Enhancing the immune responses using the synergistic effects of TLR4 and NOD2 agonists for multifaceted vaccine development

by

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Abstract

Effective and defined adjuvanted vaccines can provide enhanced immunity to intra-cellular pathogens, which disproportionately affect certain immunocompromised individuals. Also, the importance of T-cell mediated immunity is vital in reducing the severity of intra-cellular infections. Strategies to increase vaccine immunogenicity include the addition of innovative adjuvants, which can help to achieve robust and persistent immune responses against infectious diseases. Despite the indispensable importance of adjuvants, only an extremely limited number of adjuvants have been included in licensed vaccines. That is why a search for more effective adjuvants has become one of the primary concerns in medical research.

Simultaneous stimulation of germ-line encoded pattern-recognition receptors of the innate immune system, particularly Toll-like receptors (TLRs) and Nucleotide oligomerization domain (NOD)-like receptors (NLRs) can augment the cellular immune response. With this in mind, I hypothesized that adjuvants that activate TLR of NLRs would enhance vaccine response. To study this we developed a bioassay model of cytokine secretion using murine immortalized and primary antigen presenting cells(APCs), which were stimulated with diethanolamine-based lipid A (A1), a synthetic lipid A TLR4 agonist, and muramyl dipeptide (MDP),a NOD2 agonist in the presence or absence of the currently available high-dose influenza vaccine, Fluzone®. We evaluated the immune response by measuring cytokine secretion, protein cell signaling, and cell surface markers. We showed that A1 in combination with MDP was able to induce enhanced early innate immune responses synergistically by increasing ERK1/2 phosphorylation and increasing proinflammatory cytokine secretion. Next, we assessed the A1-MDP adjuvanted-Fluzone vaccine formulation against drifted influenza virus A/H3N2 variants in an antigen challenge protocol on human peripheral blood mononuclear cells (PBMCs). There were significant enhanced releases of IFNγ, IL-6, and IL-1β with no significant changes in IL-10 secretion and

increased expression of CD8+ T (cytolytic T cell) cell surface markers, which suggested a polarized antigen-specific Th1 immune response. In summary, our combined adjuvant approach will ameliorate current vaccines especially for intracellular infections.

Keywords

Adjuvant, Lipid A, TLR4 ,NOD2, MDP, proinflammatory cytokine, innate immunity, adaptive immunity, APCs, PRR, PAMP, Influenza

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Abbreviation

ANOVA	Analysis of Variance
Ag.	Antigen
APC	Antigen Presenting Cell
BCA	Bicinchoninic Acid
BMDCs	Bone Marrow-Derived Dendritic Cells
BMMϕs	Bone Marrow-Derived Macrophage Cells
BSA	Bovine serum albumin
^{o}C	Degree Celsius
CD	Cluster of Differentiation
CpG ODN	CpG oligodeoxynucleotides
CTL	Cytotoxic T Lymphocyte
DAMPs	Damage-Associated Molecular Patterns
DCs	Dendritic Cells
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
ERK1/2	Extracellular signal-regulated kinases
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
h	hours
HBSS	Hanks Balanced Salt Solution

HSV:	Herpes simplex virus
HRP	Horseradish Peroxidase
IAV	Influenza A virus
IFN	Interferon
SDS	Sodium dodecyl-sulfate
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
MFI	Mean Fluorescence Intensity
MHC	Major HistocompatibILity complex
MIP	Macrophage inflammatory protein
mg/ml	Milligrams per mililitre
g/mL	Microgram per mililitre
min	Minute
NFκB	Nuclear factor *kappa*-light-chain-enhancer of activated *B* cells
NK	Natural Killer
ng/ml	Nanogram per mililitre
PAGE	Polyacrylamide Electrophoresis
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PGN	Peptidoglycan
PRR	pattern-recognition receptors
RPMI	Roswell Park Memorial Institute media

1 General introduction

1.1 General aspects of vaccines

Immunization is the second most excellent public healthcare intervention next to providing safe drinking water (1).

A vaccine is any biological preparation intending to provide adaptive immunity to a disease. Vaccines usually contain antigens resembling components of a disease-causing organism which can stimulate the host's immune responses (2). In 1776, British physician Edward Jenner first inoculated the cowpox virus into persons to confer protection against smallpox(3). Vaccination has provided a revolutionary medical achievement by reducing human suffering and mortality rates to various infectious agents (4).

Interestingly, a report by the World Health Organization (WHO) indicates that mandatory vaccination may be required to significantly improve health, indicating that "Nearly nine million children under 14 years of age die every year from infectious disease and at least a third of them could be saved if existing vaccines were more widely used, but the rest (could be saved) only if suitable new vaccines were developed "(1). Although vaccines have led to the eradication of smallpox, have nearly eradicated polio, and have significantly improved protection against measles, mumps, tetanus, and whooping cough, specific individuals are still unable to get the direct benefits from vaccination because of age or health complications(5). For many infectious diseases, there is no or insufficient vaccine to confer protection. Therefore infectious diseases remain the most common cause of death in children under five years of age(5). It is a primary concern to develop efficient vaccines to overcome these limitations.

Initial approaches to vaccination relied on using killed or attenuated heterologous pathogens (first-generation vaccines), which possess inherent properties, including endogenous adjuvants, and induce high immunogenicity (6).. Nevertheless, these traditional vaccines occasionally had nonspecific reactogenicity and safety issues due to residual virulence. To avoid the virulence factors of these pathogens, second-generation vaccines are made of conjugated and highly purified pathogen subunits comprised of synthetic toxoid peptides or recombinant microbial antigens (proteins/ DNA). They are often predicted by computer databases to represent pathogens(7). Despite being considered safer, second-generation vaccines exhibit less immunity due to the lack of endogenous markers of pathogenicity. This means that the innate immune response was not appropriately activated and, therefore, cannot activate an effective downstream adaptive response on their own. Increasingly, the coadministration of novel adjuvants with the vaccine-antigens may be a way to augment, reinforce, or modulate the immune responses to modern vaccines (8). Despite the growing importance of adjuvant usage, only a small number of adjuvants are included in currently licensed vaccines, and research on their development is very limited limited(9).

1.2 The immune system: an overview of innate and adaptive immunity

The immune system is composed of a network of lymphoid organs, cells, humoral factors (e.g., secreted antibodies, complement components, specific antimicrobial peptides, and cytokines (peptides with immunomodulating and cell signaling properties), which can be divided into the innate and the adaptive immune response (10).

Innate immunity- The earliest immune response against invading pathogens occurs via the innate immune system, which recognizes specific pathogen components to induce a nonspecific response to create our first line of defense. Innate immunity relies on pre-existing effector cells and provides immediate host defenses within hours to days, which is beneficial for responding to an urgent crisis(11). One of the significant responses of the innate immune system is that it leads to a rapid burst of inflammatory cytokines and activation of antigen-presenting cells (APCs) such as macrophages(Mϕs) and dendritic cells (DCs). To distinguish pathogens from self-components, APCs use a wide variety of relatively invariable receptors, called pattern recognition receptors (PRR), that detect evolutionarily conserved signatures from pathogens called pathogen-associated molecular patterns (PAMPs). This highly conserved response can be seen in the simplest of animals, confirming its importance for host survival(11),(12). That is why potentiators of the innate response are widely used as therapeutic agents(12).``

Adaptive immunity- The innate immune system reacts quickly (within minutes) against molecular patterns found in microbes. Then it activates processes to eradicate infections that develop more slowly and target specific pathogen components, called the adaptive immune response(10). Adaptive immunity is modulated by intrinsic immune activities. It is a characteristic of higher animals. This response is precise, possesses made-to-order somatically rearranged receptor genes, and involves highly selective clonal expansion, which can take several days to weeks to mount a response with specificity and long-lasting immunological memory(13). This response is mediated by antigen-specific lymphocytes, T and B cells(14).

1.2.1 Linking the innate and adaptive by antigen-presenting cells (APCs)

. The innate and adaptive immune responses are interrelated by two primary antigen-presenting cells (APCs) - macrophages (Mϕs) and dendritic cells (DCs), that all vertebrates have evolved for defense

mechanisms(15). In order to grasp the physical links between these two immune systems, primarily when it is associated with an adjuvant's efficacy, an understanding of the performances of DCs and Mϕs gets the priority (16),(17). Several types of cells, but most prominently, APCs such as DCs, respond to many adjuvants by mediating the upregulated expression of activation markers or the secretion of cytokines from leukocytes after exposure. Therefore, treatments with adjuvants can directly activate APCs which, in turn, can activate leukocyte responses and modulate the adaptive immune response. These parameters and substances can easily be observed, quantified and are often used to study the effects of vaccine adjuvants.

DCs are the most potent sentinels of the hematopoietic immune system and play a significant role in shaping adaptive immunity by recognizing PAMPs, such as bacterial components, LPS (18). Also, DC maturation is vital in promoting adjuvant-mediated vaccine formulation immunogenicity. Because maturation involves the expression of lymphocyte co-stimulatory molecules, activation of different intracellular signaling protein kinases such as extracellular signal-regulated kinase (ERK), and upregulation of proinflammatory cytokines, it can initiate a specific immune response and modulate polyfunctional Th cell polarization(19,20). Based on their central role in modulating immunity, DCs are targeted in many clinical experiments requiring T-cell recruitment, such as during vaccination (21)

Macrophages (Mϕs) are another vital inflammatory APCs with similar cell phenotypes, including mononuclear phagocytosis. Peripheral blood monocytes and tissues Mϕs play diverse functions following LPS stimulation, including the secretion of proinflammatory mediators, antigen presentation, and co-stimulation of lymphocytes(22,23)

1.2.2 Pattern recognition receptors (PRRs) and Pathogen-associated molecular patterns (PAMPs)

In the past two decades, advancement in recognizing the pharmacology of the innate immune response and its influences on the adaptive immune response has paved the way for the design of vaccine-adjuvants. In 1989, Charles Janeway JR. first introduced the immunostimulatory activities of the germ-line encoded PRRs, expressed by the diverse group of APCs that recognize PAMPs (24). These PRRs are differentially expressed on many immune cells, like Mϕs, DCs, natural killer cells, T cells, and B cells (25). PRRs are mainly divided into four groups: 1) **Toll-like receptors (TLRs)** that recognize bacterial components such as LPS, protein, nucleic acid, and flagellin; 2) **C-type lectin receptors (CLRs)** that recognize glycan from the wall of fungi; 3) **Nucleotide-binding oligomerization domain (NOD)-leucine-rich repeat-containing receptors (NLRs)** that recognize peptidoglycan of bacterial cell walls, viral, parasitic, and fungal PAMPs; and, 4) **Retinoic acid-inducible gene-I (RIG)-like receptors (RLRs)** that recognize viral RNAs (26).

Type-1 immune response is led by PRR-mediated recognition of functional features of microbes such as bacteria, viruses, etc. Type-2 immune response depends on PRR-mediated recognition of functional features of microparasites. APCs such as Mϕs and DCs are involved in type-1 immune responses, and type-2 immune responses are promoted by epithelial and mast cells (15). In recent years, the properties of PAMPs have been getting more attention, and they are considered strong immunostimulants, as they have been purposefully used in traditional vaccines (12). Also, the induction by PAMPs covers the broad spectrum of pathogens. Studies that use PRRs agonists as adjuvants unequivocally support the idea that they can direct and induce long-lasting, broad-spectrum immune responses against a non-immunogenic vaccine antigen (7). In mammals, several classes of PRRs have been identified, although the TLRs and NLRs are the most studied. Ligands for these PRRs are recognized

to be effective immunostimulants to incorporate into vaccines to promote immune responses and confer immunities(21-24).

1.2.3 Orchestrating the adaptive immune response by engaging the innate PRRs of APCs

According to Derek T O'Hagan, perfect adjuvants should ensure the clearance of the pathogen of interest through three steps of signal (27).

Signal 0 –Adjuvants associated with antigens involve "danger signals" and bind to PRRs expressed by APCs that trigger multiple molecular events. PRRs-PAMPs mediate enhanced phagocytosis by APCs based on the type of immune potentiators (28) (Fig. 1).

***Signal 1- APCs present antigen*s:** APCs present antigen-specific signals in conjunction with major histocompatibility complexes (MHC) class I/II to T cell receptors (TCRs) on naive T (T0) cells to prime the cells to differentiate into effector T cells such as CD4+ T helper (Th) or CD8+T cells /cytotoxic.

APCs present antigen-specific signals in conjunction with major histocompatibility complexes (MHC) class I/II to T cell receptors (TCRs) on naive T (T0) cells to prime the cells to differentiate into effector T cells such as CD4+ T helper (Th) or CD8+T cells /cytotoxic lymphocytes(CTLs)(20). CD4+T cells are crucial in suppressing or regulating an effective immune response by releasing T cell cytokines. CD4+ Th cells are involved in B cell antibody class switching and activation and are essential in increasing the antimicrobial activity of phagocytes such as Mϕs (29). CD8+T cells /CTLs are responsible for the clearance of intracellular pathogens, including malignant cells, by promoting the lysis of cells expressing the target antigen. These mechanisms depend on APCs activated by TLR-PAMP mediated signaling (15) (Fig. 1).

***Signal 2* – *Co-stimulation of immune cells*:** APCs deliver signals through surface molecules CD80/86 on the APC surface and mediate co-stimulation through interaction with the CD28 receptor on the T cell surface to effectively activate T cells (15) (Fig. 1).

Signal 3 - Immune modulation: APCs induce the first level of cytokines IL-12, IL-23, IL-6, and IL-1β for type-1 immune responses and IL-4, IL-10, and IL-13 for type 2 immune responses. Type 1 immunity can be induced by PRR signaling from IFNγ–producing cells, CD8+T cells, and CD4+ T cells, which involve clearing intracellular microbes through degranulation and phagocytes (30). Type 2 immunity is mainly mediated by Th2 cells, which activate mast cells, basophils, and eosinophils, thus providing immunity against allergens and microparasites(15). These cytokines are secreted by the activated APCs and act on lymphocytes, such as T cells and polarize the Th phenotype into different subsets of T cells. According to the signature cytokine secretion and related to protection against specific pathogens, Th cells are subdivided into Th1, Th2, Th17, and Treg subsets (15,32) (Fig. 1). Th1 cells are the principal regulators of type 1 immunity and secrete the proinflammatory cytokines IFNγ, TNFα, and interleukin-12 (IL-12). Th2 cells, conversely, stimulate high titers of antibody secretion. In particular, secretion of IL-4, IL-10, and IL-13 activate B cells proliferation, antibody secretion, and class-switching, thereby stimulating antigen presentation to T cells (30). The regulatory T (Treg) cells' key role in suppressing the activity of Th1, Th2, and Th17 cells by producing IL-10 (31). Human Th17 priming is stimulated by the combined activity of IL-1β, IL-23, and IFNγ in addition to IL-17 in the presence of IL-12, suggesting a close developmental relationship with Th1 cells. Th17 cells are vital in host protection against extracellular bacteria and fungi and are associated with autoimmune diseases (15).

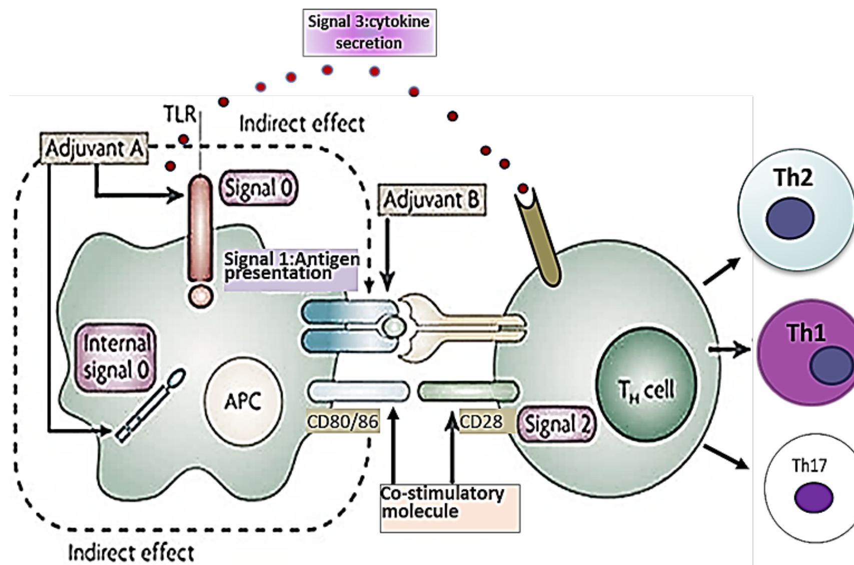

Figure 1 Overview of the three signals activation by adjuvants and polarization of naive T-Cells[Taken with adaptations from (O'Hagan 2019)](28).

1.3 Adjuvants

Adjuvants are defined by their mode of action instead of their molecular structure or origin. Immunologic adjuvants are non-specific immunostimulants that are added to vaccines to potentiate or modulate the immune response to the target antigen and generally do not exhibit immunity themselves(32). The term "adjuvant", coined by Gaston Ramon, comes from the Latin word "*adjuvare*", which means to aid (33). Adjuvants can be very diverse without common chemical features and may derive from a wide range of sources. For example, many classes of compounds have been assessed as adjuvants, including mineral salts, microbial products, detergents, emulsions, saponins, cytokines, polymers, microparticles, and liposomes(34). The different groups of adjuvants significantly affect the patterns of cytokine secretion and thus shape the efficacy of immune responses(36,13).

1.3.1 Adjuvants positive contribution towards vaccines

Adjuvants increase immune activity to overcome the weakened immunity present in immunocompromised individuals due to age (elderly and infants), treatments, or diseases (32).

These agents can qualitatively modulate immunogenicity. For example, adjuvants can induce a bias in the immune response by driving the response towards an inflammatory, Th1, response versus a regulatory T cell, Th2, response. They can also induce CD8+ T cells, which help to generate effective vaccines against intracellular pathogens(8).

Adjuvants can rapidly enhance the immunity of a vaccine by broadening the antibody titer and promoting longer-lasting immunity in the general vaccinated population by increasing the breadth of the heterologous protective response by promoting the formation of prolonged immune memory (especially T cells) cells(27) (Fig. 2).

Adjuvants can facilitate the reduction of target antigen in the vaccine (called dose sparing), which could be crucial in the case of a pandemic, where the secretion of large-scale vaccine batches is urgent. Because adjuvants can induce comparable responses with a reduced amount of vaccine-antigen, thereby, more vaccines can be made from the same amount of antigen. In addition, it can reduce the number of booster immunization required to induce immunity, which may benefit public health costs in a country with logistic challenges(32).

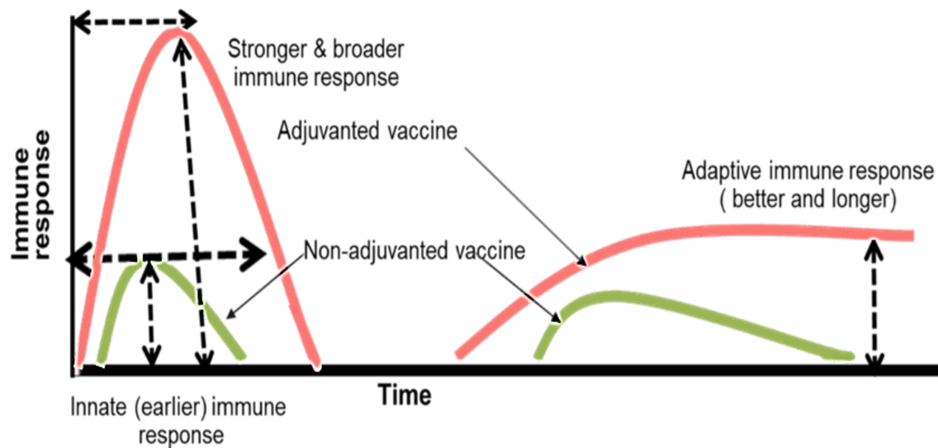

Figure 2 Benefits of an adjuvanted vaccine- compare between with and without adjuvant

1.3.2 Challenges in adjuvant development

In the 21st century, the obstacles to adjuvant development are primarily four-fold: pathogen-related challenges; challenging populations; excess reactivity, and confinement in adjuvanticity (2),(37).

Pathogen related challenges-- Finding the suitable adjuvant relevant to the pathogen of interest is an ongoing challenge. Some pathogens are challenging targets for vaccine development. For example, intracellular pathogens such as Mycobacterium tuberculosis, pathogens with multifaceted lifecycles such as the malaria virus, pathogens that can suppress immune responses by the host such as HIV, and pathogens with a latent disease phase such as human papillomavirus are all difficult targets for vaccine development (12). Many viral pathogens (influenza) undergo continuous evolution due to antigenic drift. Notably, new strains of influenza emerge every year and require new vaccines for each influenza season (38)(Fig.3). These pathogens have developed a sophisticated mechanism to evade the human immune systems, which making it possible to completely prevent their spread by antibodies alone(2).

Challenging Populations - Increasing age is accompanied by a reduced immune response and a diminished generation of effector memory and CTLs (39) (Fig.3). This translates that licensed vaccines that are made of live attenuated pathogens, such as the yellow fever vaccine, are best able to generate cell-mediated immunity, which is not safe for the aged or other vulnerable groups. (immunocompromised, patients receiving therapeutic interventions, and individuals with chronic diseases)(40) (Fig.3). Therefore, finding the right adjuvants that will generate potent and durable effector T cell immunity with current vaccines is a challenge(2).

