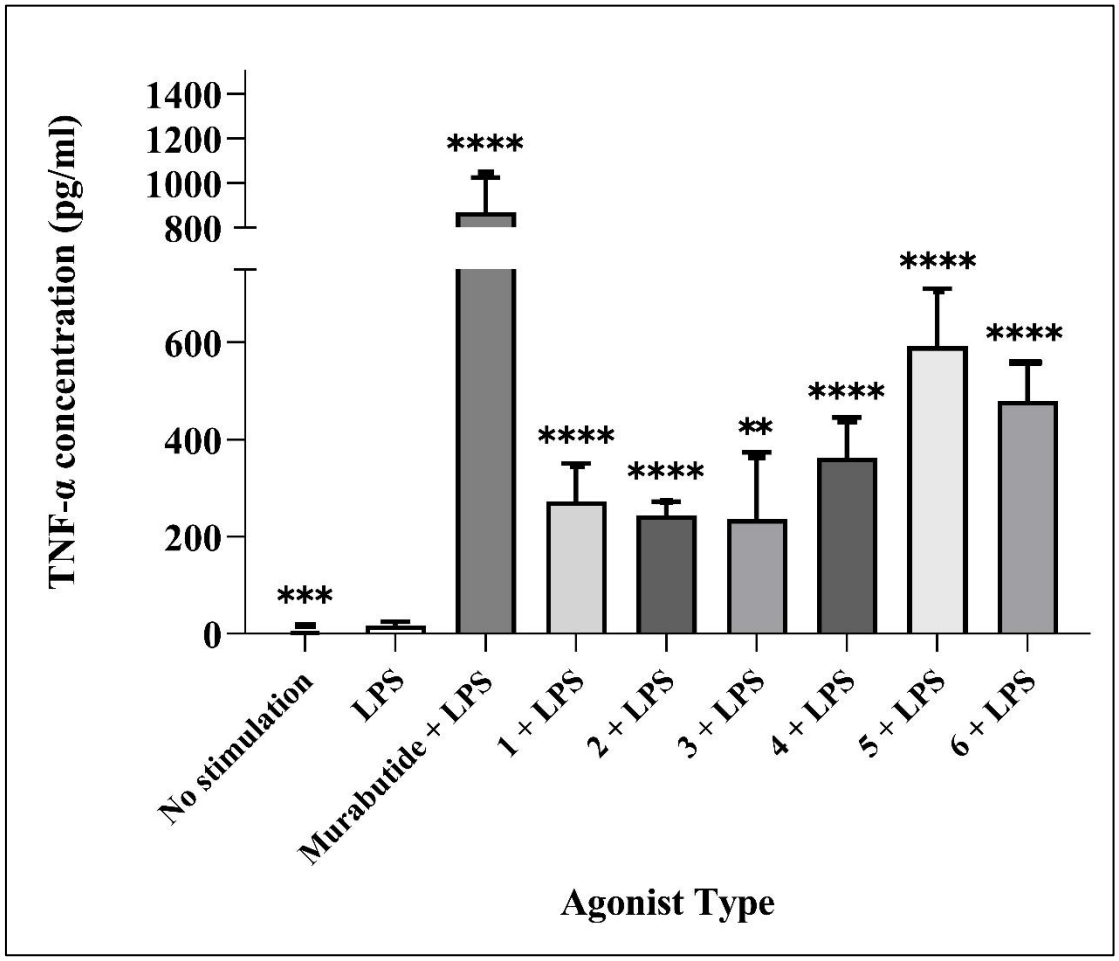

Figure 12: The concentrations of TNF-α secreted by THP-1 macrophages treated with 20 µM MDP analogues in co-stimulation with LPS (100 ng/ml). ELISA test of supernatants collected from THP-1 macrophages treated with MDP analogues **1-6** (20 µM) plus LPS (100 ng/ml), positive controls; Murabutide (20 µM) plus LPS (100 ng/ml), LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SD) with three replicates each. The concentrations of TNF-α results are presented in pg/ml. One-way ANOVA determined statistical

significance where, **** p<0.0001, *** p=0.0004 and ** p=0.001 difference compared to LPS (100 ng/ml) treated supernatants of THP-1 macrophages.

- *Summary*

From the previous section on TNF- α secretion, it can be observed that MDP analogues **1-5**, Murabutide and LPS induce minimal to no secretion of TNF-α by THP-1 macrophages (Figure 11). On stimulating THP-1 macrophages with MDP analogues **1-5** alone, there is no secretion of TNF-α, compounds **1-5** are seen to be inactive on their own to induce TNF- α production but they can induce a significant amount of TNF- α when co-stimulated with LPS that is evident from the statistical analysis above, thus showing a synergistic effect between each of compounds **1-5** and LPS.

Compound **6** induces the release of TNF-α at 1963±16 pg/ml on its own, but when co-stimulated with LPS, TNF-α decreases to 480±79 pg/ml. So, in this case, there is a 75% decrease in the concentration of TNF-α secreted between THP-1 macrophages stimulated with compound **6** plus LPS vs. compound **6** alone. Therefore, in this case, LPS showed an inhibitory effect on the release of TNF-α by THP-1 macrophages stimulated by compound **6**. The massive production of TNF-α by compound **6** can be controlled by combining it with LPS. Compound **6** potentiates TNF-α production in THP-1 macrophages and is a potent immunostimulatory agent activating the innate immune response.

3.3.3 Induction of IL-1β secretion

IL-1β is essential for host response and resistance to pathogens and exacerbates damage during chronic disease and acute tissue injury and is produced and secreted by cells of the innate immune system, such as monocytes and macrophages (Lopez-Castejon & Brough, 2011). IL-1β is produced as an inactive 31 kDa precursor called pro-IL-1β in response to molecular motifs carried by PAMPs that act through PRRs on macrophages to regulate pathways that regulate gene expression (Takeuchi & Akira, 2010). NLRs expressed by THP-1 macrophages cause the inflammasome to activate caspase-1, which induces proinflammatory cytokine IL-1β (Chen et al., 2009). The induction of pro-IL-1β expression is generally referred to as the priming step. It is an ineffective secretory stimulus that further needs to be exposed to PAMP or DAMP to stimulate the processing and secretion of the active IL-1β molecule (Lopez-Castejon & Brough, 2011 that initiates adaptive anti-tumour responses (Kaneko et al., 2019) along with inflammatory responses. Thus, it would be interesting to study the release of IL-1β by THP-1 macrophages stimulated by MDP analogues to increase our information on the immunomodulatory activity of the novel compounds.

- *Induction of IL-1β secretion by **1-6***

An ELISA test was used to determine the concentration of IL-1β secreted by THP-1 macrophages stimulated by compounds **1-6** alone (Figure 13). The positive controls Murabutide and LPS induce concentrations of IL-1β secretion by THP-1 macrophages in this present study, Murabutide shows a concentration of 67±12 pg/ml

($p<0.0001$) and LPS shows 268±37 pg/ml ($p<0.0001$) compared to untreated THP-1 macrophages. It can be noted that compound **1** alone induces only 2±0.6 pg/ml and is not statistically significant to the concentration of IL-1β secreted by untreated THP-1 macrophages. In comparison, compound **2** shows a slightly higher concentration of IL-1β of 9±4 pg/ml ($p=0.0002$) compared to untreated THP-1 macrophages. There is a further increase in the concentration of IL-1β by compound **3** of 21±3 pg/ml ($p<0.0001$) compared to untreated THP-1 macrophages. Compounds **4** and **5** also induce 4±1 pg/ml ($p<0.0001$) and 4±2 pg/ml ($p=0.2$) of IL-1β by THP-1 macrophages respectively. Compared to compounds **1-5** show minimal activity in IL-1β secreted by THP-1 macrophages compared to compound **6** ($p<0.0001$) in Tukey's multiple comparisons tests. Compound **6** shows a drastically high concentration of IL-1β of 862±172 pg/ml, which is significantly higher ($p<0.0001$) than the positive control Murabutide, and 3X times higher in concentration of IL-1β than the positive control LPS ($p<0.0001$) (Figure 13).