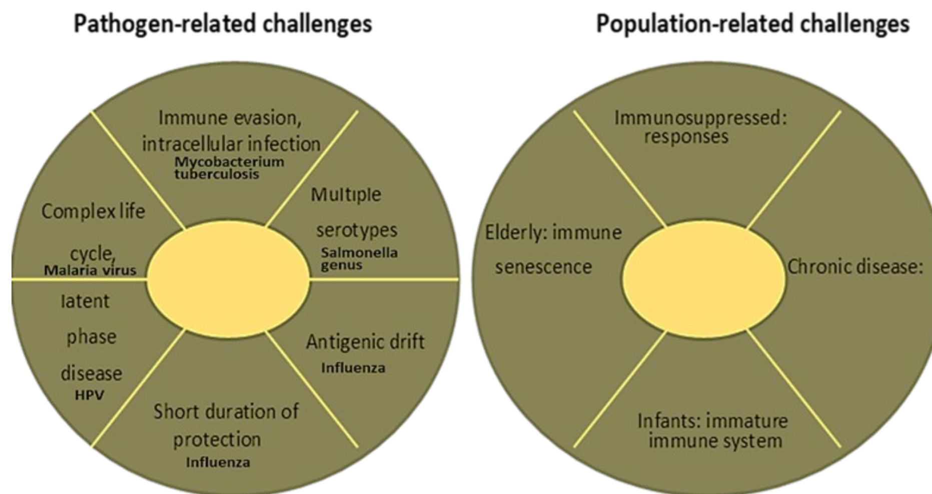

Figure 3 Challenges for modern vaccine development[Taken with adaptations from (Pasquale A *et al*.2015)] (2)

Confinement in adjuvanticity- The "one size fits all" adjuvant is not ideal for all vaccines. Antigen-specificity criteria of adjuvants makes it difficult to predict the ultimate outcome (36). A study by Knudsen *et al*. 2015, reported that the effect of each adjuvant is specific for a single disease target. The results described in Fig. 4, showed that alum and MF59® induced a Th2- biased response, but that GLA-SE and IC31® expressed strong IFNγ secretion with a clear Th1-biased response, whereas CAF01 induced a mixed Th1/Th17 phenotype (36). Despite having many assays for quantitative analysis, there is no current standardized assay and definitive animal model established for adjuvant development (38).

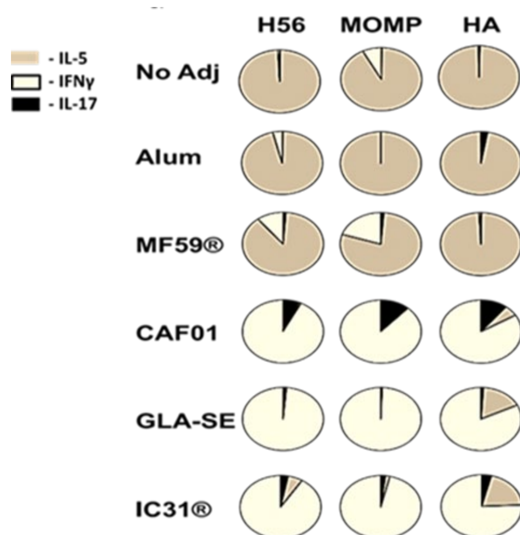

Figure 4 H56, MOMP, and HA specific cellular immune response induced by vaccination with different adjuvant[Taken with adaptations from (Knudsen NPH *et al*.2016)] (36)

E*xcess reactogenicity* - Adjuvant development relies primarily on empirical experiments and may have substantial safety implications, which strikes a significant challenge in developing new adjuvants (42). Primarily adjuvants are designed for prophylactic vaccines and are associated with adverse side effects such as local reaction at the site of injection, sickness, and chronic inflammation (5), such as QS-21 (saponin), which caused severe red cell lysis in human (5). Since young children with no pre-existing

medical complaints and other vulnerable groups are the primary consumers of vaccines, the balance between potency and safety is always the most significant concern (38).

1.3.3 Slow path to the discovery of adjuvants

In 1920, the first scientific report that documented the addition of an exogenous adjuvant (Aluminum salt) to a vaccine was published by Ramon; it was licensed for human use in the USA in the 1920s (9). Jules Freund developed the second adjuvant, Freund's complete adjuvant (FCA), in 1974. It is highly toxic to humans and has limited clinical benefit (41). It took 70 years to develop the next suitable adjuvant for humans. In 1997, the squalene-based oil-in-water emulsion MF59 was used in a flu vaccine (FLUADTM, Sequirus) for individuals over 65 years in Europe and 2015 in North America (Fig.3). AS03, an oil-in-water emulsion adjuvant was approved for addition to a pandemic flu vaccine (Prepandrix), while in 2008 ASO4,[monophosphoryl lipid A (MPLA) with alum salt], was first approved for addition to a hepatitis B virus (HBV) vaccine (Fendrix) in Europe in 2005 (42) (Fig.5). Zoster Vaccine (SHINGRIX), a recombinant glycoprotein E subunit vaccine with AS01B (QS21 and MPL with liposomes) was approved in the USA in late 2017(27,30). At present, GlaxoSmithKline (GSK) Biologicals is manufacturing ASO1(liposomes mixed with MPLA and QS21)(42).

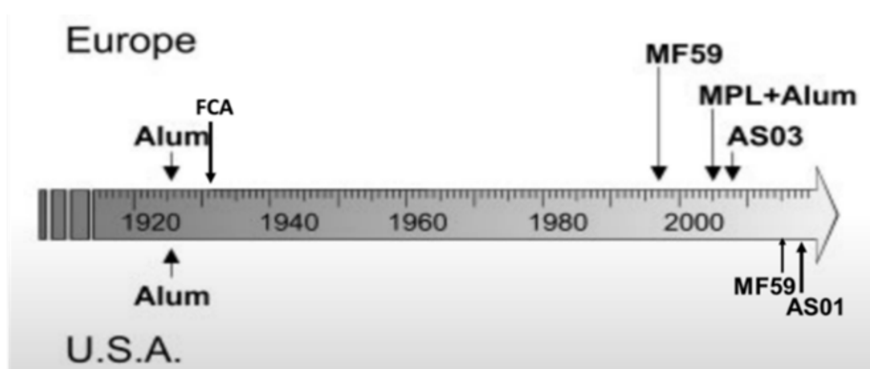

Figure 5 The slow process in adjuvant development[Taken with adaptations from (Awate S *et al*. 2013)](42).

1.3.4 Vaccine-adjuvants development based on PRRs

Current novel adjuvant candidates were identified after the fundamental discoveries of receptors on APCs in immunology as possible targets that are tailored to stimulate immune responses (40). PRRs-PAMPs trigger intracellular signaling pathways by culminating in an inflammatory microenvironment and steers subsequent modulation of the adaptive immunities. It is well established that rapid advances in developing defined vaccines encompassing TLRs and NLRs ligands indicate there will be more valuable molecules in the near future (43). Novel insights into harnessing new adjuvants began with the discovery of TLRs in 1990. Since then, ten TLRs have been found in humans (Fig.6) and thirteen in mice. Altogether, TLRs can sense various microbial components not ordinarily present in mammalian hosts, such as bacterial lipopolysaccharide (LPS), flagellin, and CpG DNA (44). These receptors appear to be evolutionary conserved from *Caenorhabditis elegans* through to mammals (45). There are 22 cytoplasmic receptors in the NLRs family. It is mostly expressed by hematopoietic and non-hematopoietic cells of the intestine and epithelial cells in mammals (46). Also, among all PRRs in mammals, TLR4 and NOD2 receptors (Fig.6) are the best characterized. Currently, most of the adjuvants in widespread clinical and experimental use target these two receptors (4).

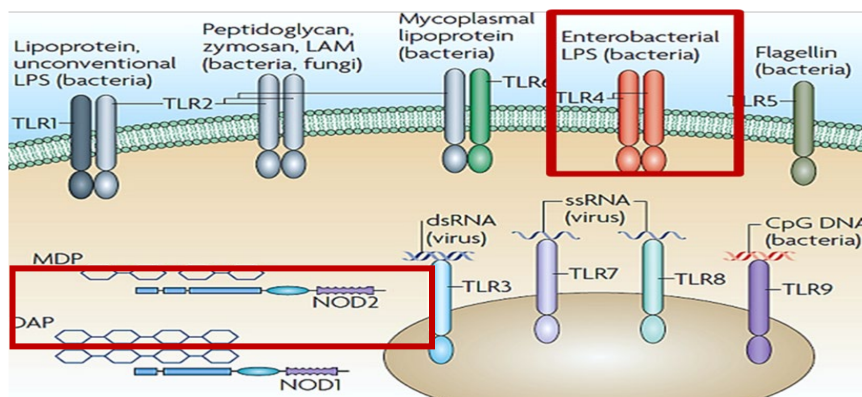

Figure 6 PRRs and PAMPs: TLR4 and NOD2 of nonphagocytic receptors, recognize PAMP - lipopolysaccharide (LPS) and muramyl dipeptide (MDP) [Taken with adaptations from (Pulendran B *et al.*2004)] (35).

1.3.5 TLR4 agonists-Lipopolysaccharide (LPS): as an adjuvant

Lipopolysaccharide (LPS) is a complex glycoprotein, major amphiphilic heat-stable endotoxin, and an integral part of the outer membrane of Gram-negative bacteria. LPS is considered one of the most potent stimulators of the host innate immune system [Fig.7(a)](47)]. LPS consists of three main biologically and chemically distinct subunits: (I) The lipid A domain is embedded in the bacterial outer membrane and is recognized as a PAMP by the innate immune system; (II) the core oligosaccharide (O) region linked with lipid A; and, (III) the O-antigen or O-specific polysaccharide, with the hydrophilic inner membrane [Fig.7(b)](48). LPS is involved in many defense responses, such as phagocytosis and secretion of inflammatory mediators. Using the natural derivative, bacterial LPS, as an adjuvant was precluded due to its undue pyrogenicity in humans (48).

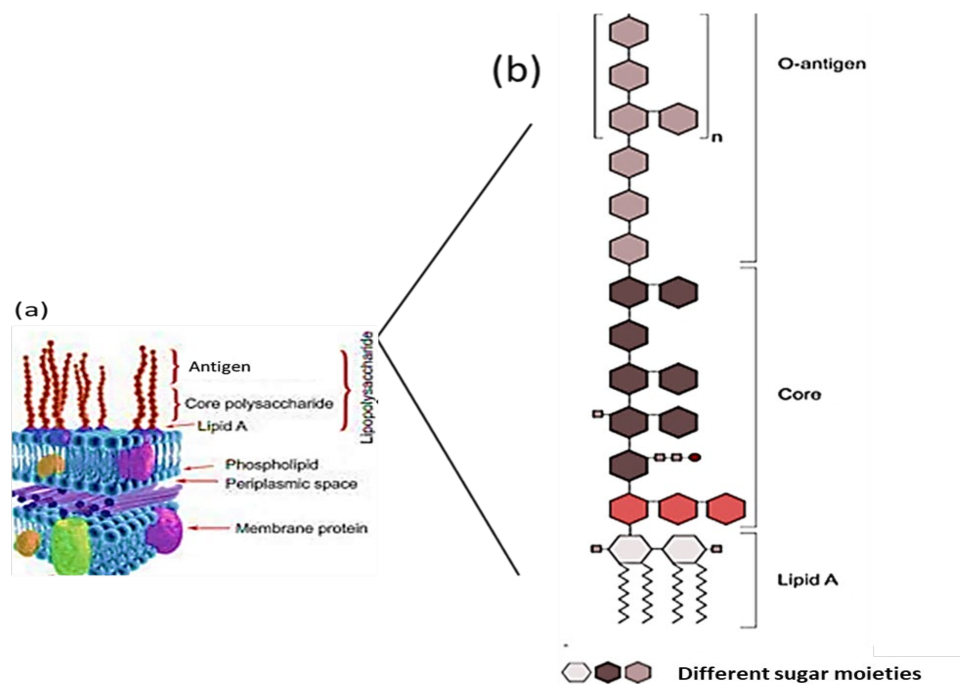

Figure 7 (a) Cell wall of a Gram-negative bacteria[Taken with adaptations from (Huber M *et al*.2006)] (47), (b) Scheme of LPS structure[Taken with adaptations from (Ranf. S.2016)](48).

1.3.6 Molecular Basis of TLR4 Activation

The highly conserved lipid A structure in nature plays a key role in the case of primary stability and host receptor recognition. Lipid A is one of the most potent agonists of the human innate immune system. The central structure of lipid A is a di-glucosamine core carrying four to seven fatty acids. Escherichia coli lipid A structure is considered the ideal of all lipid A structures (49) [Fig. 8(a)]. The number of lipid A acyl chains and the number of phosphate groups varies among different bacterial species. Natural Lipid A activates both the MyD88-dependent and MyD88-independent pathways versus a synthetic lipid A analog, which activates only the MyD88-independent pathway fully and partially activates the MyD88-dependent pathway (50). Edgar Ribi developed the clinical-grade, less toxic derivative of endotoxin LPS from Gram-negative bacteria (Salmonella Minnesota R595), monophosphoryl lipid A (MPLA) by reducing one or more acyl chains, polysaccharide side groups, and anomeric phosphate groups [Fig. 8(b)]. MPLA has the same level of immune-stimulating activity but only 0.1% toxicity of LPS (51),(52) . The synthetic hexaacylated lipid A derivative, glucopyranosyl lipid adjuvant (GLA) [Fig. 8(c)] was synthesized by Avanti Polar Lipids and was developed by Steve Reed and his group at the Immune Design. GLA lacks one phosphate group and has no attached residues on the hydroxyl group, but still shows less endotoxicity compared to naturally occurring lipid A (53),(55).

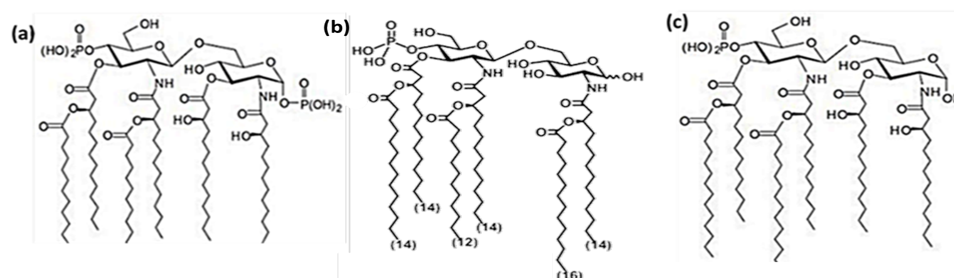

Figure 8 Structure of Lipid A (a), Lipid A from E. coli [Taken with adaptations from (Lewicky JD et al. 2012) (54) (b) MPLA[Taken with adaptations from (Bohannon JK et al. 2014) (55) and (c) GLA [Taken with adaptations from (Coler RN et al. 2006)](53).

1.3.7 Immunogenic activities of TLR4 agonists

TLR4 agonists have potent immunomodulatory activity and can promote adaptive immune responses to co-administered antigens; consequently, they have been exploited as adjuvants in vaccines for infectious diseases and immunotherapeutic purposes.

Adjuvant activities- Several synthetic TLR4 ligands, have been developed as immunomodulators. MPLA is a component of the Hepatitis B and Human Papillomavirus vaccines by enhancing T-cells development(56),58). GLA combined with split-virus vaccines (SVVs), was shown to stimulate CTLs responses which is required for clinical protection against influenza in peripheral blood mononuclear cells (PBMCs) *in vitro*, and which induced potent Th1 immune response(59). Another TLR4 agonist, aminoalkyl glucosaminide phosphate (AGP), and their mimics can provide protection against *Listeria monocytogenses*(59).

Therapeutic activities- Lipid A-based TLR4 agonists OM-174 and AS15 have been shown to exhibit anti-cancer effects (60). LPS absorbed GVAX (GMCSF- secreting whole cell tumor cell vector), a TLR4 ligand, as a tumor cell vector, improved the anti-tumor immunity in mouse models (61). In addition, TLR4 involves controlling the infectious diseases that are associated with *N. meningitis*, *S. pneumoniae, M. tuberculosis,* and *H. influenza,* etc.(62),(63).

1.3.8 Design of a novel TLR4 agonist family

We examined a novel multifaceted-adjuvant formulation using a synthetic TLR4 agonist, diethanolamine-based lipid A (A1) (54) developed and prepared by Dr. Zi-Hua Jian and his research team at Lake head University, Thunder Bay, Ontario. It reserves the elements that are shown to be critical for TLR4 activation, such as the number and length of lipid chains, acylation pattern, and phosphorylation. In new lipid A mimics, the reducing D-glucosamine of the natural disaccharide Lipid A framework has

been replaced by a diethanolamine-based acyclic scaffold (Fig. 9), envisioned as a simple and efficient molecular mimic framework to offer synthetic ease. In addition to the glycosidic linkage, the diethanolamine-based acyclic scaffold allows the preservation of the functional groups essential for TLR4/MD-2 ligand binding in an appropriate location, namely the fatty acid chains and the phosphate on the non-reducing sugar (54). The hexa-acylated A1 which contains three identical bilipid chains, was one of the first members of this lipid A mimic family synthesized (54).

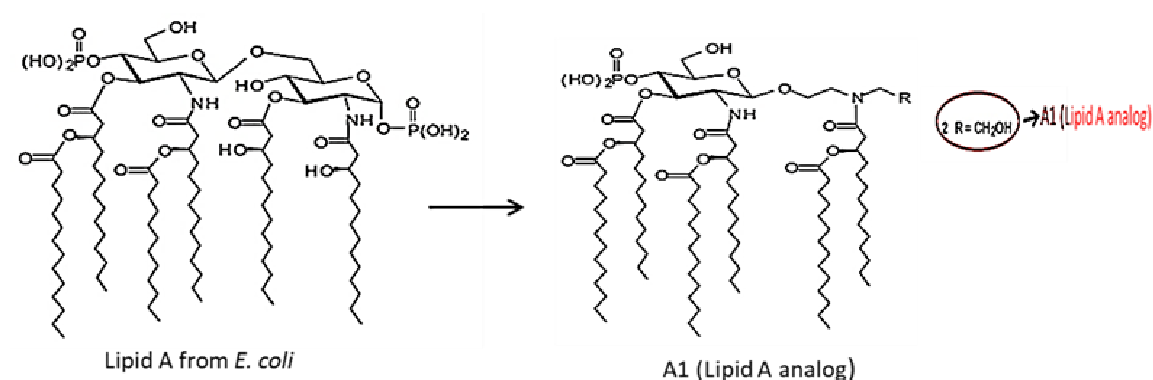

Figure 4 Structural simplification of the natural disaccharide lipid A framework via diethanolamine-based acyclic scaffold [Taken with adaptations from (Lewicky JD. *et al.*2012)] (54)

1.3.9 Immunostimulatory activity of novel TLR4 agonist family

Researchers evaluated the immunostimulatory properties of compound A1 (Fig.9) by measuring TNFα, IL-6, and IL-1β cytokine secretion in human THP1 cells, which expresses the TLR4 receptor. A1 was a potent activator of TLR4, inducing significant levels of TNFα, IL-6, and IL-1β at a 9µM maximum tested concentration (54),(64).

1.3.10 Toll-like receptor 4 (TLR4), ligand recognition and activation of TLR4

TLR4 was the first characterized TLR and the only receptor that can signal through both the MyD88 dependent and independent pathways (65).The TLR4 receptor complex consists of type-1 transmembrane receptors (TIR) that are composed of a ligand-binding domain of TLRs that bears

leucine-rich repeats (LRRs) in the extracellular domain in combination with the cluster of differentiation14 (CD14) antigen and the myeloid differentiation factor2 (MD2) (Fig.10). Prior to stimulation, TLR4s exist as monomers in cell membranes (66). At first, LPS binds with CD14. CD14 transfers LPS to MD2. Then the LPS: MD2 interaction causes dimerization of TLR4 and forms a TLR4:MD2: LPS complex (66) (Fig.10). This complex then interacts with membrane-associated co-receptors like MAL, initiating the downstream intracellular signaling cascade that triggers expression of pro-inflammatory mediators. (67). Given the fundamental role of TLR4 in controlling many infectious diseases, it is not surprising that TLR4 agonists are the most studied adjuvants (62).

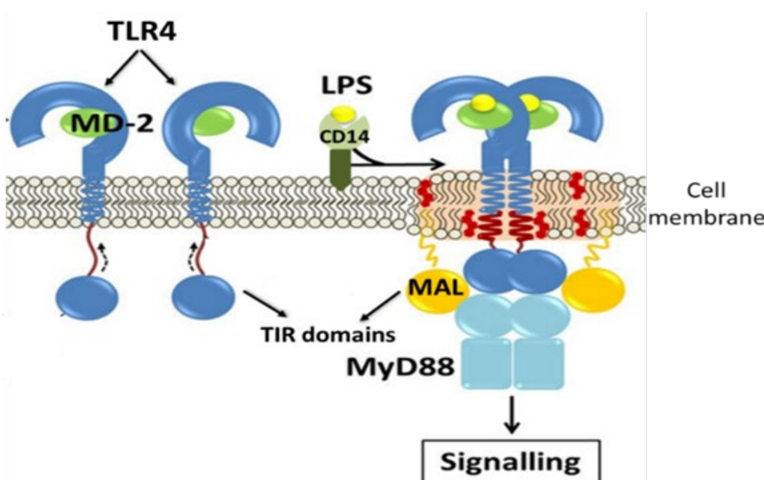

Figure 5. A schematic view of the TLR4 receptor activation process[Taken with adaptations from (Awasthi S *et al*. 2019)] (66).

1.3.11 Muramyl dipeptide (MDP): as an adjuvant

MDP is the minimum biologically active peptide that acts as an immunoreactive PAMP of peptidoglycan, that has conserve motifs of Gram-negative and Gram-positive bacteria, and is comprised of alternating residues of N-acetyl muramic acid link to a short dipeptide chain of L-alanine-D-

isoglutamine (68) (Fig.11). MDP, as an immunogen, was first recognized by E. Lederer and other associates in 1970 (69).