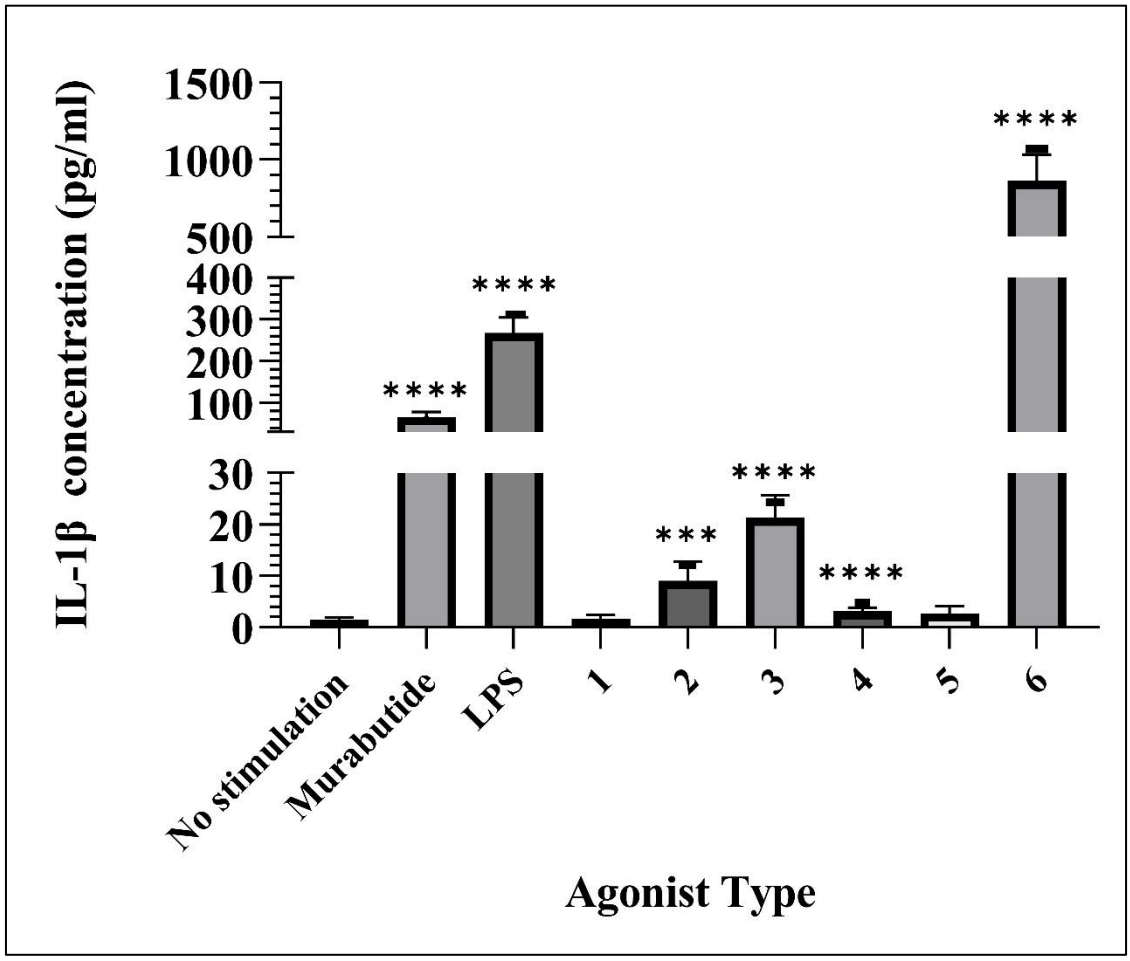

Figure 13: The concentrations of IL-1β secreted by THP-1 macrophages treated with 20 µM MDP analogues. ELISA test of supernatants collected from THP-1 macrophages treated with MDP analogues **1-6** (20 µM), positive controls; Murabutide (20 µM), LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SD) each with three replicates. The concentrations of IL-1β results are presented in pg/ml. One-way ANOVA determined statistical significance, compared to untreated supernatants of THP-1 macrophages. One-

way ANOVA determined statistical significance where, **** $p<0.0001$ and *** $p=0.0002$

- *Induction of IL-1β secretion by **1-6** plus LPS*

The next set of experiments is to measure the concentration of IL-1β secreted by THP-1 macrophages by compounds **1-6** in combination with LPS (Figure 14), Murabutide in co-stimulation with LPS shows concentration of 765±46 pg/ml ($p<0.0001$) compared to macrophages treated with LPS alone at 276±39 pg/ml which means there is a significant increase in agonist activity of MDP compound in co-stimulation with LPS and also depicts a synergistic effect between Murabutide and LPS. Compounds **1**, **2** and **3** in co-stimulation with LPS depict a significant increase in the concentration of IL-1β secreted by THP-1 macrophages to 3517±623 pg/ml ($p<0.0001$), 2740±157 pg/ml ($p<0.0001$) and 1969±527 pg/ml ($p<0.0001$), respectively compared to positive control LPS (100 ng/ml) treated THP-1 macrophages. Compounds **4-6** in co-stimulation with LPS (100 ng/ml) depict a concentration of IL-1β that amounts to 3438±670 pg/ml ($p<0.0001$) by compound **4**, 3041±211 pg/ml ($p<0.0001$) by **5** and 2950±456 pg/ml ($p<0.0001$) by compound **6** compared to macrophages treated with LPS alone. Thus, the MDP analogues show a significant increase in the amount of IL-1β secreted by THP-1 macrophages indicating a synergistic effect between these MDP analogues and LPS. The data suggests that there may be a synergistic and complementary nature between NOD-like receptor (NLR) signalling and Toll-like receptor (TLR) signalling and the reason for multiple receptors would be to increase the inflammatory response to an infection. The

combined effect is commonly observed with TLR and NLR signalling in response to PGN and TLR and NOD2 agonists which has been demonstrated with IL-1β secretion (Figure 14). The IL-1β pathway involves both NOD2 and TLR signalling. Stimulation of TLRs or NOD2 stimulates the production of pro-interleukin-1-beta (pro-IL-1β) by activating nuclear factor-kappa B (NF-κB) signalling leading to IL-1β gene transcription. In contrast, NLRs detect the presence of microbial molecules in the cytosol, causing inflammasome formation and caspase-1 activation. Activated caspase-1 cleaves pro-IL-1β and leads to secretion of mature IL-1β. This TLR-NLR interaction in response to intracellular bacteria helps prepare the immune system to fight invading pathogens (Chen et al., 2009).

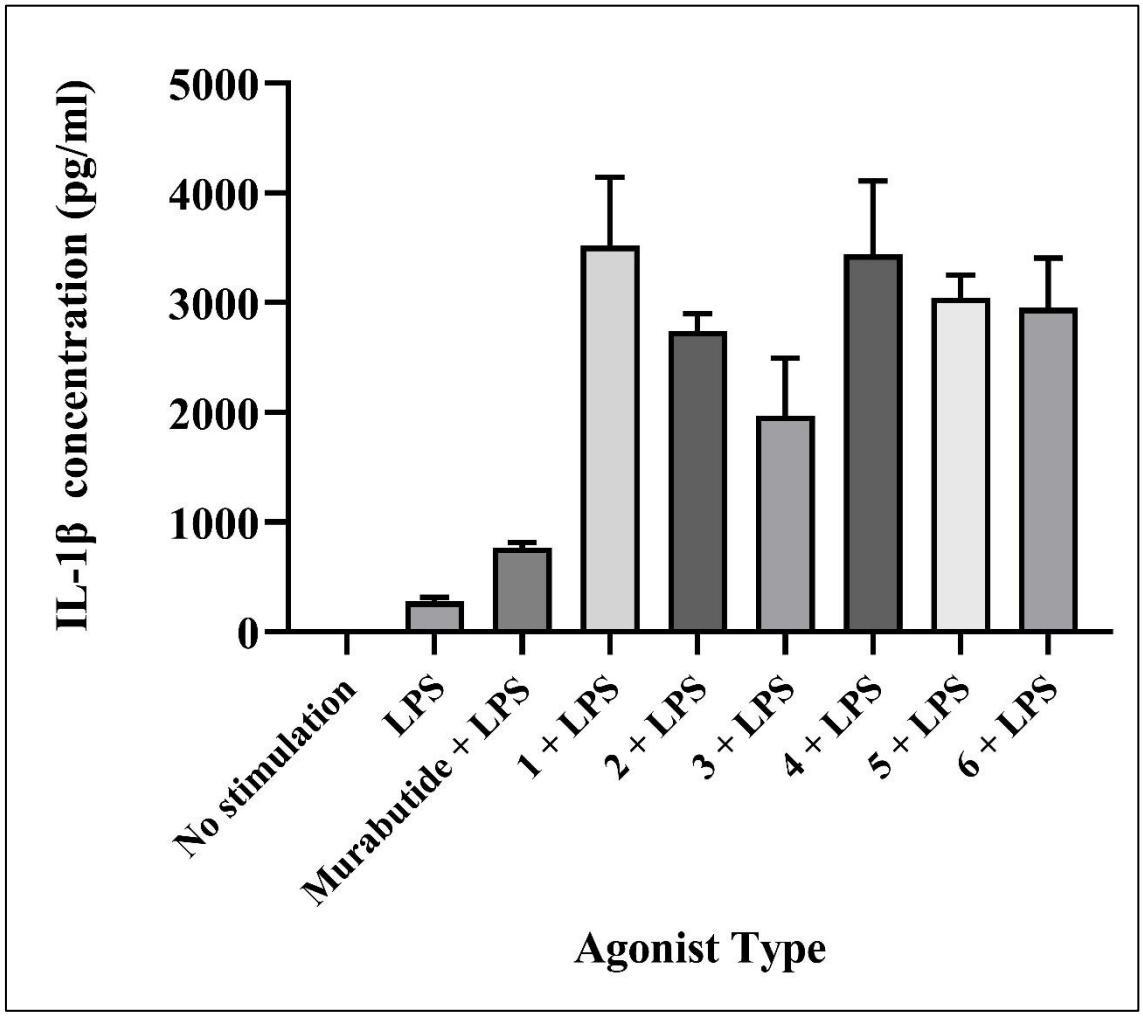

Figure 14: The concentrations of IL-1β secreted by THP-1 macrophages treated with 20 µM MDP analogues in co-stimulation with LPS (100 ng/ml). ELISA test of supernatants collected from THP-1 macrophages treated with MDP analogues **1-6** (20 µM) plus LPS (100 ng/ml), positive controls; Murabutide (20 µM) plus LPS (100 ng/ml), LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SD) with three replicates. The concentrations of IL-1β results are presented in pg/ml. One-way ANOVA determined statistical significance, where negative control, Murabutide plus LPS and compounds **1-6** plus LPS

depict **** $p<0.0001$ difference compared to LPS (100 ng/ml) treated supernatants of THP-1 macrophages.

- *Summary*

From the previous section on IL-1β, it can be observed that MDP analogues **1-5** show no induction of IL-1β. On stimulating THP-1 macrophages with MDP analogues **1-5** alone, there is no secretion of IL-1β, compounds **1-5** are seen to be inactive on their own to induce IL-1β production but induce a significant amount of IL1β when co-stimulated with LPS, thus showing a synergistic effect between each of compounds **1-5** and LPS. Similarly, compound **6** induces the release of IL-1β at 862±172 pg/ml. However, when co-stimulated with LPS, IL-1β increases to 2950±456 pg/ml, indicating a significant synergistic effect between compound **6** and LPS. This study also depicts that apart from compound **6** alone inducing high levels of IL-1β, the other compounds **1-5** alone show minimal activity compared to in co-stimulation with LPS, thus indicating that immune responses by MDP- mediated activation of the NF-κB pathway via the NOD2 receptor are much weaker than the overall immune response by NF-κB activation during co-stimulation with LPS and MDP analogue. The synergy between TLR4 and NOD2 is evident in the results found in Figure 14, where THP-1 macrophages are simultaneously stimulated with TLR4 and NOD2 agonists.

3.3.4 Mechanistic studies: inhibition of NOD2

THP-1 cells express high levels of NOD1, NOD2, and TLR4 receptors and any dysregulation or mutation of these receptors and their downstream signalling pathways

can lead to various infectious diseases, chronic inflammatory and autoimmune diseases (Jakopin 2014). Therefore, selective inhibition of NOD2 signalling can be useful in the treatment of many acute and chronic diseases and GSK717 is a commercially available NOD2 inhibitor that has been used in many studies to investigate the negative regulation of MDP mediated NOD2 signalling pathways (Alyami et al., 2019; Guzelj et al., 2020; Rickard et al., 2013). The developed in-vitro method in this present study can also be used to characterize NOD2 antagonists like GSK717. MDP analogues Murabutide and compound **6** could induce ICAM-1 expression and the release of proinflammatory cytokines TNF-α and IL-1β on their own and in combination with LPS therefore, it is interesting and important to investigate the possibility of inhibition of NOD2 activation that may be achieved with NOD2 antagonists. These compounds interfere with MDP binding to NOD2 or disrupt downstream signaling pathways and this can be tested by measuring the effect of NOD2 antagonism on ICAM-1 expression and further examine the extent to which TNF-α and IL-1β secretion by THP-1 macrophages is inhibited by GSK717 to determine if the biological activity of these MDP analogues is mediated by NOD2 receptor. ICAM-1 is relatively easy to detect and measure using methods such as flow cytometry, while TNF-α and IL-1β can be easily detected through ELISA which makes it a practical choice for evaluating NOD2 activity in laboratory experiments and clinical trials.

- *NOD2 inhibitor GSK717*

The commercially available NOD2 inhibitor GSK717 is a cell-permeable benzimidazole diamide compound that selectively inhibits MDP-induced, NOD2

mediated cytokine production and though the exact mechanism is still being researched, multiple studies have tested the inhibition of pro-inflammatory cytokines in macrophages pre-treated with GSK717 (Guzelj et al., 2020; Jakopin, & Corsini, 2019) and was therefore used in this present study.

Differentiated THP-1 macrophages were treated with GSK717 at various concentrations to test for cell viability. The concentration range of 20 μM to 30 μM was suitable for our studies, with cell viability at an average of over 80%. The maximum cell viability was seen at 25 μM at 92%; therefore, the highest safe concentration the inhibitor could be tested at is 25 μM.

A few studies (Khan et al., 2017, Khan et al.,2021) have reported the surface glycoprotein ICAM-1 expression by differentiated THP-1 cells induced by MDP analogues. To expand our knowledge of the relation between NOD2 receptor expressed in THP-1 macrophages and MDP analogue-induced ICAM-1 expression, pharmacological inhibition of the NOD2 receptor was done by pre-treating THP-1 macrophages with GSK717 for an hour prior to stimulating the macrophages with NOD2 agonist Murabutide.

- *Effect of GSK717 on ICAM-1 expression induced by Murabutide*

A single set of experiments to measure the ICAM-1 expression in macrophages treated with GSK717 at 20 μM (most used concentration in studies related to GSK717) was done using Murabutide as the positive control (Figure 15). When GSK717-treated THP-1 macrophages are stimulated along with Murabutide at 20 μM, an increase in ICAM-1 expression is observed compared to macrophages treated with Murabutide alone (Figure 15) indicating that GSK717 does not exert antagonistic activity against Murabutide in inducing ICAM-1 expression. This contrasts with earlier reports that GSK717 inhibits

MDP-induced response and that the inhibitor GSK717 has a competitive interaction to bind to the same site on NOD2 as MDP or any other NOD2 agonist (Rickard et al.2013). The observed effect depends on the concentrations of agonist and antagonist used in the assay, which in turn depends on how strongly each one binds to the receptor. In this case, however, there seems to be a statistically significant increase in response with the antagonist alone, which may be an issue, but it could explain the increased response upon co-administration.

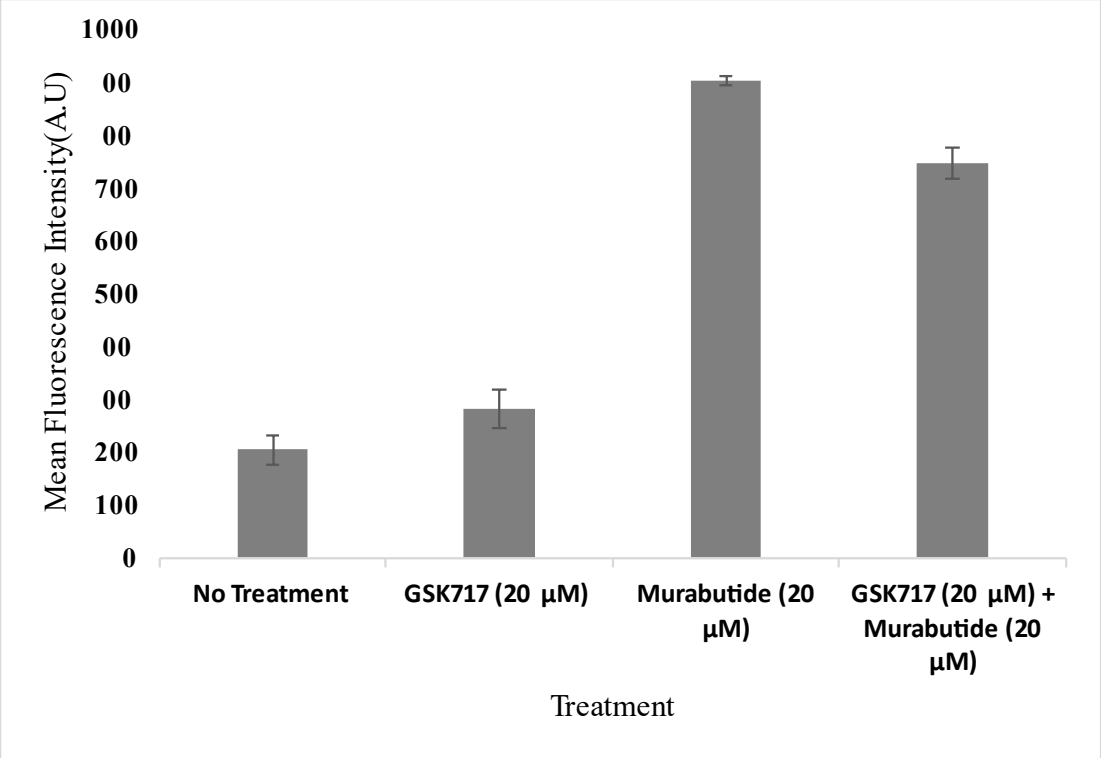

Figure 15 : ICAM-1 expression by THP-1 macrophages treated with NOD2 inhibitor GSK717 at 20 µM and Murabutide at 20 µM. FACS analysis of THP-1 macrophages treated with GSK717 (20 µM) plus Murabutide (20 µM), Murabutide (20

μM), GSK717 (20 μM) and unstimulated macrophages, measured via immunostaining and flow cytometry, and the results are expressed as the mean of an experiment ± (SEM) with three replicates. Cell viability was determined by incubating unstained THP-1 macrophages with 1 μg/mL (8 μL) Propidium Iodide (PI) for one minute and then subjected to flow cytometry; cells with viability over 90% are used in this study. The ICAM-1 expression results are presented as the Mean Fluorescence Intensity (MFI) on the FL2 channel acquiring 10,000 events.