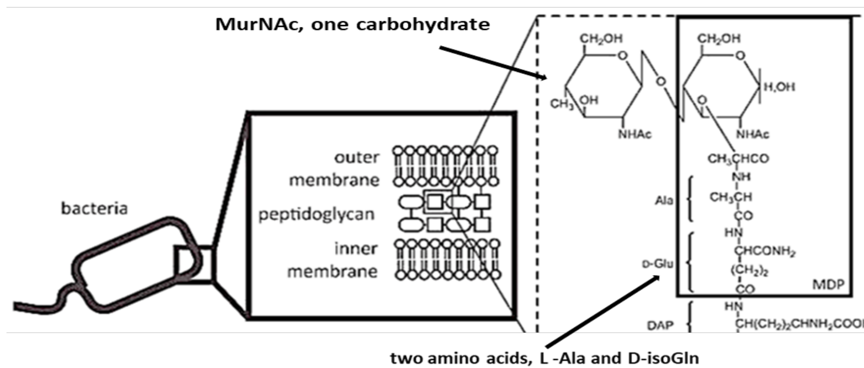

Figure 6 MDP is a component of bacterial cell wall peptidoglycan[Taken with adaptations from (Jahn and Scheller 2006)] (41)

1.3.12 Immunogenic activities of MDP

Adjuvant activities- MDP boosts phagocytic, anti-microbial activity and increases antibody-mediated cytotoxicity of vaccine-antigens by increasing the surface marker expression required for cellular adhesion, processing, and co-stimulating antigen presentation by antigen-presenting cells (70),(71). In addition, MDP stimulates the cell to mediate immunity by delayed-type hypersensitivity and augments the secretion of cytokines such as IL-6, IFNγ thereby accelerating lymphocyte generation (72),(74).

Synergism with LPS- MDP showed strong immune amplifying effects with LPS than each alone would in different human and murine primary cell lines(75),(76).

Priming effects- MDP is involved in the priming of cells of animals towards anaphylactic reactions and lethal toxicity in response to bacterial endotoxins (77).

Therapeutic activities- MDP modulates the non-specific reaction of bactericidal and viral infections without pyrogenic effects and reduces the LPS lethality *in-vivo* (78) In addition, Paclitaxel, conjugated with MDP, showed significant upregulation of IL-12 and TNFα secretion and worked as an immunotherapeutic agent (75) .

1.3.13 Mechanisms of MDP internalization

The route of MDP internalization into eukaryotic cells are still poorly understood. Even so, multiple routes of entry for MDP to get into cytoplasm have been discovered.

MDP can be internalized by phagocytosis of invasive bacteria such as *Shigella flexneri* (76)(Fig.12). MDP can get to the cytosol by clathrin-mediated endocytosis followed by transportation out of the endosome via oligopeptide transporters- SLC46A2, SLC15A3, and SLC15A4, (77), (78),(Fig. 12). Also, host cells absorb peptidoglycan (PGN) fragments of Gram-negative bacteria from outer membrane vesicles (OMVs) and also can uptake MDP from the shedding PGNs (79),(80) (Fig.12). Transportation from the extracellular matrix to the cytosol is another way of introducing of MDP inside the cell directly by transmembrane channels such as hPepT1(SLC15A1) (81),(78),(82).

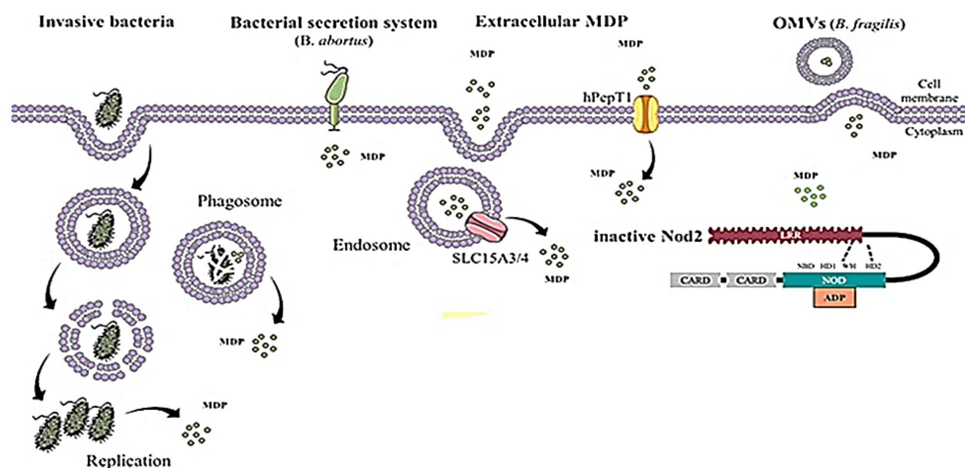

Figure 7 Mechanisms by which MDP enters cells to trigger Nod2 signaling[Taken with adaptations from (Nabhani ZA *et al*.2017)] (82).

1.3.14 NOD2 (MDP receptor) and signaling interaction

S.E. Girardin and N. Inohara, first delineated that MDP was the ligand for the NOD1 and NOD2 receptors (83),(84). NOD2 is the major cytosolic detector in sensing MDP. It is the second member of the NLRs family (84). NOD2 protein has three different domains. **1. CARD,** Caspase-recruitment domain; **2. NOD,** Nucleotide-binding oligomerization domain; and **3. LRR,** Leucine-Rich Repeat, the site of ligand binding [(Fig. 13(a)]. NOD2 receptors remain autoinhibited by MEKK4: RIP2 complex under basal conditions [(Fig. 13(c)]. Upon MDP stimulation, NOD2 directly binds to NALP1 and caspase1 via its CARD domain, forming a complex that triggers IL-1β secretion (85). Finally, a few studies showed that NOD2 also binds to procaspase-1 through CARD-CARD interactions and converts pro-caspase-1 into caspase-1, which is required for processing pro-IL-1β to mature IL-1β (86)[(Fig. 13(b)], in an intracellular complex termed the inflammasome. NOD2 plays a crucial role in gut-microbiome homeostasis and is an ideal target for the stimulation of mucosal immunity by sensing both commensal and pathogenic microbes and modulating TLR signaling pathways (87),(88).

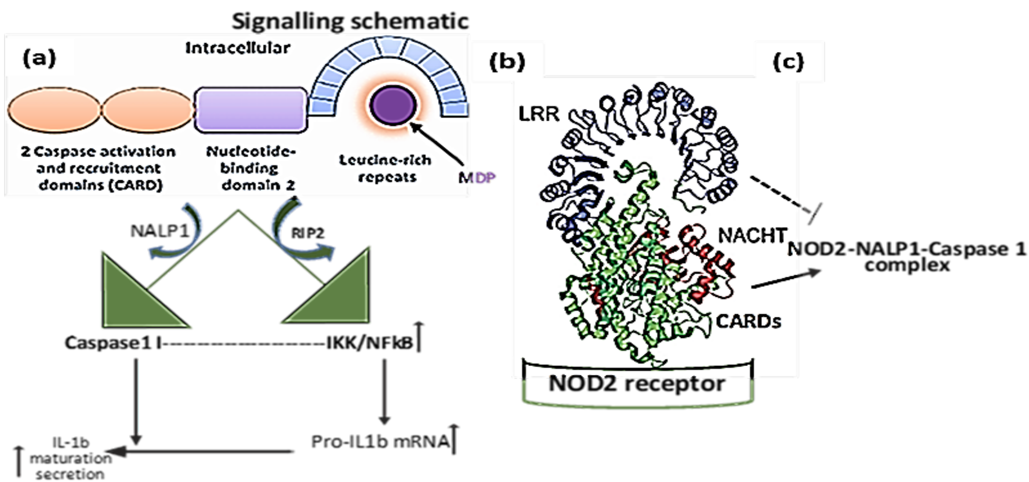

Figure 8 (a) NOD2 receptor protein [Taken with adaptations from(Salazar JC *et al*. 2002)] (89), (b) NOD2 signaling diagram and (c) Activation of NOD2 receptor [Taken with adaptations from (Matsushima N *et al* 2015)] (90).

1.4 Synergism: TLR4 and NOD2 agonists

During any infection, the immune systems encounter a diverse group of PAMPs. So, TLR4 and NOD2 agonists may interact simultaneously in the same infectious event, which reinforces the potent physiological importance of the perception of synergism even more (91). Also, several studies described applying different conditions, such as cellular models, stimulus conditions, and different endpoints. However, results consistently showed that LPS always exerted a remarkable synergistic effect with MDP (92),(93),(94).

1.4.1 The interrelationship of NOD2 and TLR4: Signaling pathways

TLR4 has been shown to utilize both the MyD88 and TRIF signaling adaptors for inducing the secretion of both proinflammatory cytokines and type I interferons (IFN1) by way of the nuclear factor kappa-light chain enhancer of activated B-cells (NFκB) and interferon regulatory factor 3 (IRF3)(49) (Fig.14). Several studies have shown that LPS is able to stimulate NFκB activation in MyD88- deficient murine macrophages suggesting the existence of a MyD88-independent pathway(95). Subsequent

research demonstrated similar findings, whereby MyD88-deficient DCs were still able to express costimulatory molecules in response to MPLA stimulation (96,97). Additional findings have suggested that the TIR domain-containing adaptor protein, TIRAP/Mal as well as the IRF3 adaptor proteins are involved in the MyD88-independent signaling pathway(98,99). TRIF has also been described as a signaling adaptor molecule in the MyD88-independent signaling pathway, stimulated by both GLA and MPLA (100,101) .

Kim *et al*. described that LPS stimulated MD2/TLR4 homodimers to cause conformational changes and promote MYD88 to recruit and phosphorylate IL-1 receptor-associated kinase (IRAK), a central mediator of the TLR signaling pathway. IRAK is associated with tumor necrosis factor receptor (TNFR)-associated factor (TRAF) 6 and TRAF3 cofactors, respectively(102). TRAF6 dissociates to form a large complex with Transforming growth factor β (TGFβ) activated kinase 1 (TAK1). TAK1 recruits and phosphorylates the IKK complex and activates NFkB through IKKα/β and other protein kinases, such as extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinases (p38) (103). Once activated, these kinases phosphorylate nuclear transcription factors. Then, these transcription factors translocate into the nucleus and ultimately induce MyD88-dependent inflammatory cytokine secretion (100). In the case of the MyD88-independent pathway, LPS stimulation involves TRIF and TRIF-related adaptor molecule (TRAM). TRAM stimulates sustained NFκB activation and a different signaling pathway, leading to activation of IRF3 (104), recruits TBK1, and induces expression of a set of genes distinct from that of NFκB, phosphorylation of IRF3 and followed by IL-12p35 and type I interferons (IFNs) IRF3 secretion (95,105,106) .

Abbott *et al.* described that, in the cytosol, MDP: NOD2 complexes induce NOD2 to undergo self-oligomerization, which attracts RICK (CARD containing serine/threonine kinase) via CARD-CARD homotypic interaction and forms NOD2: RICK. This complex leads to the recruitment of the IKK complex

and thereby allows the downmodulation of the TLR4 pathway(107). The recruitment of NOD2:RIP2/TAK1 complexes is always involved in controlling bacterial infection (82). Previous research by Kobayashi *et al.* 2006, demonstrated that TLR4 could augment the expression of NOD2 and vice versa (108). Abbott *et al.* and Hasegawa *et al*. added their research in this cascade by outlining that stimulation of NOD2 by MDP activated the NF-kB and MAPK pathways through TRAF6- complex molecules(107). Surprisingly, TLR4 and NOD2 agonists synergize both the MyD88 and TRIF pathways for maximal downstream immune responses (64). This pathway is overviewed in Fig.14.

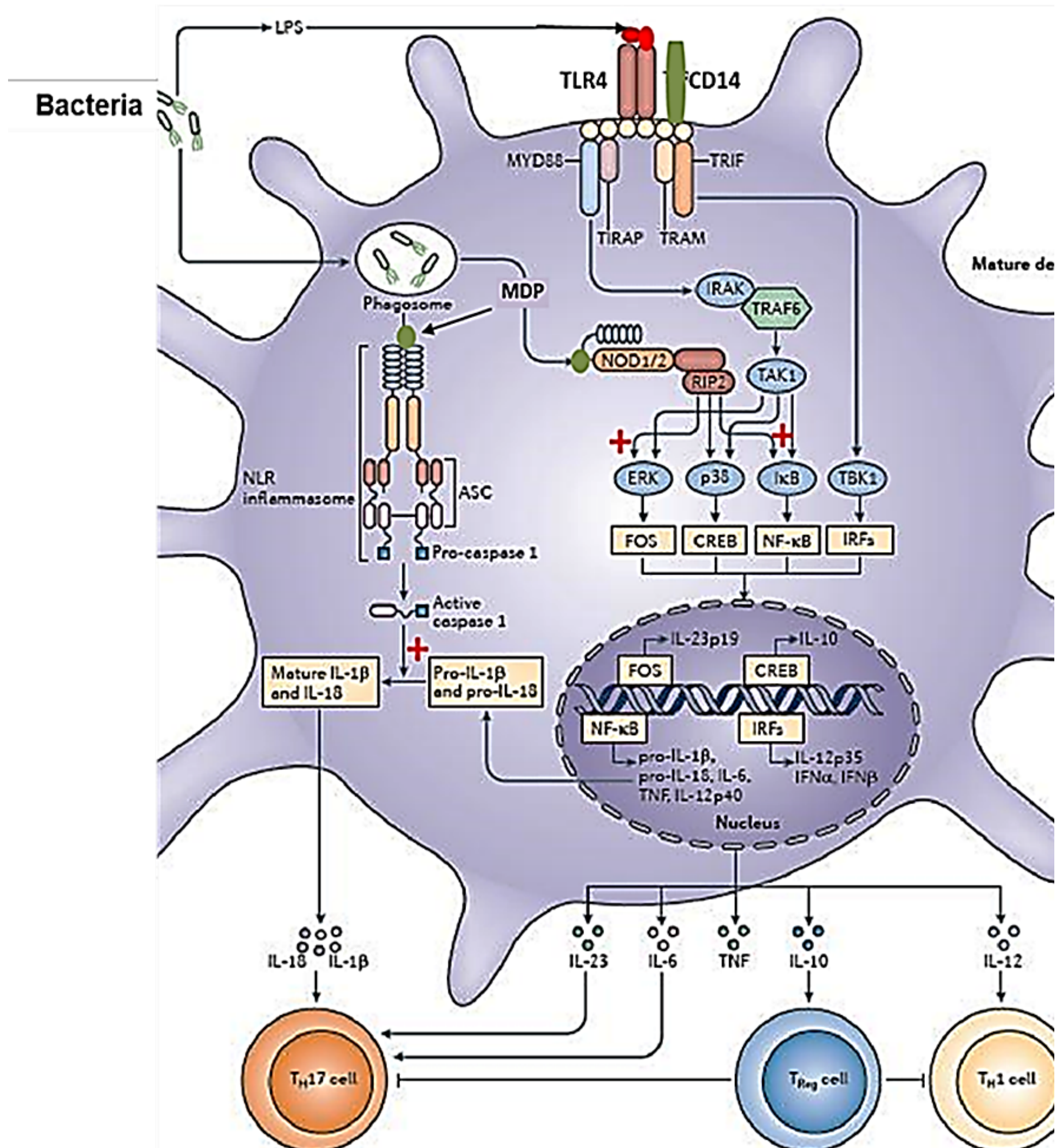

Figure 9 Model of molecular interactions of TLR4 and NOD2signaling pathways[Taken with adaptations from (Mills KHG 2011)](110).

1.5 Assessment tools

Identifying suitable biomarkers that accurately assess the immunogenicity and correlate with adverse side effects is crucial for vaccine development.

1.5.1 Cytokines

According to Bermann-Leitner *et al.,* cytokines have been implicated as critical modulators by participating in a complex network of interacting and maturating stimuli of immune cells during the event of infections (5). A panel of proinflammatory cytokines (IL-1β, TNFα, IL-6, and IL-12) produced by APCs are considered to establish thresholds for "Safe-adjuvanticity"(7).

TNFα-TNFα shares the most effector functions as an endogenous amplifier with TLR4 agonists (111), central to the acute inflammatory action of the innate immunity, with diverse functions in cell differentiation and apoptosis (112).

IL-6-IL-6 produces an acute phase-level induction in response to LPS and MDP in whole human blood (35). It significantly differentiates the Th2 and regulatory T cell (T reg) phenotypes (113). It is also a hallmark of expanding effector memory CD8+T cells (114).

IL-12- IL-12 is synthesized by various TLRs containing APCs, such as Mϕs, DCs, and B cells. Upon IL-12 stimulation, naïve CD4+ Th cells undergo differentiation to become Th1 effectors at the expense of Th2 cells. The interplay between TLR4 and NOD2 stimulants can significantly elevate IL-12 induction and activate CD8+ Th cells with increased cytolytic potential in different cellular models (115).

IFNγ- The importance of IFNγ is indispensable concerning its ability to inhibit viral replication directly(116). CD4+ Th1 and CD8+ effector T cells can produce IFNγ after developing an antigen-specific immunity against an intracellular pathogen (117). Researchers showed that GLA, combined with split-

virus vaccines (SVV), significantly increased IFNγ secretion in PBMCs, promoted Th1 biased immune responses to influenza virus by suppressing the IL-10 (118).

IL-1β- IL-1β, is a strong endogenous pyrogen. It principally synthesized (cleaved from pro-IL-1β) in response to microbes by TLRs and NOD2 receptors(119).

IL-10- IL-10 is a pleiotropic anti-inflammatory cytokine synthesized by Th2 cells, CD4+CD25+Foxp3+ regulatory T(Treg) cells, and APCs. IL-10 works as a feedback regulator of diverse immune responses. Jiang et al. and Wang et al. showed that IL-10 predominantly inhibits LPS-mediated induction of the proinflammatory cytokines from whole human blood cells. However, in the case of coadministration of LPS and MDP, IL-10 secretion was suppressed, and proinflammatory cytokines secretion was increased, respectively (120,121). In the end, IL-10 balances proinflammatory cytokine secretion. Even though the amount of IL-10 secretion may remain the same, there could be an increase in the other cytokines; therefore, overall immune responses shift towards pro-inflammation in a controlled manner.

IL-17--Dampening of IL-17 was correlated with IFNγ reduction, which suggested that IL-17 was involved in the recruitment of Th1 cells. A moderate level of IL-17 is critical for vaccine-induced protective/cellular responses during infections (122).

Also, several reports point to the role of different endpoints, primarily cytokines, which have been measured as a prime biomarker to analyze the potential of vaccine-induced immune protection (116).

1.5.2 Transcription proteins

Early research by Arrighi *et al.* showed that dendritic cells maturation following treatment with lipopolysaccharide (LPS) led to the induction of mitogen-activated protein kinases (MAPK)/extracellular

signal-regulated kinases (ERK) pathways (123).). ERK/MAP kinase1/2 is the most evolutionary conserved signaling regulator activated by PAMPs such as LPS. Later, An *et al.* demonstrated that inhibition of ERK remarkably suppressed TLR4, TLR2, and TLR9 expression, which proved that LPS-induced activation of TLR4 involved the ERK1/2 signaling pathway in murine BMDCs (124). The significant transcripts include signaling molecules such as ERK/MAP kinase ½, cell surface markers, and cytokines/chemokines (125). These works showed that MDP alone could stimulate the NFkB and ERK1/2 pathways of BMDMs, peritoneal macrophages, and THP1 monocyte (126). However, subsequent studies by Fritz *et al.* and Park *et al*. showed that BMDCs could not exert any inflammatory expression following MDP treatment alone (125). Current findings suggested that activation of NOD2 by MDP also depended on the type of cell line.

It was stated that the MPLA could upregulate the ERK1/2 and p38 pathways through MyD88 and TRIF (127) in multiple cell lines. In addition, Coler *et al.* showed that GLA-induced transcriptional upregulation, which is Caspase-11-dependent and TLR4-independent but could not activate the MyD88 pathway similarly (53). However, minor changes in the structure of the ligands can determine which pathway is favored (60). Based on that, and given the different requirements of TLR signaling, it is likely that the activities of distinct types of lipid A can differ markedly at the TLR4/MD-2 complex. So far, the novel TLR4 agonist A1 has not been thoroughly investigated for activating molecular pathways.

1.5.3 Design principle of immune responses: using TLR and NLR agonists to modulate the CD4+ Th cell responses

Contemporary trends in adjuvant development solely rely on synthetic PRR agonists. TLR4 receptors involve cell-extrinsic recognition and do not require the cell to be infected, whereas NOD2, cell-intrinsic recognition, needs intra-cellular sensors. TLR4 and NOD2 receptors involve cells being

infected, which mimics a natural infection state, and initiates an early immune response through a cascade of pivotal events for the appropriate effector T and B cells. APCs, DCs, and Mφs expressing TLRs and NLRs work as sensors for antigens and provide type-1 immunity by secreting IL-1β, TNFα, IL-12, and IL-23 to prime naïve CD4+ T cells toward specific Th1 phenotypes (15). TLR4s promote the cross-presentation of antigens to T cell receptors (TCRs) on naive CD4+T (T0) cells to activate cytotoxic CD8+ T cells. NOD2s present antigens through autophagy and are shown to upregulate CD4+ T-cell responses in DCs (128) (Fig.16). Furthermore, DCs induce Th17 cell secretion in the presence of IL-23 and IL-6. Th1 and Th17 polarization is mediated by TLR4 stimulation, while, in the nonhematopoietic compartments, NOD2 stimulation induces a Th2 polarized response *in vivo*. Remarkably, simultaneous stimulation of TLR4 and NLR synergizes to exert Th1 and Th17 immune responses (Fig.15). The outcome from the immune response never deviates entirely in one direction (cell-mediated or humoral) (129).