It was assumed that decreasing the concentration of NOD2 agonist Murabutide and increasing the concentration of the antagonist GSK717 would inhibit or decrease the ICAM-1 expression. Further studies were done to test this idea. The concentration of GSK717 in THP-1 macrophages was increased from 20 μM to 25 μM and additionally stimulated with a concentration of Murabutide decreased from 20 μM to 12.5 μM. The MFI of THP-1 macrophages, when stimulated with 12.5 μM of Murabutide alone, is 339±66. In comparison, when pretreated with 25 μM of GSK717 and stimulated with 12.5 μM of Murabutide, similar results of an increase in ICAM-1 expression can be observed as indicated by an increased average MFI of 1213±86 (Figure 16). This variation in ICAM-1 is different from what was anticipated. Due to time constraints, this experiment was not repeated to confirm the findings.

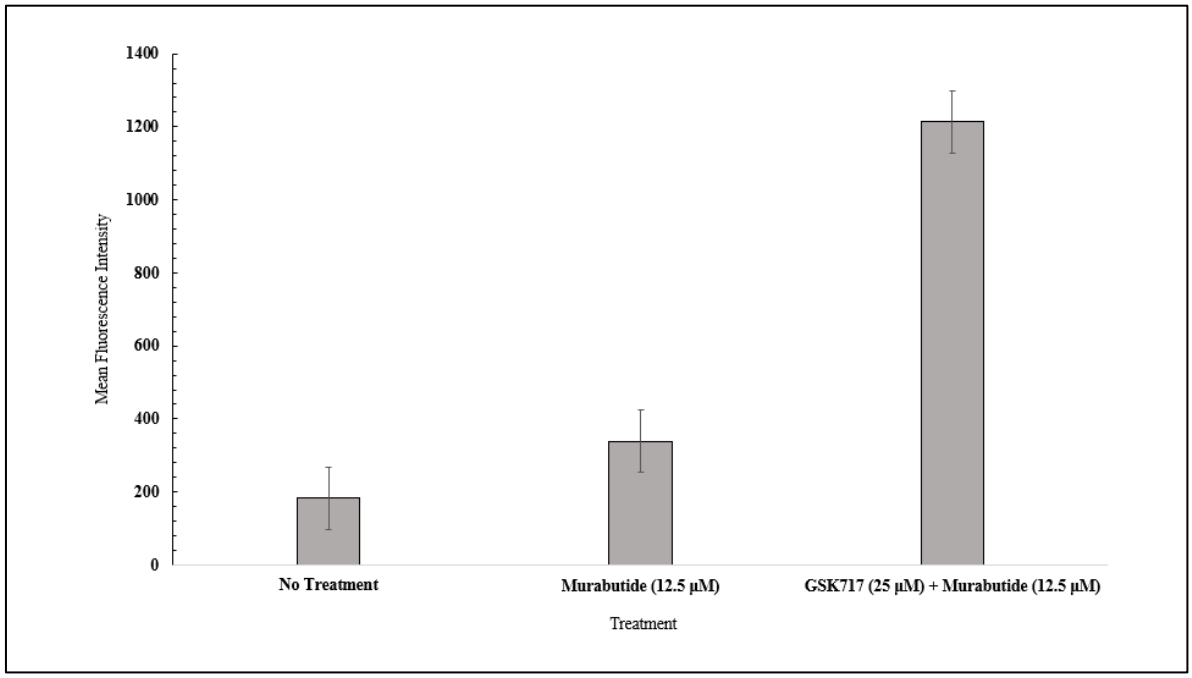

Figure 16: ICAM-1 expression by THP-1 macrophages treated with NOD2 inhibitor GSK717 at 25 µM and Murabutide at 12.5 µM. FACS analysis of THP-1 macrophages treated with GSK717 (25 µM) plus Murabutide (12.5 µM), Murabutide (12.5 µM) and unstimulated macrophages, measured via immunostaining and flow cytometry, and the results are expressed as the mean of an experiment ± (SEM) with three replicates. Cell viability was determined by incubating unstained THP-1 macrophages with 1 µg/mL (8 µL) Propidium Iodide (PI) for one minute and then subjected to flow cytometry; cells with viability over 90% are used in this study. The ICAM-1 expression results are presented as the Mean Fluorescence Intensity (MFI) on the FL2 channel acquiring 10,000 events.

Murabutide was reported to be a NOD2 agonist and activates the NOD2 signalling pathway when it binds to the NOD2 receptor (Rickard et al.,2013). GSK717 has been shown as an inhibitor of MDP-induced responses and an antagonist of the

NOD2 receptor (Rickard et al.,2013). Our findings indicate that GSK717 does not inhibit the induction of ICAM-1 expression in THP-1 cells stimulated by Murabutide. The increased level of response in cells co-stimulated with GSK717 and Murabutide can be attributed to the dual action of GSK717 and Murabutide. Such dual action can only be realized when GSK717 and Murabutide have different targets. If both molecules act on the NOD2 receptor, they will have two different binding sites on NOD2. Alternatively, one of the two molecules binds to the NOD2 receptor and the other acts on another component in the NOD2-mediated signalling pathway. Their concerted action leads to an enhanced response. The crystal structure of the NOD2 receptor with a bound ligand in the receptor binding site is not yet available (Guzelj et al., 2022). Therefore, there has been no direct evidence of how a NOD2 ligand binds to the receptor. Further studies are needed to provide evidence in support of Murabutide and GSK717 binding to NOD2 as an agonist or antagonist. To further support the data collected, additional tests need to be conducted with additional controls to conclude that GSK717 enhances ICAM-1 expression by direct interaction with NOD2 receptor. The supernatants collected during this experiment need to be further tested for the inhibition of pro-inflammatory cytokines to understand the interaction better, due to time restriction, this was not completed in this study.

- *Effect of GSK717 on TNF-α secretion induced by **6** alone and **6** plus LPS*

To further study the inhibition of cytokines by GSK717, ELISA tests were conducted to measure the TNF-α concentration by THP-1 macrophages pre-treated with GSK717 and stimulated by compound **6** alone and in combination with LPS (Figure 17).

It is interesting to note the decrease in pro-inflammatory cytokines TNF-α by THP-1 macrophages pre-treated with GSK717 and stimulated with compound **6** (Figure 17). Compound **6** alone secreted TNF-α at 1963±16 pg/ml, but when compound **6** was used to stimulate macrophages pre-treated with GSK717 at 20 μM, a drastic decrease in the concentration of TNF-α to 15±1 pg/ml ($p<0.0001$) can be seen (Figure 17). Similarly, GSK717 treated macrophages, when stimulated with LPS and compound **6**, show a decrease in the concentration of TNF-α from 996±4 pg/ml to 58±4 pg/ml ($p<0.0001$). The observed diminished levels of TNF-α production in the current study shows that THP-1 macrophages pre-treated with GSK717 and further stimulated with Compound **6** and LPS does not bring the level of cytokine back down to the LPS baseline, as seen in the study by Jakopin et al., 2019. This suggests that compound **6** may activate other pathways, or perhaps the concentration of antagonist used is not sufficient for full NOD2 inhibition. The above data also indicate that GSK717 can completely inhibit the stimulatory effect of compound **6** on inducing the production of TNF-α either alone or in combination with LPS. The results suggest that compound **6** binds to NOD2 and further experiments need to be done to create a concentration curve that shows the signal is inversely proportional to the concentration of GSK717 at a fixed concentration (EC50) of compound **6** to conclude competitive antagonism between NOD2 and compound **6**.