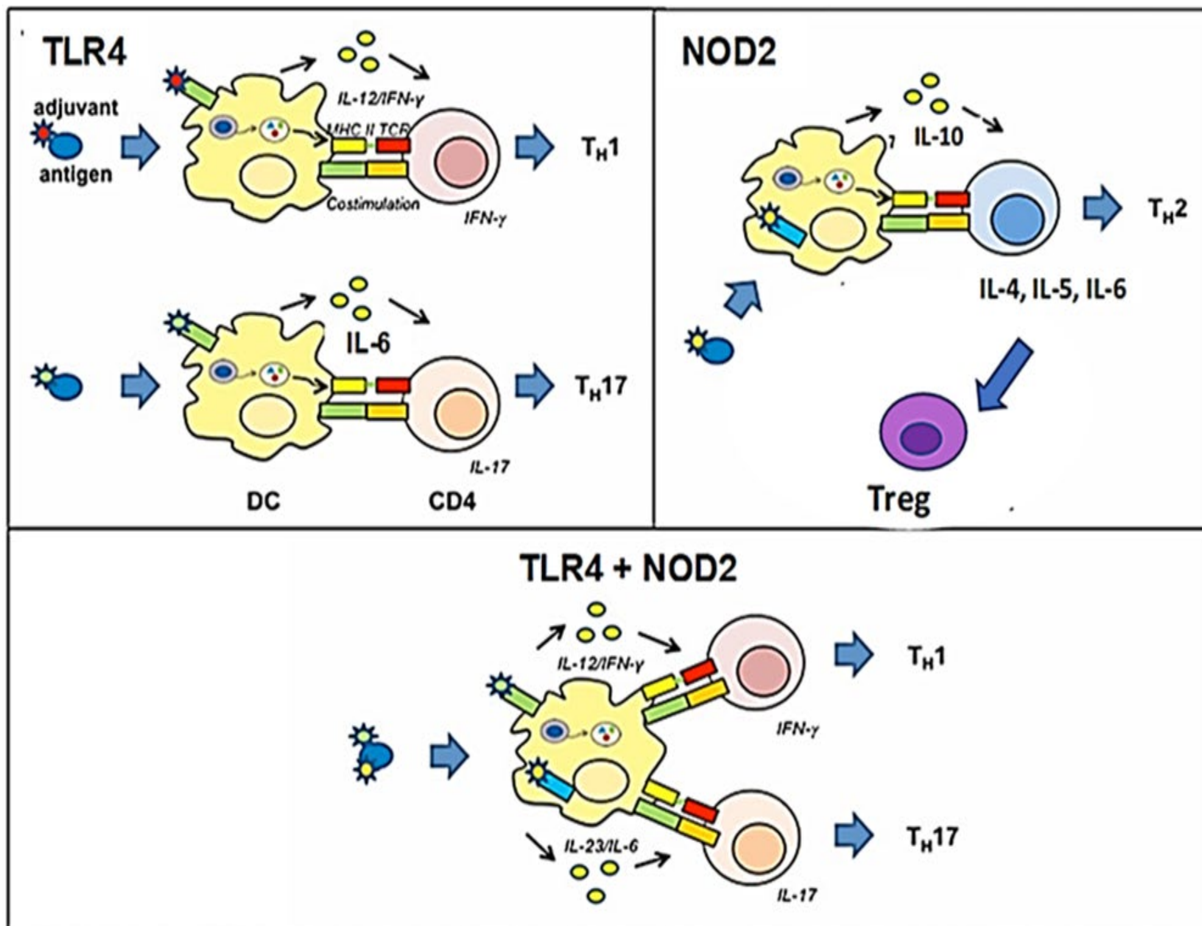

Figure 10 TLR4 stimulation mediates Th1 responses. NOD2 stimulation induces the Th2 response. Costimulation with TLR4 and NOD2 elicit Th1 and Th17 immune responses [Taken with adaptations from (Jahn and Scheller 2006)](4).

1.5.4 *In-vitro* evaluation of TLR4 agonists

Performances of an adjuvant are initially identified by *in vitro* transmitting the correspondent cell lines expressing conserved innate immune receptors. Such approaches optimize adjuvant activities in a clinical trial by improving statistical power and providing a more definitive outcome. Also, *in vitro* studies help in the early phase of adjuvant discovery by ensuring the purity and consistency of heterogeneous compounds, which rationally modify an ideal adjuvant candidate (130). Successful evaluation of vaccine-adjuvants by *in vitro/ ex vivo* studies help magnify the screening outcome, which

could be later validated in *in vivo* studies. The robust *in vitro* assays offer the benefits of replacing animal models, and specific criteria of adjuvants can be assessed within a shorter time frame (131). Mainly, evaluating the biocompatibility of inflammatory diseases has always exploited several *in vitro* and *ex vivo* studies as the first step before any pre-clinical trial.

The mouse (*Mus musculus*) has been extensively used in pre-clinical *in-vivo* studies for mammalian immunological research. Primarily, mice share many characteristics with humans at the level of pathology, species-specific probes, and cost-effectiveness. Immortalized and relatively stable, J774A.1 macrophages and JAWSII dendritic cells are well-known murine i*n vitro* models for assessing inflammatory drugs to profile cytotoxicity, inflammatory response, and biocompatibility (132). Both cell lines have retained most of the significant features of their natural counterparts *in vivo*. Also, both express TLR4 and NOD2 and, upon interaction with LPS, leading to the activation of downstream signaling. JAWSII cells had previously been reported to be able to mature and subsequently have T-cell stimulatory abilities (133).

Experiments in vitro/ex vivo have limitations regarding evaluating an adjuvant's inflammatory reaction. The *in vitro* response of individual cells is mostly downstream aspects, which usually relates to *in vivo* adjuvanticity(132). Further, utilizing primary-murine bone marrow-derived immune cells and human peripheral blood mononuclear cells (PBMCs) helps demonstrate the cell lines' equivalencies (144). To compare the immune complexity of *in vivo* side by side, upgraded *in vitro* models are profoundly needed.

1.5.5 Immune cell model for the intracellular pathogen of interest

Currently, vaccine designs against an intracellular virus are based on the primary cell-mediated immune (CMI) responses of TLR4/NOD2 agonists, which are tailored to stimulate immune responses or might be optimal for pathogens.

Initially, immunosenescence, defined as "*inflamm-aging*," described the dysfunction of TLR4 receptors in innate immunity in the elderly (134). Initially, immunosenescence, defined as "*inflamm-aging*," described the dysfunction of TLR4 receptors in innate immunity in the elderly (134). This senescence state puts the elderly at high risk of fighting against viral infections, especially influenza, due to its antigenic drift for each season. Antibody-mediated protection is, therefore, inadequate against heterologous strains with distinct coat proteins. This distinction is the consequence of antigenic shift since many viruses rapidly mutate their coat proteins; an effective humoral response–a based vaccine against a particular virus may be ineffective against next season's variant (135). On the contrary, T cells, which mediate cellular immune responses, can target internal proteins common to heterologous infectious strains (129). In comparison, TLR4 adjuvants promote vaccines to induce protective cellular immune responses against heterologous viral strains. Besides, TLR4 ligand stimulations restore innate immune responses with the expression of TLR4 on APCs, which seems well-maintained with aging (136).

Antigen challenge- Ex vivo studies have been performed to assess the effector CD8+ T cell generation against intracellular pathogens by analyzing the responses to antigen challenge. The model we chose for this study was the influenza A virus (IAV). The flu vaccine was used as an antigen to stimulate virus-specific CTLs from human PBMCs. Here we evaluate the competency of A1 and MDP on the population of human PBMCs for priming either Th1 or Th2 T cells. In primary responses that result in a state of immunologic memory, A1 and MDP prime naive T cells and lead

to rapid, specific cell expansion with a designated T helper phenotype and usually produce large amounts of cytokine promptly upon antigenic challenge**.**

GLA was used as a positive control. GLA could activate and enhance the performance of myeloid DCs to induce a higher ratio of Th1 to Th2 proinflammatory cytokines (IFNγ to IL-10) in response to influenza A/H3A2 challenge in cells pre-stimulated with TLR4 agonist and SVV in PBMCs(118).

The *in vivo* requirements for the reactivation of these cells have yet to be made clear. A mouse model (C57BL/6-B6, H2b) of IAV pneumonia provided a well-developed experimental system to analyze T cell-mediated immunity. However, influenza infection in mice did not precisely replicate the natural infection in humans(137). Most importantly, this practical approach of assessing novel adjuvant (TLR4 +NOD2)-vaccine formulations will be more effective, and the proinflammatory cytokines will synergize their immune response by increasing Th1 mediated- CD8+ T- cell (CMI) against a number of viruses (62).

1.6 Rationale

It is known that attenuated or subunit vaccines are inevitably associated with lower immunogenicity and generate $CD4^{+}$ T cell responses in addition to antibodies. So, these vaccines are able to induce exceedingly lesser amounts of CTLs. due to the lack of cell permeability. They do not have the ability to continue the infection, such as the live attenuated Flu Mist® vaccine, which triggers only local and limited infections. Furthermore, despite containing the same antigens as in the SVV, e.g. Fluzone® and Live attenuated intranasal vaccine (LAIV), they induce different immune responses(135). Thus, the goal of modern vaccines is to achieve high immunogenicity by selecting the right adjuvants that can promote strong CD8+ T cell induction(135). Improved mechanisms for prophylaxis and therapy are needed for influenza because control of the respiratory infection in older adults is not readily achieved through current approaches to vaccination, which is a significant cause of morbidity in elderly(138). At

present, an ever-increasing immunocompromised population needs the novel adjuvanted vaccines(134). So, the need for new adjuvants will continue to grow.

Several new vaccines in clinical trials are also relying on adjuvants with desirable capabilities(139). Besides, growing evidence shows that during any infectious event, the importance of the synergistic effects of TLRs and NLRs are: survey extracellular and intracellular environments for PAMPs; and, upregulate the immune molecules to drive adaptive immunity towards different T-helper cells profiles which will ensure a complete protective immunity against a given pathogen (140). Also, TLR4s are better suited for targets as the only prototypic TLR4 agonists activate early and can stimulate two signaling pathways considered initiators of pathologic conditions (13). NOD2 receptor agonist, MDP combined with TLR4 activation, arouses a significant cellular response. Furthermore, TLR4 is considered to be able to restore the age-related decline in T cell immunity in the elderly and in populations with reduced immunity (141).

With these increasing expectations, the most advanced approach for developing a novel adjuvant formulation, now means that combined effects of rationally selected adjuvants, targeting different PRRs based on their responses. Also, they will induce optimal priming environments with broader (Th1 versus Th2-mediated) cellular immunity in healthy and immunosenescent individuals, which will be a logical and attractive approach.

1.7 Project aims and objectives

The project aims to examine the biomarkers of the immortal and primary cells in response to TLR4 ligands to develop an immune model. The synergistic immune responses between TLR4 and NOD2 receptors will be evaluated using immortalized *(in vitro*) and primary (*ex-vivo*) immune cell lines that are necessary for a successful antigen-specific response and to assess the effects of the novel adjuvant-

vaccine formulation in the presence of antigens using human PBMCs on the memory T-cell secretion. The overall objectives are as follows:

To be the first to demonstrate the effects of TLR4 and NOD2 agonists combined or alone on multiple biomarkers produced by murine *in-vitro* macrophages.

To evaluate the homemade TLR4 (synthetic lipid A analog (A1) and NOD2 (MDP) agonist adjuvant formulations and develop a novel bioassay model to establish a cytokine model of secretion from murine *in-vitro* APCs.

To characterize the response of bone marrow (BM)-derived DC (BMDCs) and BM-derived macrophages (BMMϕs) to the A1 and MDP adjuvant system stimulation in the presence of vaccine for cytokine secretion and protein kinase identification; and

Finally, conduct an *ex vivo* experiment using human PBMCs as a model to test general and antigen-specific immune responses to define the simultaneous immune response of A1 and MDP in the presence of a vaccine.

2 Material and methods

2.1 Tissue Culture

The J774A.1 cell line, [American Type Culture Collection (ATCC ® TIB-[67]™) Manassas, VA], isolated from female BALB/c mice is a reticulum cell sarcoma tumor-derived monocyte-macrophage cell line. J774A.1 cells were cultured in the complete medium [Dulbecco's Modified Essential Medium (DMEM, Hyclone, Logan, UT) with high glucose supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) (Gibco, Grand Island, NY) and 100 U/ml Penicillin-Streptomycin (pen.strep.) (Hyclone, Logan, UT)] in T75cm2 flasks (Biolite). J774A.1 cells were dislodged with a cell scraper, collected and centrifuged at 1000 rpm for 5 min.

An immature monocyte dendritic cell (DC) line termed JAWSII (ATCC® CRL-11904™, Manassas, VA) derived from bone marrow cells of C57BL/6 $p53^-$ *Mus musculus*, mice were used. JAWSII cells were cultured in the complete medium [RPMI-1640 (Hyclone, Logan, UT) with L-glutamine supplemented with 10% HIFBS and 100 U/ml pen.strep. and 5 ng/ml murine recombinant Granulocyte-macrophage colony-stimulating factor [(rmGM-CSF) from Invitrogen], in T75cm2 flasks (Biolite). Cells were released by treating attached cells with 0.25% trypsin-0.03% EDTA (Gibco) at 37°C for 2-3 min.

J774A.1 and JAWSII cells were re-seeded for sub-culture and maintained at 37C in 5% CO2. Both cultures were grown to confluency before each experiment. No cell culture was used which exceed a passage number of more than 24.

2.1.1 *In vitro* cell cultures treated with commercially available adjuvants

J774A.1 and JAWSII cells were seeded in 24 well tissue culture plates (Biolite, USA) at a density of $0.5X10^6$ cells/mL in triplicate and left untreated or treated with or without either 10ng/mL ultrapure

LPS (TLR4 agonist) [(TLRL-3 PEPLPS, Sigma, St. Louis, USA)] and MDP 1 and 10µg/mL of synthetic MDP(prepared by Dr. Zi-Hua jian and Dr. Thanh's Lab's collaborators in Thunder Bay). LPS was diluted to 10ng/mL in PBS. MDP was diluted to 1 and 10µg/mL in distilled water(dH2O). Non-treated (NT) groups were treated with the reagent diluent (1X PBS). Supernatants were collected at different time points (6, 12, 24, and 48 h) and stored at –200C. *Experimental replicates* were defined as cells harvested from different passages.

2.1.2 Cell enumeration and viability assessment

Counts and viability of cells were assessed using a hemocytometer and trypan blue exclusion test. This test is based on the principle that viable cells with intact membranes can actively exclude the dye, whereas dead cells are unable and appear blue when viewed under the microscope. An aliquot of gently agitated cell suspension mixed 1:1 in trypan blue solution (Gibco, USA). Cells were gently applied to a Brightline™ Neubauer hemocytometer (Sigma, USA) and counted under a phase-contrast microscope. The cell viability cut-off point was >80%.

Cells per ml = N (average cell number) x DF (dilution factor) x 10^4 (constant)

2.1.3 Evaluation of the novel adjuvant system

TLR4 agonist, A1, is less potent compared to LPS. Based on that, Dr. Le's laboratory researchers have selected a dose of A1 at 1µg/mL. GLA (Invivogen, San Diego, CA) 500 ng/mL was used as a positive control18. To determine the best relative A1/ MDP dose ratio, J774.A1, and JAWSII cells were seeded in 24 well tissue culture plates at a concentration of 5 x10^5 cells/mL/well and left untreated or treated with either 1µg/mL A1 or 500ng/mL GLA and in the presence or absence of 100ng/mL and 1 µg/mL MDP. Supernatants were collected after 24h and assayed for the cytokines (TNFα, IL-6, IL-10, IL-1β, and IL-12p40) secretion by ELISA. Further, to determine the immune responses of A1 on different cell lines would be used. Cytotoxicity Assay was performed following Methyl Tetrazolium Blue (MTT)

Non-treated (NT) groups were treated with the reagent diluent 1X PBS. A1 was diluted to 1μg/mL in DMSO(0.5%) in PBS. 500ng/mL GLA and 1μg/mL MDP were diluted in MiliQ water.

To measure the cytotoxicity of the novel adjuvant formulation and viability of cells treated with adjuvant formulation, a methyl tetrazolium bromide (MTT) reduction assay was employed.

J774A.1 and JAWSII cells were counted and seeded at 0.1x10 5/100μl/well in triplicate, left untreated or treated with either 1μg/mL A1 or 500ng/mL GLA(positive control) and 1μg/mL MDP or both in three 96-well plates (each plate for one-time point- 6 h, 24 h, and 48 h) incubated with 5% CO2 at 37 ºC to allow sufficient time for cells to adhere. At first, for a 6 h time point, 20 μl of MTT solution was added to each well of one plate at a final concentration of 0.5 mg/mL after 3 h before the time point. Plates were incubated at 37 ºC at 5% CO2 for another 3 h. Cells were centrifuged at 1000 rpm for 2 min following the incubation period. 200μg /L DMSO was added to each well and incubated for 10 min to ensure the full dissolution of the purple formazan. A synergy H4 Multi-mode Hybrid Microplate Reader (Biotek) was used to measure the absorbance at 540nm. Experiments were repeated for 24 h and 48 h time points.

2.2 Isolation and culture of splenocytes, bone marrow-derived dendritic cells (BMDCs) and macrophages (BMMϕs)

Animal experiments were approved by the University of Laurentian Animal Care Committee and the Biosafety Committee of HSNRI. Female BALB/c mice aged 8-10 weeks were purchased from Charles River (QC, Canada) and housed under Specific Pathogen Free (SPF) conditions. Femurs and tibiae were isolated aseptically from naïve mice. Femurs and tibiae were removed from the surrounding muscles by rubbing with Kim wipes ™ and shaved with razors. The intact bones were soaked for 2 min in 70% ethanol and transferred to ice-cold Hank's balanced salt solution (HBSS, Hyclone, Logan, UT). Then both

ends of the bones were cut, and bone marrow (BM) precursors were flushed with phosphate-buffered saline (PBS) using a Syringe with a 27.5g needle. BMs were centrifuged for eight minutes at 400 *Xg*(142) Pellets were resuspended in 20 mL of HBSS to prepare a homogenous cell suspension(142). An aliquot of cells was taken for counting using the trypan blue exclusion method to ensure an adequate number of viable cells had been harvested.

2.2.1 Primary cell harvest and evaluation of A1 formulation with the vaccine

To generate BMDC and BMMϕs, leukocytes were incubated with 10 mL of complete (c)medium in Petri dishes (sarstedt – 100 x 15 mm standard bacteria culture plates) at a density of 4 x 106cells/mL. For BMDCs phenotype, BM leukocytes were incubated with complete (c) DMEM at a density of 4 x 106/mL viable cells/petri dish in a 37°C incubator with 5% CO2. On day 3, 10 mL of cRPMI 1640 with 25 ng/mL rmGM-CSF was added to Petri dishes containing BMDCs(143). For BMMϕs, 10 mL of cDMEM with 25 ng/mL macrophages-colony-stimulating factor (M-CSF, Invitrogen, USA) was added (144). On day 7, the semi-adherent cells were removed by gently pipetting. Loosely adherent BMDCs get easily dislodged into suspension by this process. Primary BMMϕs were adherent cells dislodged with cell scrapers from the bottom of the Petri dishes and then pooled for subsequent experiments. Na YR et al. (2016) showed that some cells of GMCSF-grown bone marrow-derived cell culture differentiated into Mϕs(145). So, So there was a chance that these BMDCs population might also be contaminated with Mϕs. Both cell types were counted using the trypan blue exclusion method. The cell concentration was adjusted with appropriate media at 1x106/ml and placed in 24-well plates in triplicate, left untreated or treated with either 1µg/mL A1 or 500ng/mL GLA, in the presence or absence of 1µg/mL MDP and with or without 5µg/mL of vaccine-adjuvant, co-administered antigens, Influenza vaccine [Fluzone,(2014-2015),(Antigen only) Sanofi Pasteur, USA] for 24 h. Supernatants were collected, and cytokines (TNFα, IL-6, IL-10, IL-1β, and IL-12p40) were analyzed by ELISA. A1 was diluted to 1µg/mL in DMSO(0.5%) in PBS.

500ng/mL GLA and 1µg/mL MDP were diluted in MiliQ water. Non-treated (NT) groups were treated with the reagent diluent 1X PBS.

BMDCs were left untreated or treated with either 1µg/mL A1 or 500ng/mL GLA and in the presence or absence of 1µg/mL MDP for 30 minutes. Pellets were collected and stored at −80 °C for determining ERK1/2 concentration by Western blot analysis.

2.2.2 Isolation of Splenocytes

Spleens were removed aseptically from naïve mice and collected in incomplete (c) RPMI on ice. Then, spleens were prepared to achieve a single-cell suspension. Erythrocytes were lysed from the pellet using 3 ml ACK lysis buffer (Lonza, Walkersville, MD) for 1 minute and then resuspended in 27 mL HBSS centrifuged. Splenocytes were suspended in cRPMI 1640 medium and placed in 96-well tissue culture plates at 106cells/300µL/well in triplicate and left untreated or treated with either 1µg/mL A1 or 500ng/mL GLA and in the presence or absence of 1µg/mL MDP or both for 24h A1 was diluted to 1µg/mL in DMSO(0.5%) in PBS. 500ng/mL GLA and 1µg/mL MDP were diluted in MiliQ water. Non-treated (NT) groups were treated with the reagent diluent 1X PBS. Supernatants were collected, and the levels of cytokines (TNFα, IL-6, IL-10, IL-1β, and IL-12p40) were analyzed by ELISA.

2.3 Evaluation of A1-formulation with the vaccine in the presence of antigens for antigen-specific immune responses using human PBMCs

Peripheral blood mononuclear cells (PBMCs) are mainly lymphocytes and monocytes(146).

2.3.1 Isolating Mononuclear cells: Ficoll paque assay

The Ficoll Histopaque -a density gradient centrifugation method is widely used for separating PBMCs from whole blood. Heparinized venous human blood samples were drawn from consenting healthy adults (aged 27-37 years) subjects. The Research Ethics Board approved this protocol for

HSN(RI). In brief, blood samples were diluted in PBS at a 1:1 ratio. The blood was layered very gently on the top of Ficoll Histopaque at a ratio of 1:1 so that blood and Ficoll-paque should stay as two different layers and centrifuged for 20 min at 900 x*g.* with the brake off. The buffy coat containing PBMCs was located between the top plasma layer and the Ficoll medium, as in Fig.16. It was aspirated immediately. -The 3x volume of PBS was added based on the estimated interface volume and centrifuged in 300 *xg* 15 min.

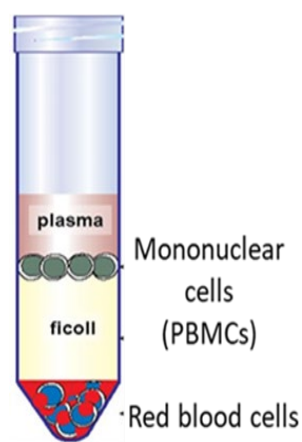

Figure 11 Isolating PBMCs: Ficoll plaque assay (146).

2.3.2 Antigen challenge: experimental protocol

In the case of the 7 days protocol, two sets of tubes (treatment groups) were needed – the pre-challenge (without virus as antigen) and the post-challenge (with the virus as antigen), for 2 separate collections of supernatants. Cells were seeded at a density of 106cells/mL in triplicate for 7 days, left untreated or treated with either 1µg/mL A1, 1µg/mL MDP and 500 ng/mL GLA and a combination of A1 or GLA and MDP, and with or without vaccine-adjuvant (co-administered influenza vaccine antigens, Fluzone)-the final concentration per well was 5 µg/mL. After 6 days, PBMC-containing plates were spun for 10 min at 1000 rpm. Cell-free supernatants were collected for the pre-challenge

treatment group. Cells/pellets were washed with media and spun at 1000 rpm for 10 min. Supernatants were removed, and pellets were re-stimulated with new media containing the influenza virus (without any treatment group) for 20 h as a post-challenge treatment group. The influenza virus A/Victoria/3/75 was prepared at an MOI of 2. A1 was diluted to 1µg/mL in DMSO (0.5%) in PBS. 500ng/mL GLA and 1µg/mL MDP were diluted in MiliQ water. Non-treated (NT) groups were treated with the reagent diluent 1X PBS.

2.3.3 Preparation for flow cytometry

Supernatants were removed from each well, placed in their respective tubes, and spun to pellet the cells after 20h. Cell-free supernatants were collected for the cytokine analysis (without disturbing the pellets). Pellets were treated with a Biolegend fixing solution (containing paraformaldehyde) to fix virus-infected cells so they could be sent to a collaborator (NOSM) for flow cytometry analysis. CD3+CD4+(for CD4+ T cells), CD3+CD8+(for CD8+ T cells), and CD3+CD4+FoxP3+ {for T reg (foxp3) cells} cell surface markers were measured to detect antigens on PBMCs. CD3-FITC (fluorescein isothiocyanate), CD4-FITC, CD8-PE (phycoerythrin), and FoxP3-APC(Allophycocyanin) (all from Cedarlane) monoclonal antibodies were used in flow cytometric analysis. Stained/fixed samples were then acquired on the FACS Canto II flow cytometer and analyzed using Kaluza software (Beckman Coulter, USA). A minimum of 10,000 cellular events were collected throughlymphocyte gate.

2.4 Enzyme-linked immunosorbent assay (ELISA)

Supernatants were measured by sandwich ELISA. Cytokines were analyzed according to the manufacturer’s description with antibody pairs supplied as capture/detection Duo-Sets from R&D Systems, as showed in Fig. 17. The cytokines IL-6, IL-10, TNFα, IL-12p40, IL-17, IL-1β, and IFNγ were quantified according to the respective ELISA kits (R&D Systems, USA) and performed according to

manufacturer’s recommendations. The limit of detection for murine IL-10, TNFα, and IL-12p40 was 15.625 pg/mL, and for IL-6, and IL-1β, it was 7.8 pg/mL. For human cytokines IL-10, IFNγ, and IL-17 the limit of detection was 15.75 pg/mL, and for IL-1β, it was 1.95pg/mL and for IL-6 it was 4.69 pg/mL.

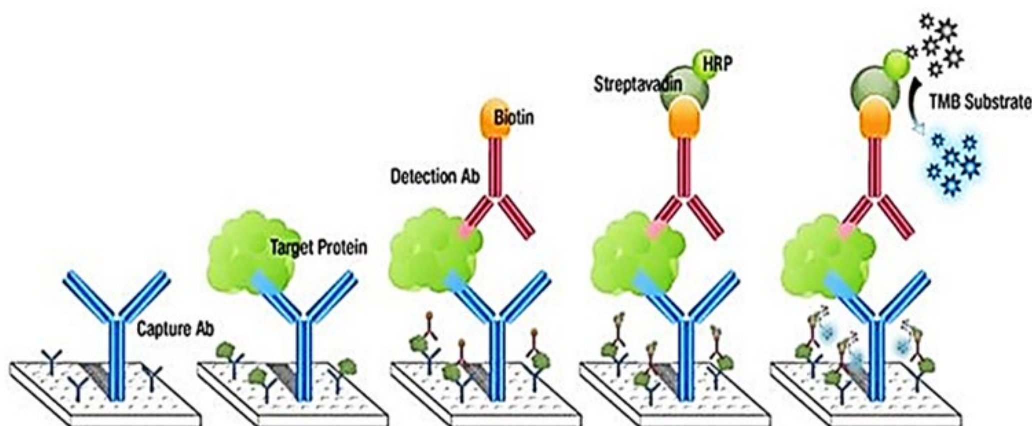

Figure 17 Illustration of the principles behind the sandwich ELISA [taken with adaptations from http://www.epitomics.com].

2.5 Western blotting

Western blotting is an important technique for identifying specific proteins from the complex protein mixture of cell extracts in cell and molecular biology.

2.5.1 Isolation of whole-cell lysates, protein extraction, and quantification

JAWSII DCs and BMDCs were counted and seeded at 1x106/mL/well in triplicate and left untreated or treated with either 1µg/mL A1, 1µg/mL MDP and 500ng/mL GLA (positive control), or both in 24-well plates for 30 mins. Cells were treated with RIPA lysing buffer [see Appendix] with protease and phosphatase inhibitor cocktails. Lysates were collected, and the protein quantification was carried out using a BCA assay kit (Thermo Scientific, USA). The proteins were mixed with 2X SDS sample buffer at a ratio of 1:1.

2.5.2 SDS denaturing polyacrylamide gel electrophoresis

For performing sodium dodecyl sulphate (SDS)-Polyacrylamide gel electrophoresis (PAGE), protocols by Laemmli and Towbin(147) were used: 10 µg of protein sample was loaded onto 12% polyacrylamide gel [see Appendix]. Proteins were separated by electrophoresis in 1X Running buffer [see Appendix], A molecular weight protein ladder (Amersham™ ECL™ Rainbow™ Marker) ranging from 12-225 kDa was added to one lane.

2.5.3 Protein transfer, immunoblotting, and detection

The PVDF membrane, onto which electrophoresed proteins were transferred, was incubated for 1 h in a blocking buffer [see Appendix]to block non-specific protein binding. Membranes were then washed 3x with TBS-T wash buffer [see Appendix] for 5 minutes and incubated with the primary antibody [rabbit monoclonal (anti-ERK1 + anti-ERK2) and anti-ERK1-(phosphor-T202) + anti-ERK2-(phospho-T185) Abcam, USA] in 5 % (w/v) BSA/TBS-T-20 overnight at 4ºC. After that, these membranes were incubated with the appropriate Horse Radish Peroxidase (HRP)-conjugated secondary antibody (Goat-Anti-Rabbit IgG, Abcam, USA) in 5% (w/v) BSA/TBS-T for 1 h. Membranes were washed 3x for 5 min in a wash buffer. Mouse anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (Abcam, USA) was used as a loading control. The target proteins were identified using the ECL kit (GE Health care, USA). The activation of the kinase was measured as the phosphorylation of ERK1/2.

2.5.4 JAWSII, TLR4 signaling inhibitor- *in vitro*

CLI-095 (InvivoGen, San Diego, CA), a novel cyclohexene derivative, is a known inhibitor of the TLR4 signaling pathway(148). JAWS II was seeded at a density of 0.5 x106 cells/mL/well in 24 well plates in triplicate and pretreated with 1 µg/mL CLI-095 for 6 h. The cells were then left untreated or treated with either 1µg/mL A1 or 500ng/mL GLA and in the presence or absence of 1 µg/mL MDP for another 30

minutes. Pellets were collected and stored at −80°C to determine the protein concentration by Western blot analysis (see section 2.1.5 for Western blot protocols).

2.6 Statistical analysis

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's Post -Hoc Test was performed to determine the differences between treatment group means. The Studies with 2 or more independent variables were analyzed by two-way ANOVA, followed by Bonferroni tests at individual time points where applicable. The level of statistical significance was indicated by $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$. All statistical analyses were performed using Graph Pad Prism 5.

3 Results

3.1 LPS and MDP synergistically enhance the secretion of proinflammatory cytokines by J774A.1 cell

The synergistic immune responses between the activated TLR4 and NOD2 receptors were evaluated using the immortalized murine immune cell lines *in vitro*. Alterations in cytokine expression at different times following treatments were used as a biomarker to determine the response that is necessary for a successful antigen-specific response.

3.1.1 Combined treatment of J774A.1 cells with LPS and MDP increased the level of proinflammatory cytokines in a time-dependent manner

Cells were seeded at a concentration of 5 $x10^5$ cells/mL/well in a 24 well plate and left untreated or treated with either TLR4 agonist (LPS) 10 ng/mL, MDP 1 and 10 µg/mL, or both. Supernatants were collected after 6, 12, 24, and 48 hours of treatment. In these experiments, the best relative LPS/MDP dose ratio to optimize the secretion of cytokines was determined.

Treatment with LPS alone resulted in the detectable amount of all cytokines (TNFα, IL-6, IL-12p40, IL-10, and IL-1β) at 24h time point. The combined treatments of MDP (1µg/mL or 10µg/mL) with LPS 10 ng/mL induced TNFα, IL-6, IL-12p40, and IL-1β secretion synergistically [Fig.18(a), (b), (c), (e)]. Treatments with MDP (1µg/mL and 10µg/mL) alone had no effect on cytokine secretion. Treating cells simultaneously with LPS and MDP induced TNFα secretion, which peaked at 6h and then started declining [Fig.18(a)]. In contrast, IL-6 secretion increased with time [secretion at 48h was higher than at 24h or 12h [Fig.18(b)]. The IL-1β concentration increased after 24h reaching a plateau [Fig.18(e)]. The IL-12p40 secretion was started at 12h after stimulation and was still elevated 48h post-stimulation

[Fig.18(c)]. The level of IL-10 production was detectable at every time point but without any major differences. [Fig.18(d)]. All cytokines examined were secreted at significant levels by 24h of treatment (LPS 10ng/mL + MDP 1 µg/mL), except IL-10. A1 was diluted to 1µg/mL in 20% DMSO in PBS. 500ng/mL GLA and 1µg/mL MDP were diluted in MiliQ water. Non-treated (NT) groups were treated with the reagentdiluent (1X PBS).

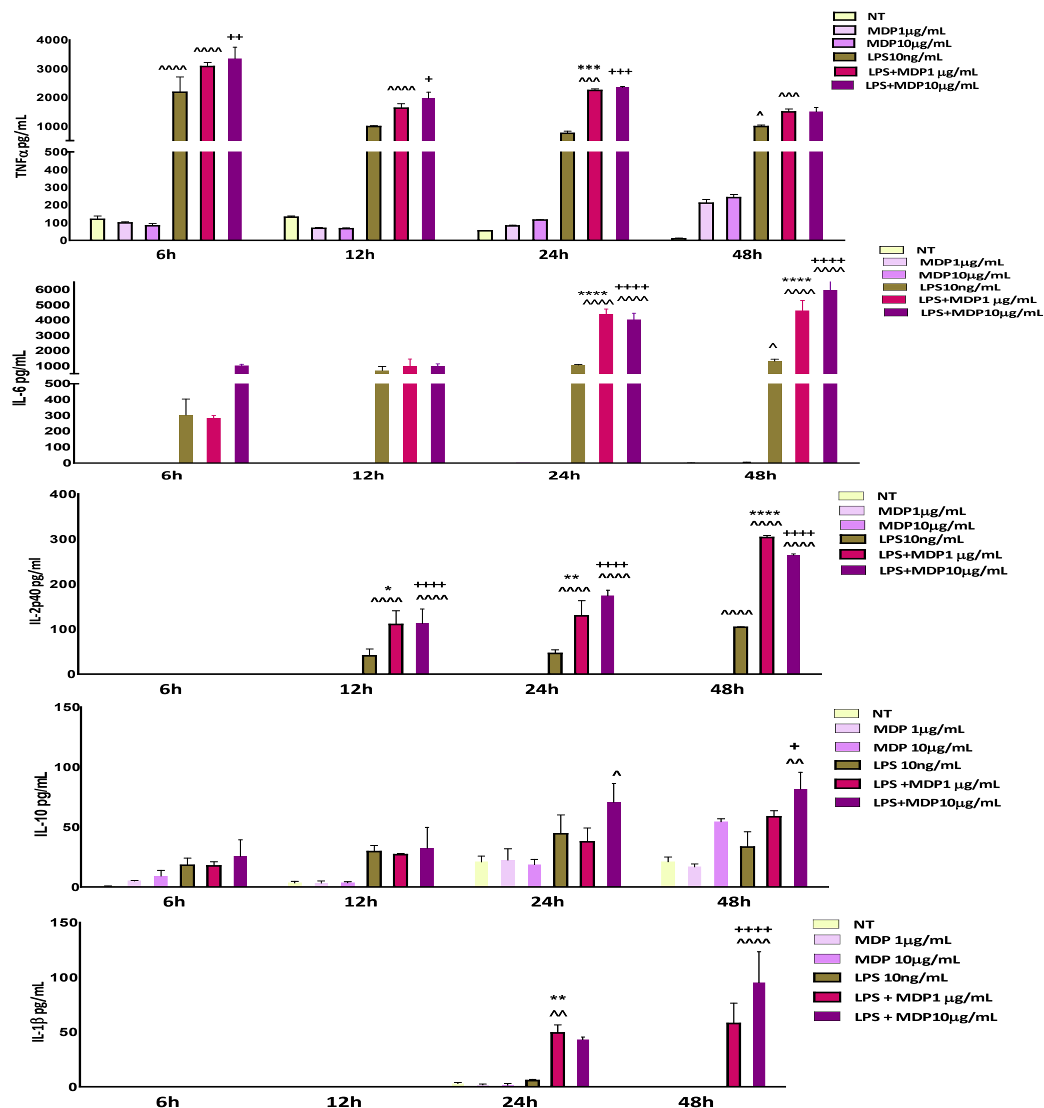

Figure 18 Determination of optimal treatment time for cytokine secretion levels. J774A.1. cells were treated with LPS with or without MDP and cytokines were determined by ELISA for **TNFα (a), IL-6 (b), IL-12p40 (c), IL-10(d)** and **IL-1β (e)**.^p < 0.05, ^^p<0.01, ^^^p < 0.001, ^^^^p < 0.001 as compared to NT group; * p<0.05, **p<0.01 and ***p<0.001,****p<0.0001 compared to the LPS 10 ng/mL with LPS +MDP1 μg/mL; +p < 0.05, ++p<0.01 +++ p < 0.001,and ++++p < 0.001, as compared to the LPS 10 ng/mL with LPS +MDP10 μg/mL Results are presented as n= 3 and ±SEM. Statistical significance was determined by two-way ANOVA.

3.2 Evaluation of a novel adjuvant system to develop a stable adjuvant

The TLR4 agonist, A1, is less toxic compared to LPS. Based on that, our laboratory team members have selected the dose for A1, at 1µg/mL, and the dose of MDP at both 100 ng/mL and 1µg/mL. An MTT viability assay was performed to determine the toxicity level of A1. In these experiments, treatment with Glucopyranosyl lipid A (GLA) at 500 ng/mL was used as a positive control.

3.2.1 Treatment of J774A.1 cells with the TLR4 agonist, A1, has no significant effect on cell viability

To compare the cytokines secretion from J774A.1 cells following stimulation with A1 and MDP alone or in combination, the potential toxicity of the treatments was first examined by exposing them to the cells. Cells were seeded at a concentration of 20,000 cells/100µL in triplicate in a 96 multi-well tissue culture plate and were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, or 500 ng/mL GLA alone or in combination for 6h, 24h, and 48h. Plates were read using a microplate reader at an O.D. of 540nm. The cell viability was assessed using the MTT cell viability assay. Incubation of J774A.1 cells with the treatment group showed no notable change in cell growth starting at 6h post-treatment (Fig.19). Furthermore, cells treated with a combination of A1 and MDP had a significant increase in cell growth ($p<0.001$) at 48h compared to 6h and ($p<0.05$) at 48h compared to 24h. Cells treated with a combination of A1 and MDP had more growth than the NT group at every time point (Fig.19). Similar effects have been seen in cells treated with 500ng/mL GLA ($p<0.001$), 500ng/mL GLA and 1µg/mL MDP ($p<0.001$) at 48h compared to 6h and 500ng/mL GLA ($p<0.001$), 500ng/mL GLA and 1µg/mL MDP ($p<0.001$) at 48h compared to 24h(Fig.19). These doses did not exhibit any significant cytotoxic effects on J774A.1 cells.

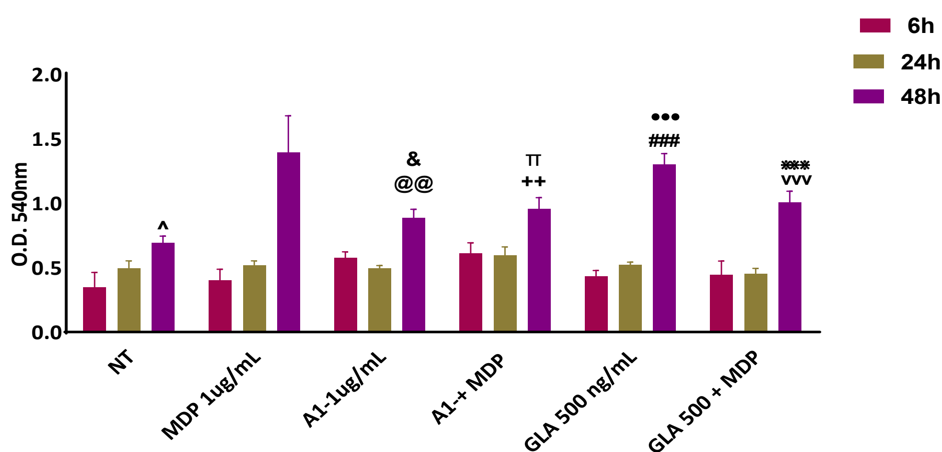

Figure 129 MTT assay to measure the viability of J774A.1 cells treated with adjuvants.
6h compared to 24 h.
6h compared to 48h: ^ p<0.01 compared to NT; @@p<0.01compared to A1-1µg/mL; ++p<0.01 compared to A1-1µg/mL+ MDP 1µg/mL; ###p<0.001 compared to GLA 500ng/mL and vvv p<0.001 compared to GLA 500ng/mL + MDP1µg/mL.
24h compared to 48h: &p<0.05 compared to A1-1µg/mL; π p<0.05 compared to A1-1µg/mL+ MDP 1µg/mL; •••p<0.001compared to GLA 500ng/mL and ⁕⁕⁕ p<0.001compared to GLA 500ng/mL + MDP1µg/mL. Results are presented as n=3 and ±SEM. Statistical significance was determined by two-way ANOVA.

3.2.2 Synthetic TLR4 agonist (A1) synergistically enhances the secretion of proinflammatory cytokines by J774A.1 cells

To determine the combined effect of A1 and MDP on the innate immune response, J774A.1 cells were seeded at a concentration of 5 x10^5 cells/mL/well in triplicate in a 24 multi-well tissue culture plate. They were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, and a combination of 1µg/mL A1 or 500ng/mL GLA and 1µg/mL MDP. Supernatants were collected after 24 h and analyzed to quantify the cytokine secretion.

TNFα, IL-6, IL-12p40, and IL-1β were not observed in the unstimulated nontreated (NT) group, except IL-10. Both A1 and GLA, in combination with MDP, induced a significant amount of TNFα (p≤ 0.001), IL-6 (p≤ 0.001), IL-1β (p≤ 0.001), and IL-12p40 (p≤ 0.001) secretion compared to NT [Fig.18(a), (b),(d),(e)]. Also, in the presence of A1 and MDP, J774A.1 cells consistently produced significantly

enhanced levels of TNFα (p≤ .05), IL-6(p≤ 0.05), IL-12p40 (p≤ 0.001), and IL-1β (p≤ 0.001) compared to A1 alone (Fig.20). In the presence of MDP, A1, and GLA secreted significant amount of TNFα, (p≤ 0.001), IL-6, (p≤ 0.001), IL-1β (p≤ 0.001), and IL-12p40(p≤ 0.001) secretion compared to MDP [Fig.18(a), (b),(d),(e)]The release of IL-10 by A1 +MDP, was significantly decreased compare to A1 alone (p≤ 0.001) and NT(p<0.05) [Fig.20(c)].