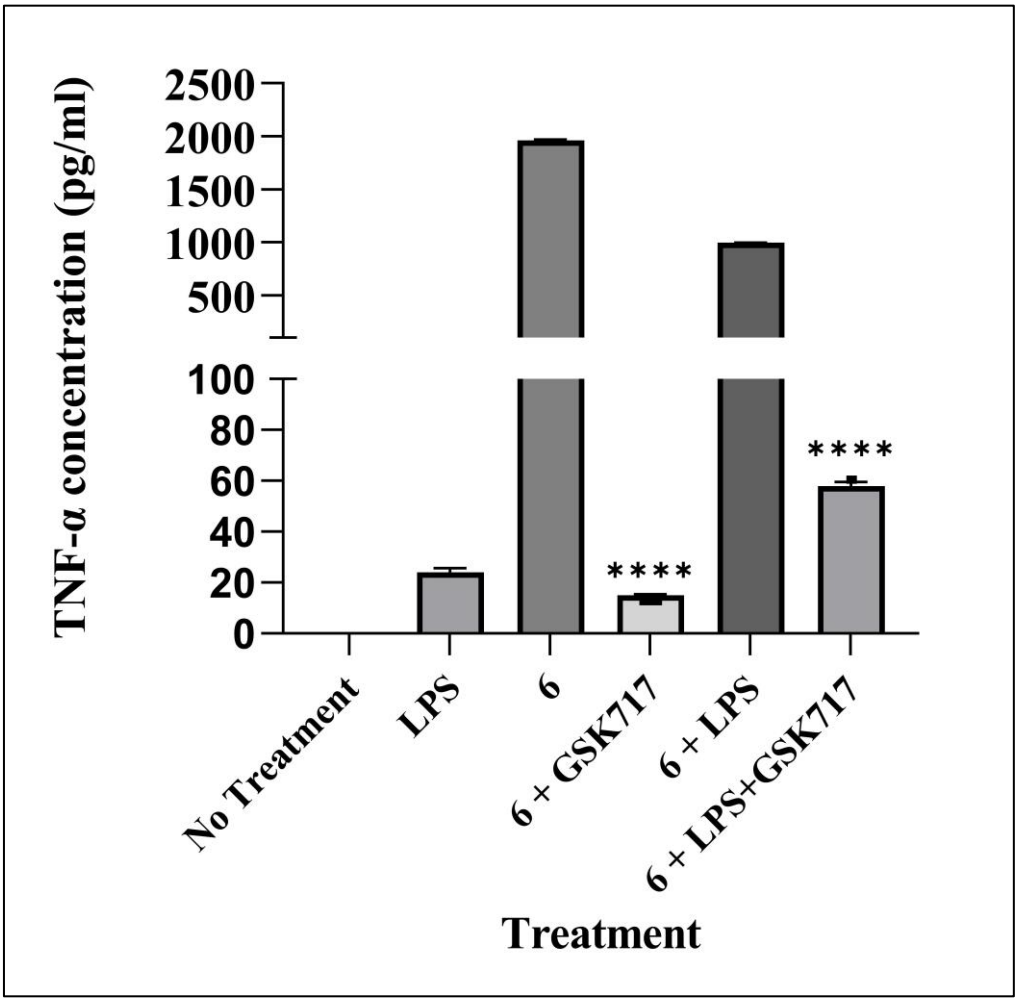

Figure 17: The concentrations of TNF-α secreted by THP-1 macrophages pre-treated with NOD2 inhibitor GSK717 and further stimulated with 20 µM MDP analogue 6 alone and combined with LPS (100 ng/ml). ELISA test of supernatants collected from THP-1 macrophages pre-treated with NOD2 inhibitor GSK717 and then stimulated with MDP analogue **6** (20 µM), positive control: LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SEM) with three replicates. The concentrations of TNF-α results are presented in pg/ml. One-way ANOVA determined statistical significance where ****

$p<0.0001$ difference compared to LPS (100 ng/ml) treated supernatants of THP-1 macrophages.

- *Effect of GSK717 on IL-1β secretion induced by **6** alone and **6** plus LPS*

In the next set of experiments ELISA tests were conducted to measure the IL-1β concentration by THP-1 macrophages pre-treated with GSK717 and stimulated by compound **6** alone and in combination with LPS (Figure 18). Compound **6** alone stimulated the macrophages to secrete IL-1β at 924±98 pg/ml, but when compound **6** was stimulated on macrophages treated with GSK717 at 20 μM, a decrease in the concentration of IL-1β to 115±5 pg/ml ($p=0.004$) can be seen (Figure 18). The activity of compound **6** alone in inducing the release of IL-1β was primarily suppressed in the presence of GSK717. On the other hand, GSK717 treated macrophages, when stimulated with LPS and compound **6**, show only a slight decrease in the concentration of IL-1β, from 3189±327 pg/ml to 2645±6 pg/ml which is statistically non-significant and more prominent than the value induced by either compound **6** alone (924 pg/ml) or LPS alone (331 pg/ml) (Figure 13). The data indicates that the synergy between compound **6** and LPS is still present when the cells are co-treated with GSK717 and the stimulants (compound **6** and LPS), and that GSK717 displays weak inhibitory activity in IL-1β production. In comparison to its strong antagonistic activity in TNF-α production with $p<0.0001$ between THP-1 macrophages stimulated with compound **6** and macrophages pre-treated with GSK717 followed by stimulation with compound **6**, GSK717 can be described as a weak antagonist against compound **6** in inducing IL-1β production in THP-

1 macrophages which could be associated with the inflammasome that activates caspase-1, an enzyme involved in inflammation that aids in the release of mature and active IL-1β and is not inhibited by GSK717, this opens a further new area of study. GSK717 is a potent NOD2 inhibitor and multi-dose studies would be required to determine the antagonistic properties of GSK717 vs compound **6**.

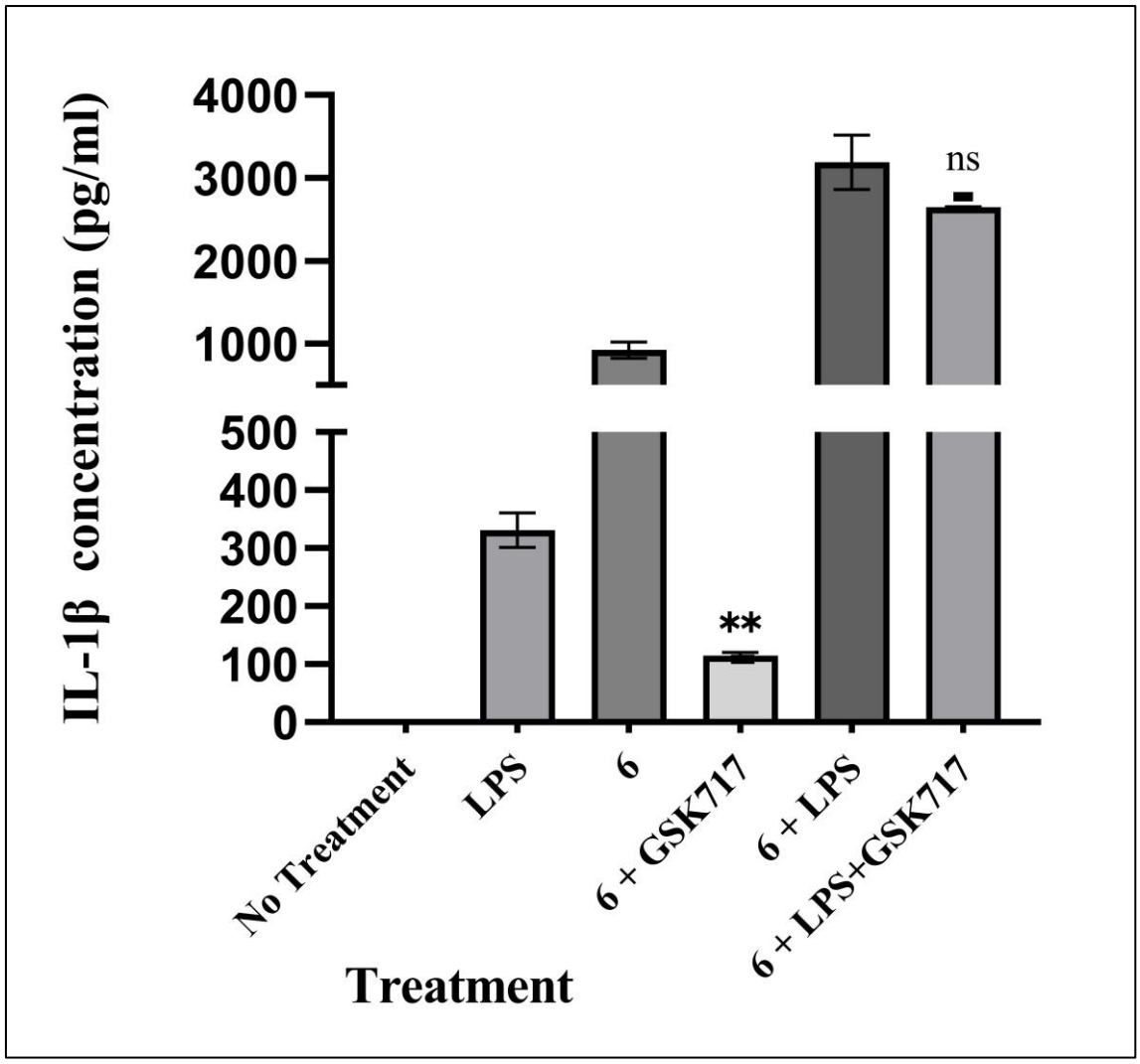

Figure 18: The concentrations of IL-1β secreted by THP-1 macrophages pre-treated with NOD2 inhibitor GSK717 (20 μM) and further stimulated with 20 μM MDP analogue 6 alone and in combination with LPS (100 ng/ml). ELISA test of supernatants collected from THP-1 macrophages pre-treated with NOD2 inhibitor GSK717 (20 μM) and then stimulated with MDP analogue **6** (20 μM), positive control: LPS (100 ng/ml) and unstimulated macrophages, and the results are expressed as the mean of three separate experiments ± (SEM) each with three replicates. The concentrations of IL-1β results are presented in pg/ml. One-way ANOVA determined statistical significance where ns is the non-significant difference, * p=0.004 compared to supernatants of THP-1 macrophages treated with compound **6** and compound **6** plus LPS.