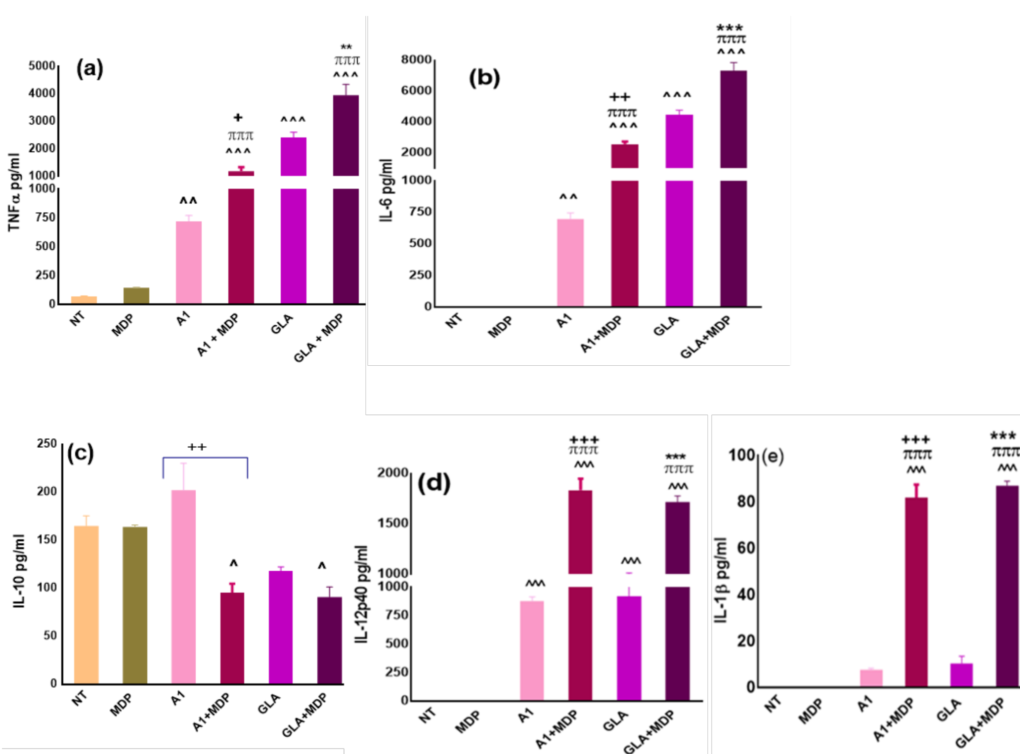

Figure 20 A1 with MDP enhances proinflammatory cytokines secretion by J774A.1.
(a) TNFα ^^ p<0.01, ^^^p<0.001 compared to NT; πππp<0.001 compared to MDP1-1µg/mL; + p<0.05 compared to A1-1µg/mL; ** p<0.01 as compared to GLA 500ng/mL. **(b) IL-6** ^^ p<0.01, ^^^p<0.001 compared to NT; πππp<0.001 compared to MDP1-1µg/mL; + p<0.05 compared to A11µg/mL; *** p<0.001 as compared to GLA 500ng/mL. 21 **(c) IL-10**; ^p<0.05 compared to NT**;** + p<0.05 compared to A1-1µg/mL; **(d) IL-12p40**. ^^^p<0.001 compared to NT; πππp<0.001 compared to MDP1-1µg/mL; +++p<0.001 compared to A1-1µg/mL; *** p<0.001 as compared to GLA 500ng/mL. **(e) IL-1β** ^^^p<0.001 compared to NT; πππp<0.001 compared to MDP1-1µg/mL; +++p<0.001 compared to A1-1µg/mL; *** p<0.001 as compared to GLA 500ng/mL. Results are presented as ±SEM and n=3. Statistical significance was determined by one way ANOVA with a Tukey HSD.

3.2.3 The Doses of the TLR4 agonist A1 used on JAWSII DCs have no significant effect on cell viability

The potential toxicity of the A1 was first examined by exposing it to the JAWSII cells. Cells were seeded at a concentration of 20,000cells/100µL in triplicate in a 96 multi-well tissue culture plate and were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, and 500 ng/mL GLA or a combination of A1, MDP, and GLA for 6h, 24h, and 48h. Plates were assayed and read by a plate reader at 540nm. The cell viability was assessed using the MTT cell viability assay, where a reduced O.D. indicates reduced cell viability. Incubation of JAWSII cells with the NT group showed no notable change in cell growth starting at 6h post-treatment (Fig. 21). Also, A1 alone caused a significant increase in cell growth ($p<0.01$) at 48h compared to 6h. Moreover, cells treated with a combination of A1 and MDP significantly increased cell growth ($p<0.001$) at 48h compared to 6h. Cells treated with the combination of A1 and MDP had more growth than the NT group at every time point (Fig.21). Similar effects have been seen in cells treated with 500ng/mL GLA ($p<0.001$), 500ng/mL GLA and 1µg/mL MDP ($p<0.001$) at 48h compared to 6h (Fig.21). These doses did not exhibit any significant cytotoxic effect on JAWSII cells *in vitro*. They thus were used at these concentrations for all subsequent experiments.

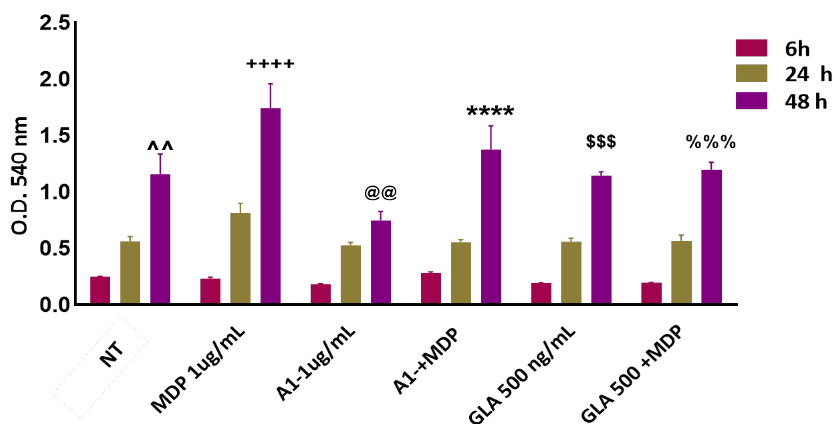

Figure 21 MTT assay of formulation A1 by JAWSII cells
. ^^ p<0.01 compared to NT; ++++p<0.0001 compared to MDP1µg/mL; @@p<0.001 compared to A1-1µg/mL; **** p<0.0001 compared to A1-1µg/mL+ MDP 1µg/mL**;** $$$p<0.001 as compared to GLA 500ng/mL; %%%p<0.0001 compared to GLA 500ng/mL + MDP1µg/mL. Statistical significance was determined by two-way ANOVA with the post Bonferroni test. Results are presented as n=3 and ±SEM.

3.2.4 Synthetic TLR4 agonist (A1) synergistically enhances the secretion of proinflammatory cytokines by JAWSII cells

The potency of A1 and MDP alone or in combination to stimulate the JAWSII cells was examined in vitro. JAWSII cells were seeded at a concentration of $5x10^5$ cells/mL in triplicate in a 24 multi-well tissue culture plate and were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, 500 ng/mL GLA, and combinations. Supernatants were collected after 24 h and analyzed for cytokine secretion.

Incubation of JAWSII cells with A1 and GLA + MDP formulations resulted in significant increases in TNFα ($p\leq 0.001$), IL-6 ($p\leq 0.001$), IL-12p40 ($p\leq 0.01$), and IL-1β ($p\leq 0.001$) secretion compared to NT (Fig. 22). In the presence of MDP, A1, and GLA secreted significant amount of TNFα, ($p\leq 0.001$, $p\leq 0.0001$), IL-6, ($p\leq 0.0001$, $p\leq 0.001$), IL-1β ($p\leq 0.001$, $p\leq 0.001$), and IL-12p40($p\leq 0.001$, $p\leq 0.0001$) secretion compared to MDP respectively. The combination of MDP and A1 enhanced synergistic IL-6 (p≤

0.001) secretion compared to A1 [Fig.22(b)]. Similar synergisms were noted regarding IL-1β ($p\leq 0.05$) after combined treatment with A1 and MDP compared to A1 alone. Although IL-12p40 secretion was increased after MDP was added to A1, the induction was not significant compared to A1 [Fig.22(d)]. Nevertheless, following stimulation with GLA and MDP, JAWSII cells had significantly upregulated IL-12p40 ($p\leq 0.05$) compared to GLA alone [Fig.22(d)]. JAWSII cells did not produce any IL-10 (Fig not shown).

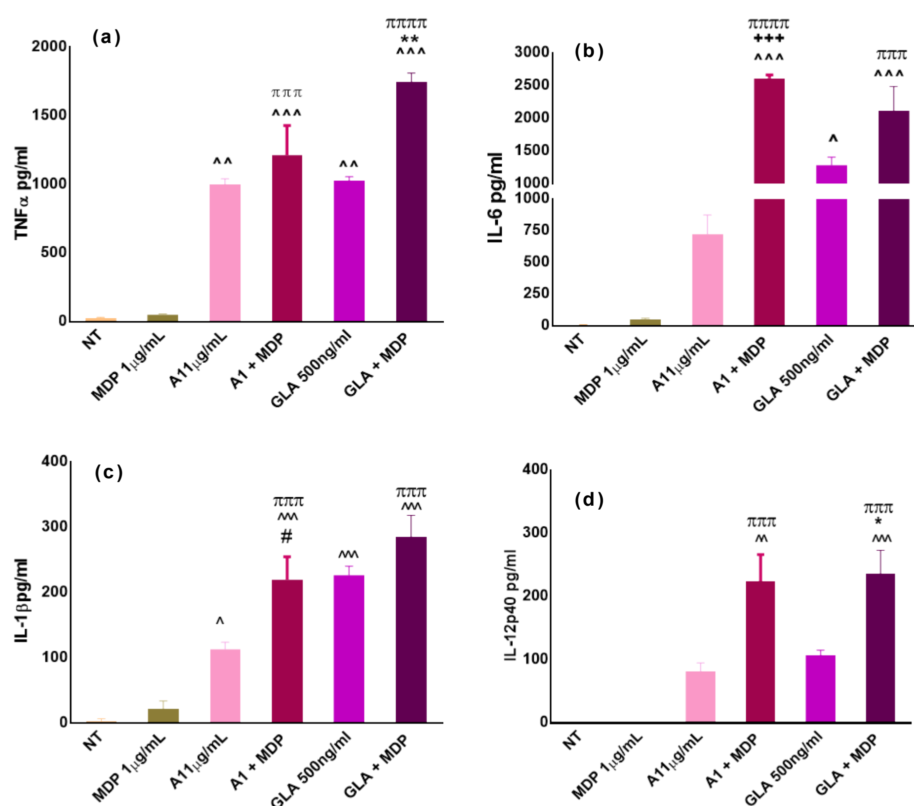

Figure 22 A1 enhanced the secretion of proinflammatory cytokines in response to MDP by JAWSII. **(a) TNFα** ^^ $p<0.01$, ^^^$p<0.001$ compared to NT; πππ$p<0.001$, ππππ $p<0.0001$ compared to MDP 1μg/mL; ** $p<0.01$ as compared to GLA 500ng/mL. **(b) IL-6** ^ $p<0.05$, ^^^$p<0.001$ compared to NT; πππ$p<0.001$, ππππ $p<0.0001$ compared to MDP1μg/mL; +++ $p<0.001$ compared to A1-1μg/mL **(c) IL-1β** ^ $p<0.05$, ^^^$p<0.001$ compared to NT; πππ$p<0.001$ compared to MDP1μg/mL; + $p<0.05$ compared to A1-1μg/mL **(d) IL-12p40** ^^ $p<0.01$, ^^^$p<0.001$ compared to NT; πππ$p<0.001$ compared to MDP1μg/mL; * $p<0.05$ as compared to GLA 500ng/mL. Statistical significance was determined by one-way ANOVA with a Tukey HSD. Results are presented as ±SEM and n=3.

3.3 Coadministration of A1 and MDP, in the presence of vaccine-antigen stimulated proinflammatory cytokines by murine primary cell lines- *ex vivo*

I sought to determine whether TLR signaling mediated the generation of cellular immune responses against the co-application of A1 and MDP to influenza vaccine-antigen(vac.Ag) formulation. The vast majority of proinflammatory cytokines were significantly upregulated in comparison to murine primary cell lines treated with formulations containing individual PRR agonists.

3.3.1 A1, enhanced the potency of immune responses in heterologous splenocytes -*ex vivo*

The trends of synergism between A1 and MDP were also observed in the murine splenocytes. Spleen cells were seeded at a concentration of 1X 10^6 cells/mL in triplicate in 96 multiwell tissue culture plates and were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, 500 ng/mL GLA, and combinations for 24h. Supernatants were collected and analyzed for cytokine secretion. A1 alone was able to induce a moderate level of cytokines from splenocytes. However, coadministration of A1 with MDP resulted in a significant level of TNFα ($p \leq 0.001$), IL-6 ($p \leq 0.05$), and IL-1β ($p<0.01$) compared to NT [Fig.23]. Addition of A1+MDP results in significant levels of TNFα ($p \leq 0.001$) over A1 alone. IL-12p40 was not increased after combined stimulation with MDP and A1. The induction of IL-10 upon stimulation with A1+/- MDP was similar to the non-treated group [Fig.23(d)].

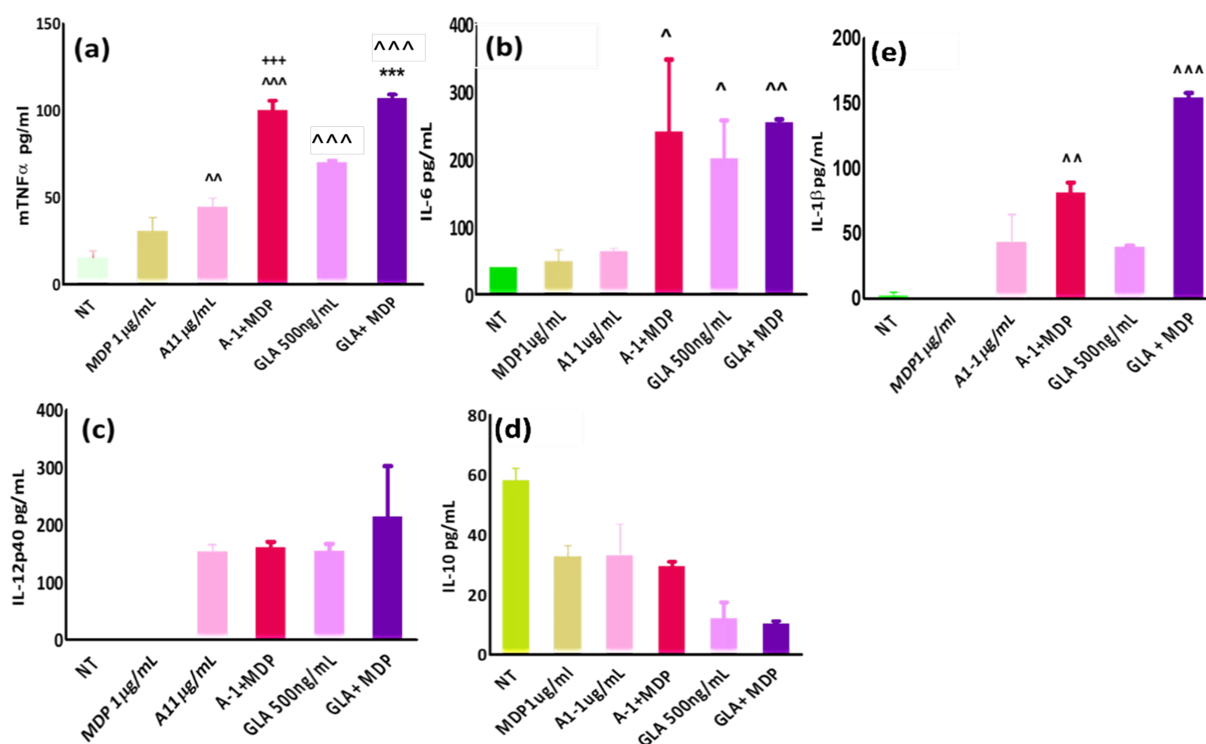

Figure 23 A1 enhanced the secretion of proinflammatory cytokines in association of MDP by murine splenocytes

(a)TNFα ^^ p<0.01,^^^p<0.001 as compared NT, +++ p<0.001 compared to A1-1μg/mL; *** p<0.001 compared to GLA 500 ng/mL (**b) IL-6** ^p<0.05, ^^p<0.01 as compared NT **(c) IL-12p40, (d) IL-10, and. (e) IL-1β** ^^p<0.01, ^^^p<0.001 as compared to NT. Statistical significance was determined by one ANOVA with Tukey HSD. Results are presented as ±SEM and n=3.

3.3.2 Coadministration of A1 and MDP, in presence of vaccine-antigen(vac.Ag) stimulated proinflammatory cytokines by murine BMDCs

BMDCs were seeded at a concentration of 1X 10^6 cells/mL in triplicate in 24 multiwell tissue culture plates and were untreated or treated with either 1μg/mL A1, 1 μg/mL MDP, and 500ng/mL GLA and combinations with 5ug/mL influenza vaccine (vac.Ag) as an antigen for 24 h. Supernatants were collected and analyzed for cytokine secretion.

TNFα - BMDCs stimulation with 500ng/mL GLA + 1μg/mL MDP in formulations resulted in significant increases in TNFα (p≤ 0.01) secretion compared to NT (Fig24). Also, BMDCs stimulation with

only 5µg/mL vac.Ag (p≤ 0.01) resulted in significant increases in TNFα induction compared with A1 +MDP (p≤ 0.001) and GLA + MDP (p≤ 0.001). The synergisms were observed when BMDCs were incubated with A1 +MDP +vac.Ag. formulation (p<0.05) compared to A1+ vac.Ag. Also, the most dramatic effect of the A1 + MDP+ vac.Ag was the significant upregulation in the TNFα (p<0.05) compared to A1 +MDP. GLA+ vac.Ag (p<0.01) was significantly increased compared to GLA only. Similarly, GLA + MDP-mediated TNFα (p<0.0001) secretion was enhanced significantly in the presence of vac.Ag (Fig24).

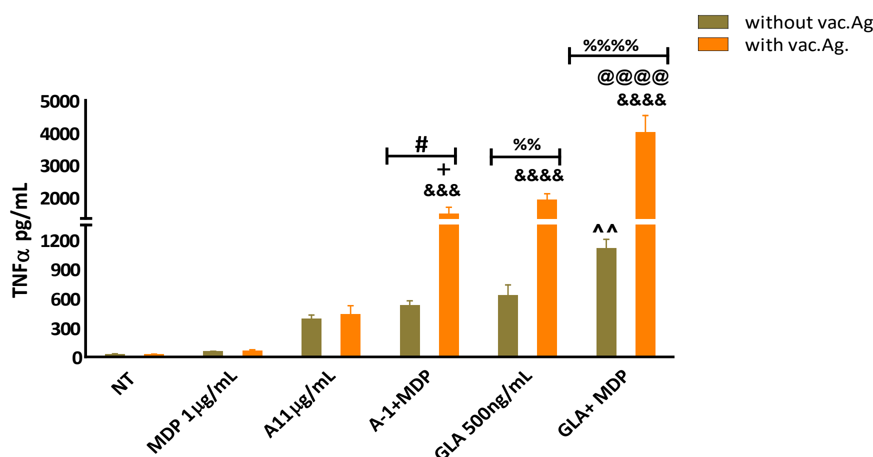

Figure 24 Enhanced TNFα secretion in presence of Influenza vaccines (vac.Ag) by BMDCs.
Before the addition of vac.Ag. (green): ^^p<0.01 compared to NT.
After addition of vac.Ag. 5µg/mL (orange): &&& p<0.001, &&&& p<0.0001compared to NT; %% compared to GLA 500ng/mL+ vac.Ag. and + p<0.05 compared to A1-1µg/mL, # p<0.05 compared to A1-1µg/mL with A1+ vac.Ag; %% p<0.01 compared to GLA 500ng/mL with GLA+ vac.Ag.@@@@p<0.0001 compared to GLA +vac.Ag with GLA+MDP +vac.Ag. and %%%% p<0.0001 compared to GLA + MDP with GLA + MDP + vac.Ag; Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-6 -Activation of BMDCs with 1µg/mL A1 + 1µg/mL MDP (p≤ 0.05) and 500ng/mL GLA (p≤ 0.001), and 500ng/mL GLA + 1µg/mL MDP (p≤ 0.001), resulted in significant levels of IL-6 compared to the NT group. In the presence of 5µg/mL vac.Ag, A1+ MDP(p≤ 0.0001) increased the IL-6 level significantly compared to A1 +MDP; also, A1+MDP with vac.Ag (p<0.001) compared to A1 only, respectively (Fig.25). Vac-Ag significantly increased the levels of IL-6 (p<0.001), (p≤ 0.001),in response to GLA+ MDP, compared to GLA+MDP without vac.Ag and GLA with vac.Ag, respectively (Fig.25).