- *Summary*

This study on NOD2 inhibitor GSK717 depicts changes in innate immune responses on cell surfaces (ICAM-1) and pro-inflammatory cytokines induced in THP-1 macrophages pre-treated with GSK717. It can be clearly noted that THP-1 macrophages pre-treated with GSK717 and stimulated with Murabutide shows significant increase in ICAM-1 expression, with a 21% increase in ICAM-1 expression between THP-1 macrophage stimulated with Murabutide (20 μM) vs. macrophages pre-treated with GSK717 (25 μM) before stimulation with Murabutide and an even drastic increase in MFI between the pre-treated and untreated macrophages when the concentration of Murabutide was lowered from 20 μM to 12.5 μM keeping concentration of GSK717 at 25 μM.

Although synergism in ICAM-1 expression between Murabutide and GSK717 can be observed, it can be noted that THP-1 macrophages pretreated with GSK717 and stimulated with the MDP analogue show lower levels of TNF-α and IL-1β. GSK717 decreases the secretion of TNF-α by THP-1 macrophages pre-treated with GSK717 before stimulation with MDP analogue **6** by 99% and secretion of IL-1β by 88%.

3.4 Structure-activity relationship (SAR) analysis

3.4.1 Lipid chain length

"Lipophilicity" describes a molecule's propensity to dissolve in lipids or other nonpolar solvents. Higher lipophilicity molecules tend to traverse biological membranes and more readily engage with lipid-based cell structures. MDP analogue's lipophilicity may impact how well they activate the NOD2 receptor. The NOD2 receptor is known to be more strongly activated by MDP analogues with increased lipophilicity. This is assumed because lipophilic MDP analogues have a higher propensity to bind to the lipid membranes surrounding the NOD2 receptor, enhancing their ability to activate the receptor and activate downstream signalling cascades (Ogawa et al., 2011). According to biological testing of MDP analogues, the molecule's lipophilicity has considerable implications on biological activity by boosting adjuvant efficacy and reducing pyrogenicity, one of MDP's main adverse effects (Ogawa et al., 2011).

The change in the lipid chains does not impact ICAM-1 expression between compounds (Figure 6). Increasing the chain length from n-hexyl (**1** & **4**, n=2; Figure 4) to n-decyl and n-hexadecyl (**2** & **5**, n=4; **3**, n=7; Figure 4) at the C-terminal statistically show a non-significant change in the MFI of THP-1 macrophages stimulated by these compounds alone, mainly because they are inactive on their own based on the data

collected (Figure 8, 11 & 13). In co-stimulation with LPS the MDP analogues **1-5** there is significant ($p<0.0001$) ICAM-1 expression by THP-1 macrophages but no synergy (Figure 9) which implies that adding an alkyl chain with moderate chain length (C_6 or C_{10}) does not increase the surface ICAM-1 expression (compounds **1 - 3** & compounds **4 – 5**; Statistically non-significant). The upregulation of ICAM-1 by compound **6** may be due to its increased lipophilicity due to the longer chain length (C_{16}) but cannot be confirmed without further studies since the presence of a benzyl group instead of a reducing sugar might be playing a combined role in the increase of MFI.

The increase in lipid chain length for the MDP analogues does not increase the secretion of TNF-α by THP-1 macrophages when stimulated with the MDP analogues alone (Figure 11), again with compound **6** being an exception considering the concept mentioned before. In co-stimulation with LPS and MDP analogues (Figure 12), there is no significant increase in TNF-α between compounds **1**, **2** and **3** (Statistically non-significant); therefore, increase in the lipid chain length from C_6 to C_{10} to C_{16} does not impact the release of TNF-α by THP-macrophages. Compounds **4** and **5** show an increase in TNF-α by 56% ($p=0.01$), but a further increase in lipid chain length from C_{10} to C_{16} between compounds **5** and **6** statistically show non-significant secretion of TNF-α but the concentration of TNF-α stimulated by compound **5** is the highest at 592±118 pg/ml , indicating that a moderate chain length might possibly be optimal in inducing TNF-a release when the cells are co-stimulated with the MDP analogue plus LPS. Further studies are required to confirm this observation.

Interestingly the increase in lipid chain length from C_6 to C_{10} to C_{16} for the MDP analogues steadily increases the secretion of IL-1β which is evident by MDP analogues **1-**

3 alone (figure 13). There is an increase in IL-1β by THP-1 macrophages stimulated with compounds **1** and **2** ($p=0.0005$) and an increase in concentration of IL-1β between compounds **2** and **3** ($p<0.0001$) implying that a longer chain length of C_{16} in compound **3** could be optimal for MDP analogues. It can be noted that an increase in the lipid chain length from C_6 to C_{10} between compounds **4-5** shows non statistically significant change in IL-1β which might be due to the additional benzyl group (compared to compound **1** and **2**) again with compound **6** being an exception (Figure 13). In studies of co-stimulation with LPS and MDP analogues, it is interesting to see the decrease in levels of IL-1β with the increase in lipid chain length (Figure 14). Although compound **1** and **2** show similar levels; there is a decrease of 9% ($p=0.001$) IL-1β between them with a lipid chain of C_6 and C_{10} respectively and further decrease between compound **2** and compound **3** ($p<0.0001$) with a lipid chain of C_{16}; similarly, THP-1 macrophages stimulated by compound **4** shows the highest-level IL-1β and decreases by 29% with compound **5** and 27% with compound **6** though it is statistically non-significant the slight decrease might possibly indicating that moderate lipid chain of C_6 to C_{10} is ideal for MDP analogues to secrete high concentrations of IL-1β. Further studies are required to confirm this observation.

3.4.2 Reducing sugar vs. benzyl glycoside

The structural modification choice depends on the specific properties desired for the MDP analogues and the intended applications (Khan et al., 2021). The parent MDP molecule contains reducing sugar that can be replaced with benzyl glycoside to improve the properties of MDP analogues though their effects may differ depending on the

specific analogue and its intended use. For example, reducing sugar may be more effective at enhancing immunogenicity, while benzyl glycoside may be more effective at improving stability (Almzene, 2018).

Compound **1**-**3** have the original reducing sugar while in compounds **4**-**6** the N-acetylmuramic acid moiety exists as the benzyl α-glycoside that show potential immunomodulating activity and can interact with NOD2 receptor to display agonistic or antagonistic activity (Almzene, 2018; Yang et al. 2005). In terms of ICAM-1 expression, compound **1** and **4** with n-hexyl chain (n=2, Figure 4) and an additional benzyl group in compound **4** along with moderate lipid chain of C_{10} for compound **2** and compound **5** (with benzyl group) shows statistically non-significant minimal activity compared to untreated macrophages. Increasing the lipid chain to n-hexadecyl (n=7) for compounds **3** ($p=0.007$) and **6** ($p<0.0001$) with an additional benzyl group in compound **6** shows a significant increase in ICAM-1 expression by THP-1 macrophages compared to untreated macrophages. Compound **6** with the benzyl group shows an increase of 327% ($p<0.0001$) compared to compound **3** with reducing sugar. In co-stimulation with LPS, compounds **1** vs. **4** show similar MFI, but there is 7% increase between **2** vs. **5** (non-significant) while **3** vs. **6** show a drastic increase in MFI by 53% ($p<0.0001$).

Compounds **1 - 3** with a reducing sugar and compounds **4** and **5** with a benzyl group show minimal activity in the secretion of pro-inflammatory cytokines TNF-α and IL-1β by THP-1 macrophages with compound **6** being an exception. Though the activity is minimal compound **4** with the benzyl group shows a higher value than compound **1** ($p=0.002$) for TNF-α and similar for IL-1β ($p=0.001$) which means with a shorter lipid chain C_6 the benzyl group shows more agonist activity in activating pro-inflammatory

cytokines. Compound **2** Vs compound **5** with the same lipid chain of C_{10} show a decrease in concentration of cytokines due to presence of benzyl group, compound **2** shows higher activity than compound **5** for TNF-α ($p=0.006$) and similar for IL-1β ($p=0.006$). Further increasing the chain to $C_{16,}$ the presence of benzyl group shows a drastic increase in TNF-α and IL-1β, this is evident in compound **6** that increases the concentration of TNF-α and IL-1β significantly compared to compound **5** ($p<0.0001$).

In co-stimulation with LPS compounds **4 - 6** with a benzyl group show higher activity than compounds **1 - 3** with a reducing sugar. These results indicate that MDP analogues prepared as benzyl αglycosides can possibly show better immunomodulating activity compared to MDP analogues with reducing sugars and further tests would be required to confirm this trend of along with the studies on how stable the compound is with an additional benzyl αglycosides MDP analogues with benzyl glycoside modification can improve the stability of MDP analogues, which is useful in developing vaccines and immunomodulatory agents, as these compounds need to be stable to be effective.