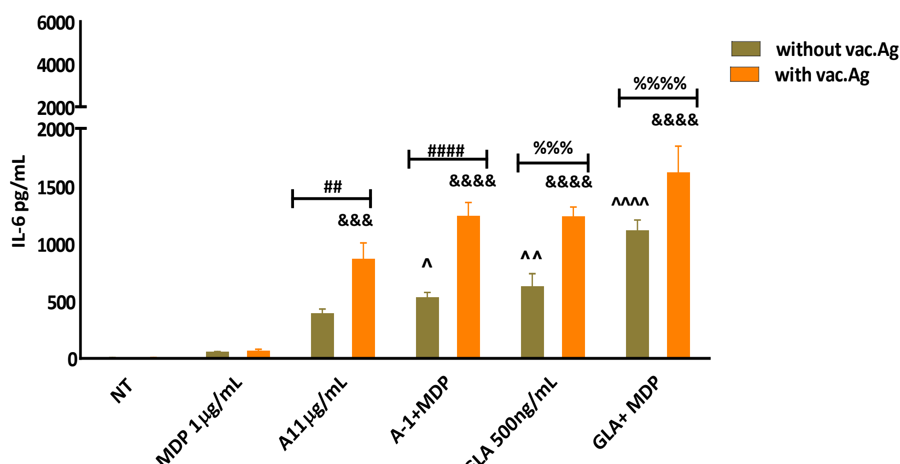

Figure 25 Enhanced IL-6 secretion in presence of Influenza vaccines (vac.Ag) by BMDCs:
Before the addition of vac.Ag. (green): p<0.05, ^^p<0.01, ^^^p<0.001 compared to NT**.**
After addition of vac.Ag. (orange): &&& p<0.001 and &&&& p<0.0001compared to vac.Ag 5µg/mL. #### p<0.0001 compared to A1-1µg/mL+ MDP 1µg/mL with A1-1µg/mL+ MDP 1µg/mL + vac.Ag 5µg/mL; and %%% p<0.001 compared to GLA 500ng/mL with GLA500ng/mL+ vac.Ag 5µg/mL; %%%% p<0.0001 compared to GLA 500ng/mL+ MDP 1µg/mL with GLA500ng/mL+ MDP 1µg/mL+ vac.Ag 5µg/mL; Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-12p40. After incubation of BMDCs with 1µg/mL A1 and 500ng/mL GLA with or without 1µg/mL MDP resulted in significant increases in IL-12p40 secretion compared to NT (p<0.001). There was no synergistic effect observed for IL12p40 secretion, when BMDCs were incubated with A1 + MDP compared to A1 alone. Similarly, with vac.Ag formulation containing A1+MDP showed slightly

increased secretion of IL-12p40 compared to A1+vac.Ag , but it was not significant. However, IL-12p40 secretion was not statistically significant either when BMDCs were treated with vac.Ag. + GLA + MDP(Fig.26).

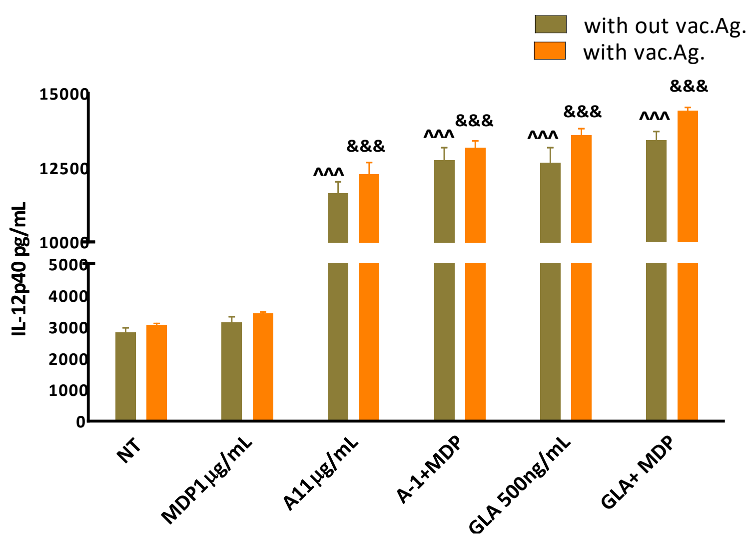

Figure 26 IL-12P40 secretion in presence of Influenza vaccines (vac.Ag) by BMDCs:
Before the addition of vac.Ag. (green): ^^^p<0.001 compared to NT**.**
After addition of vac.Ag . 5µg/mL (orange): &&& p<0.001compared to vac.Ag alone. Statistical significance was determined by two-way ANOVA with a Tukey HSD. Results are presented as ±SEM and n=3.

IL-10- To investigate the adjuvant effect of 1µg/mL A1 or 500ng/mL GLA on the IL-10 response to vac.Ag., we examined how the levels of IL-12p40 relative to IL-10. The addition vac.Ag. did alter this response. There was a significant up-regulation of IL-10 secretion in BMDCs stimulated with 1µg/mL A1 + 1µg/mL MDP (p< 0.001) (Fig.27). A similar increase was seen with 500ng/mL GLA + 1µg/mL MDP (p< 0.001). Surprisingly, BMDCs showed the most significant decreased IL-10 induction (p<0.05 and p<0.0001) after the addition of vac.Ag. to A1 and A1 +MDP compared to A1 and (A1 + MDP), respectively. Similarly, GLA + MDP-mediated IL-10 secretion (p<0.0001) was suppressed significantly in the presence of vac.Ag. (Fig27).

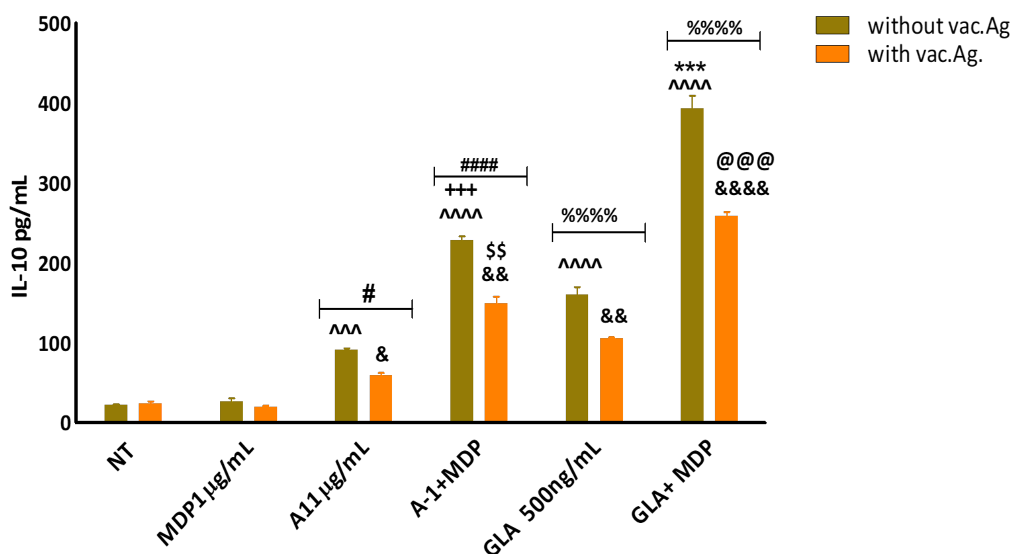

Figure 27 Enhanced IL-10 secretion in presence of Influenza vaccines (vac.Ag) by BMDCs.
Before the addition of vac.Ag. (green): ^^^p<0.001, ^^^^ p<0.0001 compared to NT; +++ p<0.001 compared to A1-1µg/mL; ***p<0.001 compared to GLA 500ng/mL.
After addition of vac.Ag. 5µg/mL(orange): & p<0.05, && p<0.01 &&&& p<0.0001, compared to NT; $$ p<0.01 compared to A1-1µg/mL+ vac.Ag 5µg/mL; @@@ compared to GLA 500ng/mL+ vac.Ag 5µg/mL.
# p<0.05 compared to A1-1µg/mL with A1-1µg/mL + vac. Ag 5µg/mL.
p<0.0001 compared to A1-1µg/mL+ MDP 1µg/mL with A1-1µg/mL + MDP1µg/mL + vac. Ag- µg/mL; %%%% p<0.0001 compared to GLA 500ng/mL with GLA 500ng/mL + vac. Ag5µg/mL and %%%% p<0.0001 compared to GLA 500ng/mL+ MDP 1µg/mL with GLA500ng/mL + MDP 1µg/mL + vac.Ag 5µg/mL; Statistical significance was determine by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-1β -BMDCs stimulation with A1-1µg/mL + MDP1µg/mL(p≤ 0.001), GLA500ng/mL and GLA500ng/mL + MDP1µg/mL (p≤ 0.001, p≤ 0.001) in formulations resulted in significant increases in IL-1β secretion compared to NT. In presence of vac.Ag. activation of BMDCs with A1-1µg/mL + MDP1µg/mL (p≤ 0.05), GLA 500ng/mL (p≤ 0.001) and GLA 500ng/mL MDP1µg/mL p≤ 0.001), resulted in significant levels of IL-1β compared to vac.Ag. BMDCs demonstrated significant up-regulation of IL-1β secretion

(p<0.001) following GLA+ MDP stimulation compared to GLA alone. Stimulation of GLA +MDP(p<0.05) significantly increased IL-1β secretion, with the vaccine formulation (Fig.28).

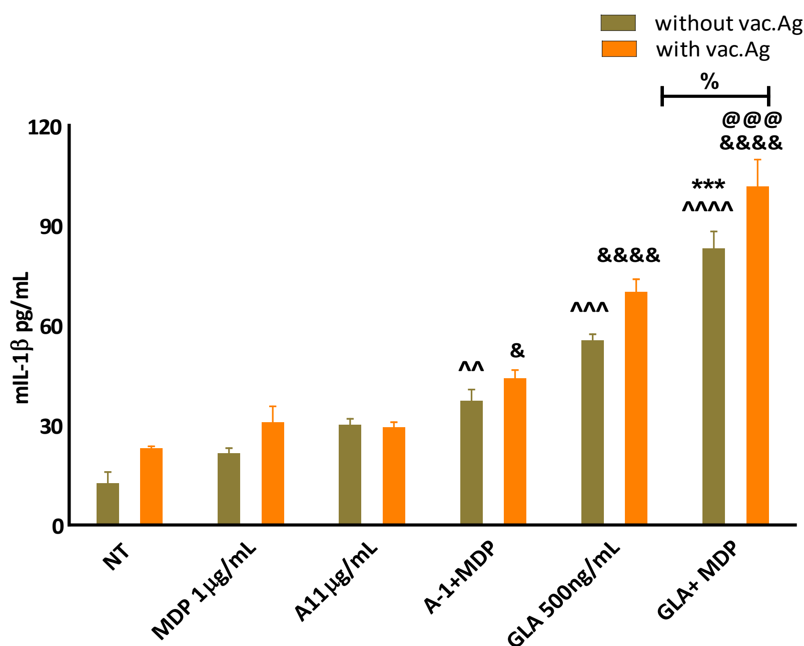

Figure 28 Enhanced IL-1β secretion in presence of Influenza vaccines (vac.Ag) by BMDCs:
Before the addition of vac.Ag. (green): ^^p<0.01, ^^^p<0.001 compared to NT. *** p<0.001 compared to GLA 500ng/mL.
After addition of vac.Ag. 5µg/mL (orange): & p<0.05, &&&& p<0.0001compared to NT; @@@ p<0.001 compared to GLA 500ng/mL.
% p<0.05 compared to GLA + MDP with GLA + MDP + vac.Ag. Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

3.3.3 Influenza vaccine-antigens promote cytokine responses by murine BMMφs.

In light of results from *ex vivo* coadministration of 1µg/mL A1 and 1µg/mL MDP in the presence of vaccine-antigens (vac.Ag), BMMφs exhibited enhanced mediator induction. Murine primary BMMφs were seeded at a concentration of 1X 10^6 cells/mL in triplicate in 24 multiwell tissue culture plates and were untreated or treated with either 1µg/mL A1, 1 µg/mL MDP, and 500ng/mL GLA and

combinations with or without influenza 5µg/mL vac.Ag for 24h. Supernatants were collected and analyzed for cytokine secretion.

TNF α -Activation of BMMϕs with A1-1µg/mL alone or combination of MDP1µg/mL, resulted in significant levels of TNFα ($p<0.05$), $p\leq 0.001$] secretion (Fig.29). Concomitant addition of vac.Ag. to A1 + MDP resulted in significant levels of TNFα ($p\leq 0.01$) secretion. Similarly, GLA + MDP-mediated TNFα ($p<0.01$) secretion was enhanced significantly in presence of vac.Ag (Fig29).

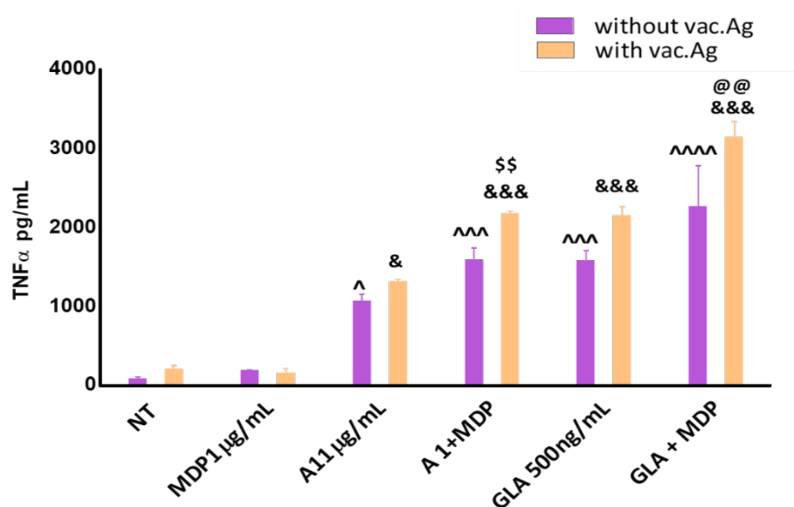

Figure 29. Enhanced TNFα secretion in presence of Influenza vaccines (vac.Ag) by BMMϕs:
Before the addition of vac.Ag. (purple): ^p<0.05, ^^^p<0.001, ^^^^ p<0.0001 compared to NT**.**
After addition of vac.Ag. 5µg/mL (yellow): & p<0.05, &&& p<0.001 compared to NT; $$ p<0.01 compared to A1-1µg/mL + MDP1µg/mL; @@ compared to GLA 500ng/mL+ MDP1µg/mL. Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-6-BMMϕs stimulation with A1-1µg/mL alone or combination of MDP1µg/mL in formulations resulted in significant increases in IL-6 ($p\leq 0.001$, $p\leq 0.0001$) (Fig.30). BMMϕs following vac.Ag to A1+MDP stimulation induced marked IL-6 ($p\leq 0.0001$) against A1 +MDP. The combined effect of GLA + MDP with vac.Ag, were able to significantly increase IL-6($p<0.001$) secretion as well.

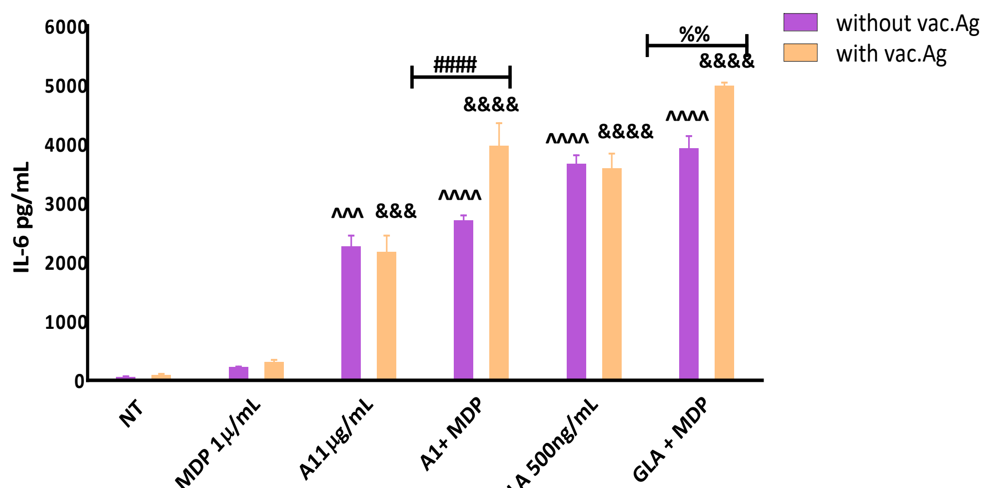

Figure 30 Enhanced IL-6 secretion in presence of Influenza vaccines (vac.Ag) by BMMϕs
Before the addition of vac.Ag. (purple): ^^^p<0.001, ^^^^ p<0.0001, compared to NT**.**
After addition of vac.Ag.5µg/mL (yellow): &&&p<0.001, &&&& p<0.0001compared to NT.
p<0.0001 compared to A1-1µg/mL+ MDP 1µg/mL with A1-1µg + MDP 1µg + vac.Ag5µg/mL, %% p<0.01 compared to GLA 500 ng/mL+ MDP 1µg/mL with GLA 500ng/mL + MDP 1µg + vac.Ag 5µg/mL, Statistical significance was determined by two-way ANOVA followed by Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-12p40-Activation of BMMϕs with A1-1µg/mL alone or combination of MDP1µg/mL, resulted in significant levels of IL-12p40 (p≤ 0.001, p≤ 0.001) (Fig.31) compared to NT group. Up on stimulation with A1 alone or A1 +MDP with or without vac.Ag, BMMϕs secreted enhanced levels of IL-12p40, but not statistically significant compared to single agonist. However, induction of GLA + MDP with vac.Ag. was not statistically significant either (Fig.31).

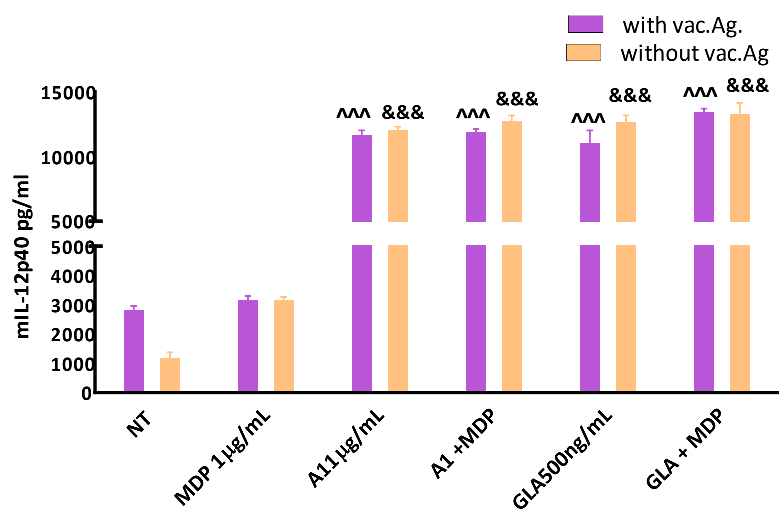

Figure 31 Enhanced IL-12p40 secretion in presence of Influenza vaccines (vac.Ag) by BMMϕs
Before the addition of vac.Ag. (purple): ^^^p<0.001compared to NT**.**
After addition of vac.Ag. 5µg/mL (yellow): &&& p<0.001 compared to NT. Statistical significance determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-10 -Upregulations of IL-10 were potentiated after the stimulation of A1 (p<0.01), A1 + MDP(p<0.001) or GLA (p<0.01) and GLA + MDP (p<0.001) relative to NT (Fig.32). The most notable effects of IL-10 were detected after addition to vac.Ag. BMMϕs showed the most significant decreased in IL-10 induction (p<0.001) after the addition of vac.Ag to A1 +MDP compared to A1 + MDP. Whereas GLA + MDP-mediated IL-10 secretion was suppressed, but not significant (Fig.32) The reduction in IL-10 resulted in significantly higher ratios of IL-12p40:IL-10 in A1+MDP/vac.Ag treated BMMϕs.

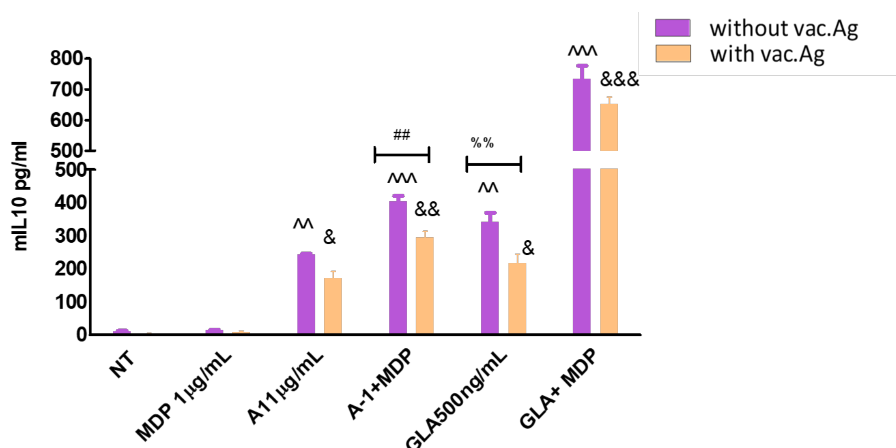

Figure 32 Enhanced IL-10 secretion in presence of Influenza vaccines (vac.Ag) by BMMϕs
Before the addition of vac.Ag. (purple): ^^p<0.01, ^^^p<0.001 compared to NT.
After addition of vac.Ag. 5µg/mL (yellow): &p<0.05,&&p<0.01and &&&p<0.001 compared to NT, ## p<0.01 compared to A1-1µg/mL+ MDP 1µg/mL with A1+ MDP + vac.Ag; %%p<0.01 compared to GLA 500ng/mL with GLA + vac.Ag. Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

IL-1β BMMϕs stimulation with A1-1µg/mL (p≤ 0.05) and A1-1µg/mL + MDP1µg/mL (p≤ 0.01), GLA500ng/mL (p≤ 0.01) and GLA500ng/mL + MDP1µg/mL (p≤ 0.0001) in formulations resulted in significant increases in IL-1β secretion compared to NT. Also, BMMϕs stimulated with A1 and A1+MDP in presence of vac.Ag induced marked IL-1β (p<0.001,p≤ 0.01)againstA1 and A1 +MDP correspondingly. The combined effect of GLA + MDP with vac.Ag, were able to significantly increase IL-1β (p<0.00001) compared to GLA + vac.Ag. as well (Fig.33).