3.4.3 3,4-diaminobenzoic acid vs. D-isoglutamine

The replacement of D-isoglutamine in the parent MDP molecule with an artificial aromatic amino acid showed agonist activity, making it a suitable replacement as for ICAM-1 expression compounds **1-5** are weakly active, but in combination with LPS, they induce a significant level of ICAM-1. Therefore, they actively activate the immune response and display immunomodulatory/immunostimulatory activity. Compound **6** alone is strongly active alone and in combination with LPS. A similar trend can be

observed by the MDP analogues in the secretion of pro-inflammatory cytokines TNF-α and IL-1β with the compounds **1-5** being inactive when stimulated alone but in combination with LPS showing significant synergism and are highly active with compound **6** again being a strong immunostimulant on its own. Compound **6** on its own can induce high levels of pro-inflammatory cytokines but in combination with LPS there is a drop in the concentration of cytokines induced which might be essential to control excess production of pro-inflammatory responses.

NOD2 inhibition studies indicated that GSK717 is a potential antagonist against compound **6** in inducing the production of TNF-α and a partial antagonist against **6** in the production of IL-1β and multi-dose studies would be required to determine the antagonistic properties of GSK717 vs compound **6**. The data suggests that compound **6** is a NOD2 agonist. In conclusion, DABA is a good replacement of D-isoglutamine in creating novel MDP analogues that potentially bind to NOD2 receptor and activate the NOD2-mediated signalling pathway.

4 CONCLUSIONS

This study can further be explored for experimental studies and clinical experience with drugs with MDP derivates as an adjuvant that can act on innate immunity receptors and modulate the production of proinflammatory cytokines can potentially influence and reduce the infectious process caused by various pathogens that is mentioned in literatures. The advantage of vaccines and drugs based on muramyl dipeptide derivatives is that it targets not the pathogens, but the inflammation caused by them; this can overcome the resistance of drugs due to variations and mutations or resistance of the pathogens to drugs over time. MDP is a vital model compound for determining the implementation mechanisms of innate and acquired immunity, with the help of NLRs, since they can detect and activate innate immune responses on encounter with the smallest fragment of PGN which is MDP.

The optimized in-vitro experimental model using THP-1 macrophages could be used to analyze new MDP analogues for their potential to induce ICAM-1 expression and production of pro-inflammatory cytokines such as TNF-α and IL-1β. In addition, a more elaborate panel of cytokines can be tested further to understand the immunomodulatory properties of novel MDP analogues.

The newly synthesized MDP analogues **1-5** in this study show minimal to weak activity in the induction of ICAM-1 expression and production of cytokines such as TNF-α and IL-1β when administered alone, but in co-stimulation with LPS, these novel MDP analogues show an increase in ICAM-1 expression and synergism with LPS for increased secretion of pro-inflammatory cytokines by THP-1 macrophages. Combined targeting of TLR4 and NOD2 signaling pathways has been studied in the context of modulating

immune responses. Several studies have investigated the use of drugs or compounds that target both TLR4 and NOD2 to more comprehensively modulate the immune system. The basic idea of this approach is to achieve synergistic or additive effects in controlling inflammation and immune responses.

The biological evaluation done on compound **6** in this study shows that **6** is highly active and a strong immunostimulant on its own. It could induce innate immune responses at low and safe doses and could be further evaluated to study its potential as immunostimulatory/immunomodulatory agents with further toxicity testing to prevent any autoimmune diseases. This study also identifies the NOD2 inhibitor GSK717 as an antagonist for NOD2 receptor that can reduce the secretion of pro-inflammatory cytokines, which could help control the toxicity due to excess production. Researchers can use NOD2 inhibitors to understand the exact molecular mechanisms and signaling pathways involved in NOD2. By blocking NOD2 activity, they can study the downstream effects on immune response and inflammation and further explore the role of NOD2 in disease development and progression. NOD2 inhibitors may be candidates for drug development in inflammatory or autoimmune diseases and their efficacy in preclinical models to assess their potential as therapeutic agents while in drug discovery efforts, NOD2 inhibitors can be used in high-throughput screening assays to identify compounds that can selectively target NOD2. Finally, this study increases our understanding of the structure-activity relationships of MDP analogues. This information can be used to design new compounds with improved properties and efficacy, with DABA a suitable replacement of D-isoglutamine and a benzyl α-glycoside in place of reducing *N*-acetylmuramic acid in addition to an n-hexadecyl lipid chain to increase the innate

immune responses. This information can be used to design compounds with specific immunomodulatory properties, which can be used to treat various diseases. Overall, the benefits of these studies can lead to the development of new and improved vaccines and immunomodulatory agents that can help prevent and treat infectious diseases and other conditions.

- *Lead compound for further studies*

This thesis highlights that compound **6** is the lead compound that can be used for further studies. Compound **6** carries an n-hexadecyl group at the C-terminus of the peptide moiety and a benzyl group at the anomeric position of the sugar. The presence of a long alkyl chain and the benzyl group render the molecule with favorably balanced hydrophilicity and lipophilicity, which results in the highest potency of activities among all compounds described here.

Compound **6** induced high level of ICAM-1 expression, TNF-α and IL-1β secretion in THP-1 cells on its own. It is a potent immunostimulant in inducing proinflammatory/inflammatory immune response. In co-stimulation with LPS, compound **6** induced ICAM-1 at higher levels and TNF-α and IL-1β at somewhat reduced but still significant levels in THP-1 macrophages. Through inhibition studies with the known NOD2 antagonist GSK717, it was concluded that compound **6** exerted its activity through NOD2 receptor and it was a potent NOD2 agonist.

Further structure modifications to compound **6** can be done, such as fine-tuning the lipid length of C_{12}, C_{14}, C_{18}, and perhaps lipophilic aromatic groups at the C-terminus;

and introducing substituent groups on the aromatic ring at the anomeric position. Further biological studies may focus on unsolved issues found in the present studies, along with specific applications such as vaccine adjuvanticity.

5 LIMITATIONS AND FUTURE WORK

The main limitation of this study is the use of *in vitro* studies with cell cultures. Though cell maintenance can be done in a controlled system and constantly monitored, it can also be of disadvantage in the time and effort that must be applied to keep the cells viable. Cell culturing also requires skilled techniques and countless repeats of the same technique. Another critical factor is the sample size, where limited samples might affect the validity of the research. The correlation between in-vitro models and clinical response is of great importance. Although immortalized cell lines are valuable for early research, it is recognized that they may not faithfully reproduce the behavior of primary cells or fully represent in vivo interactions, therefore identifying and using more physiologically relevant models, such as primary cells or organotypic in-vitro cultures could increases the translational relevance of research before advancing these compounds to clinical trials.

A wider panel of concentration-response studies of each compound can further provide valuable insights to their potential properties and their ability to initiate innate immune responses, for example NF-κB is a transcription factor involved in regulating immune and inflammatory responses and the ability of MDP analogs to activate NF-κB can be assessed using reporter gene assays or by measuring nuclear translocation of NF-κB could be explored in detail. Further tests like lactate dehydrogenase (LDH) release assays could help determine whether the novel MDP analogue is toxic to cells. The chemotactic properties of MDP analogues could be assessed through migration assays that can be performed using immune cells such as neutrophils and macrophages. The activation of NOD2 receptor by MDP analogues could further be analyzed for their downstream signaling pathways such as MAPK and IRF activation using Western

blotting or reporter gene assays and the stability of MDP analogues could be evaluated under various conditions including temperature, pH, and storage conditions to confirm their suitability for therapeutic development.

The study of NOD2 inhibitor GSK717 must be further investigated to evaluate their effectiveness as a therapeutic agent to regulate inflammation, although FACS analysis of ICAM-1 expression in present study did not provide concrete evidence as a NOD2 antagonist, a more complex study with different cell lines and a greater number of different cytokine tests is necessary.

MDP derivatives have multiple clinical uses and therapeutic potential as mentioned in the introduction and derivatives like Murabutide has been used to boost the immune response in the form of cancer treatment, the discovery of MDP analogue **6** in our study could potentially be an effective MDP derivative in clinical trials to evoke adjuvant activity in vaccines. Further in vivo studies can better determine the safety and efficiency of the new highly active MDP analogue **6**. Advancement in computational studies has molecular docking studies of receptor-ligand binding that can increase our knowledge of the structure of MDP analogue **6** and how exactly it binds to the NOD2 receptor's LRR, along with the dock scores of binding energy and the number of hydrophilic and hydrophobic interactions (Khan et al., 2021), which will help us diversify the chemical structure of MDP and explore its structure-activity relationship to optimize its desired biological activity (Ogawa et al., 2011).

6 EXPERIMENTAL SECTIONS

6.1 Biological experiments Reagents

LPS stock

The stock solution of LPS (100 ng/ml) was prepared from initial stock solution 5mg/ml of 5 x 10^6 EU ultrapure lipopolysaccharide from *E. coli 0111:B4 (*LPS-EB Ultrapure, InvivoGen) and PBS and stored in aliquots at -20 ºC.

Murabutide stock

The stock solution of Murabutide was prepared by dissolving 1097 µg of lyophilized Murabutide powder (InvivoGen) in 1000 µL of DMSO to provide 2000 µM solution and stored in aliquots at -20 ºC.

NOD2 inhibitor GSK717

The stock solution of NOD2 inhibitor GSK717 (Sigma Aldrich) was prepared by dissolving 1896 µg GSK717 powder in DMSO (1000 µL) to provide 2000 µM solution stored in vials at -20 ºC.

6.2 Preparation of novel MDP analogues 1 – 6

N. Almzene (Almzene 2018) synthesized the novel MDP analogues **1- 6** and stored them in the freezer at -20 ºC. The purity of these compounds was analyzed before their biological evaluation by thin-layer chromatography (TLC), which showed a single

spot, respectively (Table 1). TLC was performed using Silica Gel 60Å F254 (thickness 250 μm; Silicycle Inc., Canada) and detected by fluorescence under UV light (254 nm), followed by dipping into the staining reagent, the Mostain reagent [ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$, 20 g; and cerium (IV) sulphate, $Ce(SO_4)_2$, 0.4 g, in 10% H_2SO_4 solution (400 ml)] and charring at ~ 120 °C.

MDP analogues **1-6** were dissolved in freshly distilled dioxane and subjected to freeze-drying to provide a white powder. Stock solutions of the series of MDP analogues **1** – **6** were prepared by dissolving each compound (approximately 1.3 mg – 1.7 mg) in dimethyl sulfoxide (DMSO, 1000 μL) to provide 2000 μM solution, which was stored in vials at -20 ºC.

Table 1: The compound number, compound name, TLC developing solvents, and Rf values of compounds 1 – 6 have been listed in the table below.

Compound #	Compound name	TLC developing solvents	R_f value
1	4-Acetamido-3-{(S)-2-I)-2-(2acetamido- α /β-D-glucopyranos-3O-yl)-propanamido]propanamido}-N-hexylbenzamide	$CHCl_3$/MeOH 5:1	0.12
2	4-Acetamido-3-{(*S*)-2-[(*R*)-2-(2acetamido- α /β-D-glucopyranos-3*O*-yl)-propanamido]propanamido}-*N*-decylbenzamide	$CHCl_3$/MeOH, 5:1	0.12
3	4-Acetamido-3-{(*S*)-2-[(*R*)-2-(2acetamido- α /β -D-glucopyranos-3*O*-yl)-propanamido]propanamido}-*N*hexadecylbenzamide	$CHCl_3$/MeOH 5:1	0.18

4	4-Acetamido-3-{(*S*)-2-[(*R*)-2-(2acetamido-1-*O*-benzyl- α-Dglucopyranos-3-*O*-yl)-propanamido]-propanamido}-*N*hexylbenzamide	CH_2C_{l2}/MeOH/H_2O 5:0.5:0.1	0.15
5	4-Acetamido-3-{(*S*)-2-[(*R*)-2-(2acetamido-1-*O*-benzyl- α-Dglucopyranos-3-*O*-yl)-propanamido]-propanamido}-*N*decylbenzamide	CH_2Cl_2/MeOH/H_2O 5:1	0.47
6	4-Acetamido-3-{(*S*)-2-[(*R*)-2-(2acetamido-1-*O*-benzyl- α-Dglucopyranos-3-*O*-yl)-propanamido]-propanamido}-*N*hexadecylbenzamide	CH_2Cl_2/MeOH/H_2O 5:1	0.4

6.3 Cell culture maintenance

American Type Culture Collection (ATCC) provided the acute monocytic leukemia cell line THP-1 derived from the peripheral blood of a human male. The THP-1 cell line was resuspended in freezing media consisting of 90% heat-inactivated fetal bovine serum (FBS) (R&D Systems, Inc., Minneapolis, MN, USA) and 10% DMSO and stored in liquid nitrogen. In this study, the THP-1 cell line, after thawing, was maintained in an incubator at 37ºC in 5% CO_2 in T-25 flasks (Corning Incorporated, Corning, NY, USA) containing RPMI-1640 medium (Sigma-Aldrich, Oakville, ON)) supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco, Eugene, OR, USA). Maintaining a cell count of approximately 1 x 10^6 cells/mL and checking for cell morphology every four days, cells were split into new T-25 flasks. The trypan blue cellular exclusion method with a hemocytometer was used to eliminate cells with less than 90% cell

viability. The present study uses a new THP-1 cell line split into cell passages, and experiments were done using cells from cell passages 5 to 15.

6.4 Differentiation of THP-1 monocytes using Phorbol myristate acetate (PMA)

THP-1 cells were washed with 7 ml of fresh media containing RPMI-1640 medium, supplemented with 10 % FBS and 1% antibiotic-antimycotic and tested for 90% cell viability using Trypan Blue (10 µL) cellular exclusion method with a hemocytometer then plated at a concentration of 0.5 x 10^6 in a six-well cell culture plate (Corning Incorporated, Corning, NY, USA). PMA (Sigma-Aldrich, Oakville, ON) stored in stock solutions of 10 µg/mL in DMSO at -20 ºC was added at a concentration of 20 ng/ml to induce macrophage differentiation in THP-1 monocytes and incubated for 48 hours at 37 ºC in 5% CO_2. After incubation, the supernatant with unattached THP-1 cells was collected to count live and dead cells through the trypan blue cellular exclusion method using a hemocytometer to determine cell adherence and confirm macrophage differentiation (Chahal, 2021). Cell adherence and change in shape from bean-like small monocytes to irregular-shaped large macrophages could be observed under the microscope.

6.5 ICAM-1 surface expression induced in THP-1 macrophages.

Differentiated THP-1 cells plated at a concentration of 0.5 x 10^6 in six-well cell culture plates after 48 hours of incubation at 37 ºC in 5% CO_2 were washed with 1 ml PBS and refiled with 2 mL of fresh serum-free RPMI-1640 medium and left undisturbed for the next 24 hours. Once the 24-hour incubation was done, three different sets of

experiments as follows were conducted, keeping the final volume in the cell well at 2.2 mL:

1. THP-1 macrophages were stimulated with the series of novel MDP analogues **1-6** (20 μM) and incubated for 20 hours.
2. THP-1 macrophages were stimulated with the series of novel MDP analogues **1-6** (20 μM) for one hour, followed by LPS (100 ng/ml) and incubated for 20 hours.
3. THP-1 macrophages were treated with the NOD2 inhibitor GSK717 at 20 μM. The cell plates were covered in aluminum foil to avoid light and incubated for one hour, followed by stimulation with Murabutide (20 μM) and incubated for 20 hours.

In each set of the above experiments, THP-1 macrophages were treated with PBS (200 μL) for negative control and stimulated with Murabutide (20 μM) and LPS (100 ng/ml) for positive controls. The treated and stimulated THP-1 macrophages were harvested by cold shock, washed with 1 mL PBS by centrifugation at 800 x g for 5 mins, and suspended in 100 μL 1% bovine serum albumin (w/v) (BSA/PBS), followed by immunostaining with 1 μg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody (CD54, BD Biosciences, Mississauga, ON) and incubated for one hour at 4 ºC. The THP-1 macrophages were washed with 1 mL PBS by centrifugation at 1000 x g for 5 mins, suspended in 500 μL PBS, and finally subjected to flow cytometry analysis on FACS Calibur with CELLQUEST PRO software (BD Biosciences). Cell viability was determined by incubating unstained THP-1 macrophages with 1 μg/mL (8 μL) Propidium Iodide (PI) for one minute and then subjected to flow cytometry; cells with viability over 90% were used in this study. The ICAM-1 expression results are presented as the mean fluorescence intensity (MFI) on the FL2 channel (385) acquiring 10,000 events with

emission range from 490nm to 630nm (Chahal, 2021). The results for each set of the above experiments were expressed as the mean ± SD of three separate experiments run in triplicates.

6.6 ELISA Assays

The supernatants from THP-1 macrophages stimulated with positive controls, series of novel MDP analogues **1-6** and LPS from the above studies after 20-hour incubation at 37ºC in 5% CO_2 were collected and frozen at -80ºC till Enzyme-linked immunosorbent assays (ELISA) were performed using ELISA kits from Invitrogen (ThermoFisher scientific; 88-7346 and 88-7261) for cytokine quantification of human TNF-α and IL-1β, following the manufacturer's instructions. The lower limit of detection (LLOD) of the ELISA is 4pg/mL, any values falling below this threshold are reported as one-half the LLOD. The absorbance was measured at lower limit of detection at 450nm with wavelength corrections set to 540nm using a microplate reader (BMG Labtech)**.** A standard curve was generated using polynomial second-order regression analysis, and the cytokines TNF-α and IL-1β values are presented as the mean ± SD of three separate experiments.

6.7 Statistical Analysis

Statistical analyses were done using one-way ANOVA. T'e D'Agostino & Pearson test was used to determine normality and assume Gaussian distribution of residuals in GraphPad Prism version 8.3.0 for Windows, GraphPad Software, Boston, Massachusetts USA, and IBM SPSS Statistics using "he "omnibu" K2" test followed by

multiple comparisons with Dun’ett's test to determine specific differences in means and compare it to the controls while Tukey’s multiple comparisons test was done to compare readings of MDP analogues to each other. A value of $p<0.05$ was considered statistically significant.

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