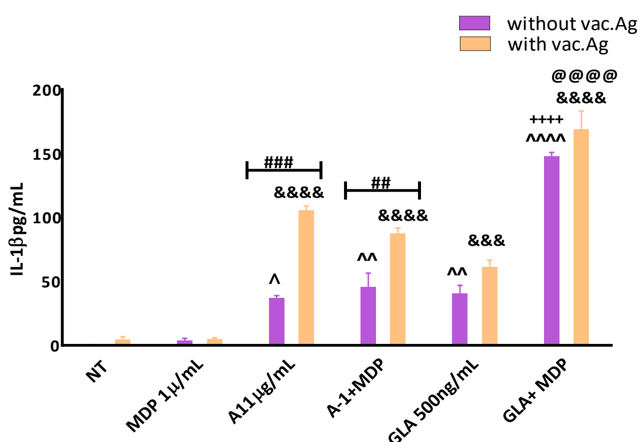

Figure 33 Enhanced IL-1β secretion in presence of Influenza vaccines (vac.Ag) by BMMϕs
Before the addition of vac.Ag. (purple): ^p<0.05,^^p<0.01, ^^^^ p<0.0001compared to NT; ++++ p<0.0001 compared to GLA 500ng/mL.
After addition of vac.Ag. 5μg/mL(yellow): &&& p<0.001, &&&& p<0.0001, compared to NT; @@@@p<0.0001 compared to GLA 500ng/mL.
p<0.001 compared to A1-1μg/mL with A1-1μg/mL + vac. Ag5μg/mL; ## p<0.01 compared to A1-1μg/mL+ MDP 1μg/mL with A1-1μg/mL + MDP 1μg/mL + vac.Ag 5μg/mL. Statistical significance was determined by two-way ANOVA followed by the Bonferroni posttest. Results are presented as ±SEM and n=3.

It was important to compare the cytokine profiles of BMMϕs with BMDCs in the induction of a Th1 polarizing pattern. Addition of the Fluzone vaccine-antigen formulation containing both TLR4 and NOD2 agonists in both murine primary cells lead to enhanced cytokine secretion in comparison to vaccine formulations with individual PRR agonists. The overall pattern and quantity as well of both cell lines in terms of the induction pro and anti-inflammatory cytokines were very similar, which were our main concern.

3.3.4 TLR4 agonist-induced enhancement of the MDP responses to cell signaling

It was of interest to assess the effects of 1µg/mL MDP in 1µg/mL A1-induced inflammatory responses on immortalized JAWSII DCs and primary murine bone marrow-derived dendritic cells (BMDCs) in order to see if it resulted in phenotype similar to the cytokine secretion. JAWSII and BMDCs were seeded at a concentration of 0 .5x10^5 cells/mL in triplicate in 24 multiwell tissue culture plates and were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, 500 ng/mL GLA or combinations. Pellets were collected after 30 minutes. To observe the inhibitory effect of TLR4 receptor activation, JAWSII cells were incubated with TLR4 inhibitor CLI-095 for 6h before the addition of any treatment. The activation of MAPK family member phosphorylated ERK1/2 (pERK) was examined by western blot analysis.

ERK1/2 with "potentiated" activities were measured qualitatively as the induction of phosphorylation level after simultaneous stimulation of TLR4 and NOD2 receptors relative to greater than cells treated with single receptor. The levels of pERK were not observed in BMDCs in MDP stimulated group. It appeared that JAWSII DCs [Fig.34-slot 3,4,5, and 6] and BMDCs [Fig.35-slot 3,4,5, and 6], both showed a slightly enhanced level of pERK after the simultaneous stimulation with A1 and MDP compared to NT. From the Fig 36, a decreased level of ERK phosphorylation was observed, after JAWSII DCs was treated with CL095, TLR4 inhibitor, suggesting that the TLR4 pathway is important for A1 mediated downstream signaling. Thus, ERK1/2 showed slightly increased phosphorylation with combined stimulation of both PRRs, but it is unclear if MDP had a synergistic effect.

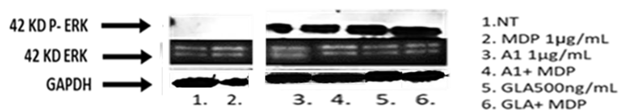

Figure 34 Western blots of TLR4 agonist, A1 regulation of ERK phosphorylation
Western blot analysis to measure the phosphorylated and total ERK levels in Immortalized murine dendritic cells JAWSII. Phosphorylated ERK and total ERK were shown for each condition. One representative experiment out of two was performed. The GAPDH served as a control.

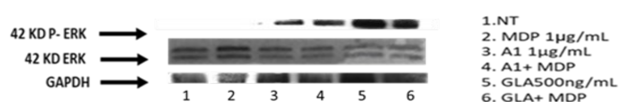

Figure 35 Western blots of TLR4 agonist, A1 regulation of ERK phosphorylation in BMDCs.
Western blot analysis to measure the phosphorylated and total ERK levels in murine primary BMDCs phosphorylated ERK and total ERK were shown for each condition. The GAPDH served as a control. Protein bands are presented from one experiment.

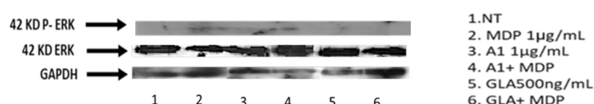

Figure 36 TLR4 inhibitor, CLI-095 inhibits the protein expression levels of ERK in JAWSII cells.
JAWSII cells were seeded at a concentration of $5x10^5$ cells/mL in triplicate in 24 multiwell tissue culture plates and incubated with TLR4 inhibitor CLI-095 for 6h before treatment with or without either A1-1μg/mL, MDP 1μg/mL, GLA 500ng/mL or combinations. Pellets were collected after 30 minutes of treatments. Phosphorylated ERK and total ERK were shown for each condition. The GAPDH served as a control. Protein bands are presented from one experiment.

3.4 Evaluation of A1-formulation with vaccine-antigen for antigen-specific immune responses in human PBMCs

Having first evaluated immune responses on the murine primary cell line, I sought to determine cytokine induction towards the A1-vac.Ag—formulation in human PBMCs. First, we evaluated the ability of TLR4 agonist (A1) and NOD2 agonist (MDP) to stimulate primary human PBMCs for the secretion of pro- as well as anti-inflammatory cytokines at 72h time point without any antigen. In order to determine whether simultaneous, consecutive application of A1 and MDP to vaccine-antigen (vac.Ag) were effective in cell-mediated immunity (CMI), we challenged PBMCs with influenza virus A/Victoria/3/75. Prior to the antigen (Ag) challenge, we determined the cytokine secretion from unchallenged PBMCs. Later, we compared the relative cytokine secretion from the antigen (Ag) challenged the group to that of unchallenged PBMCs. PBMCs seeded at a concentration of 1X 10^6 cells/mL in triplicate in 24 multiwell tissue culture plates and were untreated or treated with either 1µg/mL A1, 1 µg/mL MDP, or 500ng/mL GLA alone or in combinations and with/without flu vaccine at 5ug/mL. Supernatants were collected after 7 days, and cells were re-stimulated with the influenza virus at 2:1 MOIfor 20 h as an Ag challenge.

3.4.1 A1 and MDP enhanced the cytokine secretion in PBMCs at 72 h

Human PBMCs were seeded at a concentration of 1 x10^6 cells/mL/well in 24 well plates and left untreated or treated with either 1 µg/mL A1 and 1µg/ml MDP. Supernatants were collected from the cells after 72 hours of incubation and quantified for cytokine levels by ELISA.

A1 alone was able to stimulate significant IFNγ ($p \leq 0.05$), IL-6 ($p \leq 0.001$), IL-1β ($p \leq 0.001$), and IL-12p40 ($p \leq 0.05$) responses from PBMCs. Treatment with MDP alone resulted significant amount of

IFNγ ($p \leq 0.05$, $p \leq 0.01$), IL-6 ($p \leq 0.001$), IL-1β ($p \leq 0.001$), and IL-12p40 ($p \leq 0.001$) at 72h time point. After combined application of A1 and MDP, the significant proinflammatory cytokine secretion was observed (Fig.37). Coadministration of A1 and MDP was significant in producing IFNγ ($p \leq 0.05$), IL-6 ($p \leq 0.01$), IL-1β ($p \leq 0.001$), and IL-12p40 ($p \leq 0.05$). A1 + MDP produced slightly elevated level of IL-10 compared to A1 alone, but it was not synergistic. GLA induced PBMCs showed similar responses in case of proinflammatory cytokine secretion. Costimulation with GLA and MDP was able to secret significant amount of IL-10 ($p \leq 0.01$).

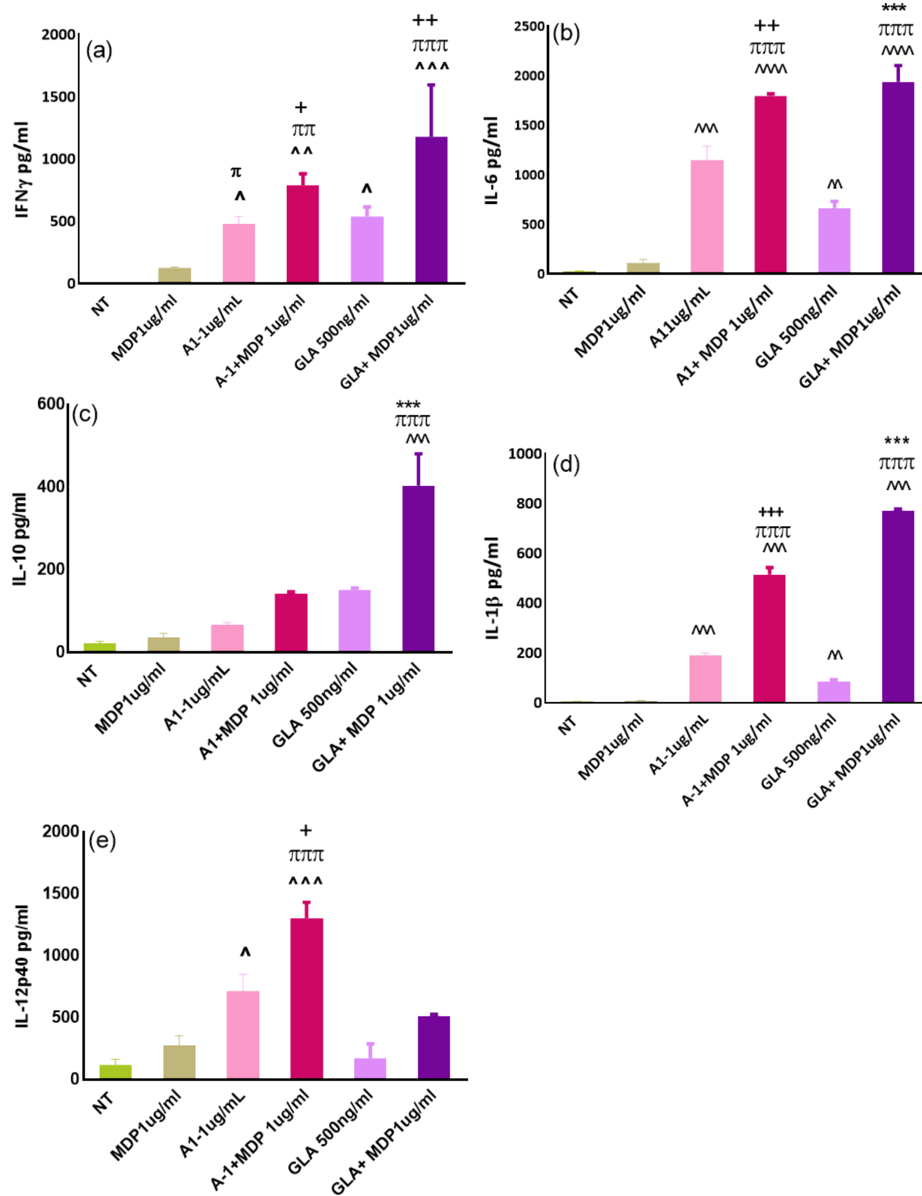

Figure 37 A1 with MDP stimulated the secretion of cytokines in human PBMCs.
Levels of **(a) IFNγ, (b) IL-6, (c) IL-10, (d) IL-1β(d) and (e) IL-12p40** produced by PBMCs. ^ p<0.05, ^^p<0.01, ^^^p<0.001 compared to NT; + p<0.05, +++ p<0.001 compared to A1-1μg/mL, ππp<0.01, πππp<0.001 compared to MDP1μg/mL *** p<0.001 as compared to GLA 500ng/mL treatment group; Results are presented as ±SEM and n=3. Statistical significance was determined by one ANOVA with a Tukey HSD.

3.4.2 A1-adjuvant formulation was potent stimulator for IFNγ secretion in human PBMCs antigen challenge - *ex vivo*

In the case of the prior antigen challenge, incubation of PBMCs with 1µg/mL A1 and 500ng/mL GLA with or without 1µg/mL MDP formulations resulted in significant increases in IFNγ secretion compared to NT ($p<0.001$). The synergistic effects were seen when PBMCs were incubated with both agonists A1 + MDP compared to A1 alone ($p<0.001$) [Fig38(a)]. Also, IFNγ induction following 5µg/mL vac.Ag. with A1 and GLA +/- MDP stimulation was significant compared to only vac.Ag. treated PBMCs ($p<0.001$). The vac.Ag. containing A1+MDP showed a synergism compared to A1 + vac.Ag. ($p<0.05$) [Fig38(a)]. The vac.Ag with GLA + MDP was also significantly increased ($p<0.01$).[Fig38(a)]. Addition of vac.Ag to A1 + MDP significantly increased the levels of IFNγ ($p<0.0001$), and similar results were seen with GLA + MDP ($p<0.0001$) in response to vac.Ag. [Fig. 38 (b)]. The data for Fig38(a) were the same as for Fig.38(b). We have decided to present the results in two different manners to simplify the graph. In Fig.38(a), the comparisons were not made between the values of with and without vac.Ag. group, which was done in Fig.38(b). The same explanation would be applied to [Fig.38(c), (d)] and Fig.39-42 as well.

After the influenza virus challenge, 1µg/mL A1 and 500ng/mL GLA alone or + 1µg/mL MDP significantly enhanced the IFNγ secretion [Fig.38 (c)]. Addition of vac.Ag to A1 + MDP significantly increased the levels of IFNγ ($p<0.0001$), compared to A1 with MDP only in the presence of influenza virus; similar with GLA + MDP in the presence of influenza virus, $p<0.0001$[Fig.38 (d)].

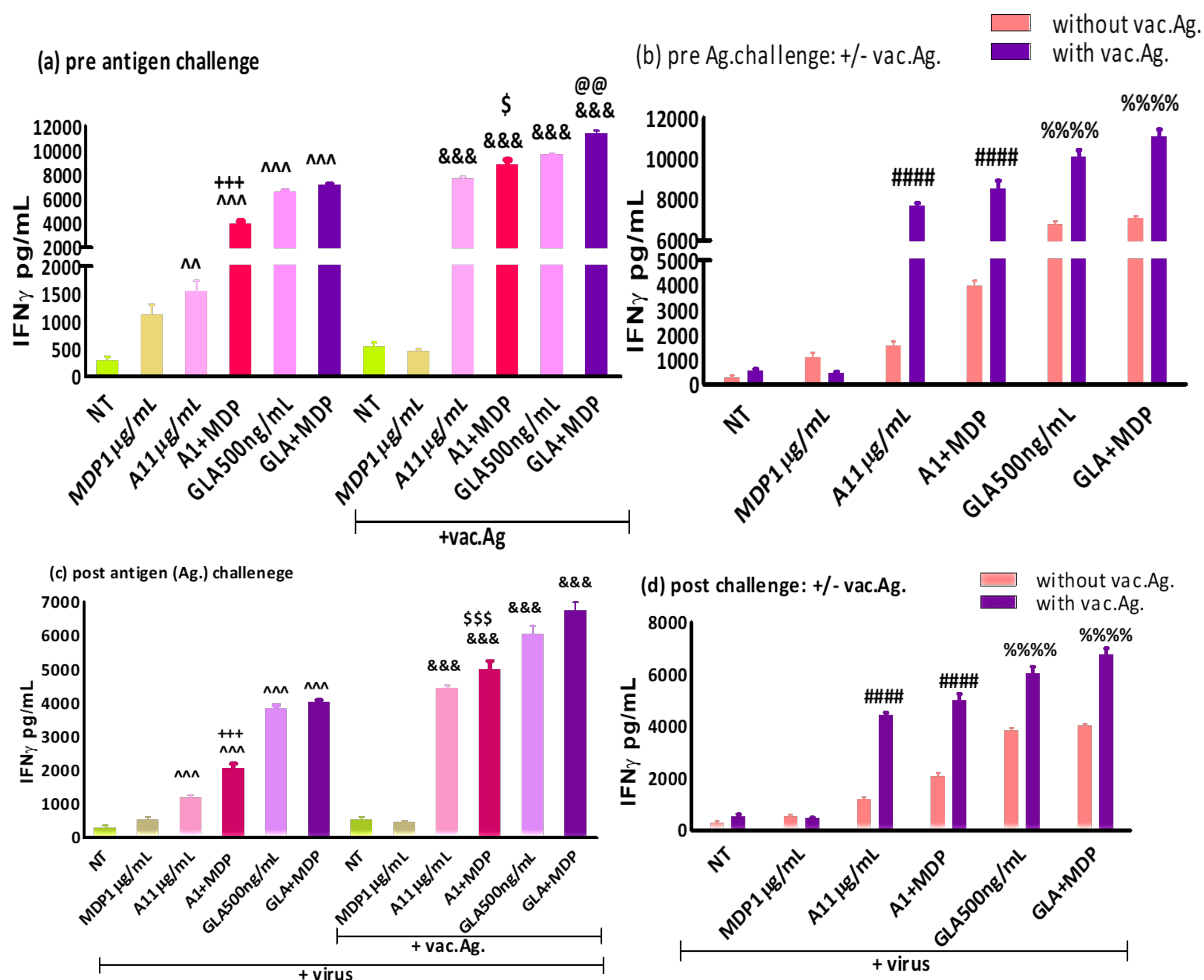

Figure 38 Influenza vaccine (vac.Ag) and +/- virus stimulated enhanced IFNγ secretion by PBMCs (a) pre antigen challenge (Ag.):.IFNγ secretion ^^ p<0.01, ^^p<0.01as compared to NT; +++ p<0.01 compared to A1-1μg/mL, **In presence of vac.Ag 5μg/mL**: &&& p<0.001 compared to NT; $ p<0.05 compared to A1-1μg/mL; @@p<0.01 as compared to GLA 500ng/mL. Statistical significance was determined by one way ANOVA with a Tukey HSD**. (b) pre antigen challenge- +/- vac.Ag**.: #### p<0.0001as compared to A1-1μg/mL and A1-1μg/mL+ MDP 1μg/mL; %%%% p<0.0001 as compared to GLA 500ng/mL and GLA 500ng/mL + MDP1μg/mL. Statistical significance was determined by two-way ANOVA with the Bonferroni posttest. **(c) post Ag. challenge:** IFNγ secretion ^^^p<0.01 compared to NT; +++ p<0.01 as compared to A1-1μg/mL. In presence of vaccine-antigen5μg/mL - &&& p<0.001 compared to vac.Ag alone; $$$ p<0.05 compared to A1-1μg/mL; Statistical significance was determined by one-way ANOVA with a Tukey HSD**. (d) post Ag challenge +/- vac.Ag 5μg/mL:** IFNγ secretion, #### p<0.0001as compared to A1-1μg/mL and A1-1μg/mL+ MDP1μg/mL; %%%% p<0.0001 as compared to GLA 500ng/mL and GLA 500ng/mL + MDP1μg/mL. Statistical significance was determined by two-way ANOVA with the Bonferroni posttest Results are presented as ±SEM and n=3.

3.4.3 A1-adjuvant formulation was a potent stimulator for IL-6 secretion in human PBMCs antigen challenge- *ex vivo*

Prior to antigen challenge, secretion of IL-6 from PBMCs treated with 1µg/mL A1 (p<0.01), 1µg/mL A1 + 1µg/mL MDP (p<0.001), 500ng/mL GLA (p<0.01), and GLA + MDP (p<0.001) formulations resulted in a significant increase compared to NT. Combined stimulation with 1µg/mL A1 + 1µg/mL MDP (p<.01) or GLA + MDP (p<0.001) resulted in significantly higher levels of IL-6 from PBMCs compare to a single agonist. Also, following 5µg/mL vac.Ag stimulation, PBMCs had significantly higher secretion of IL-6 1µg/mL A1 (p<0.01), A1 + MDP (p<0.001) and 500ng/mL GLA + MDP (p<0.001) compared to the NT group (vac.Ag). However, the conjugate A1-MDP-vac. Ag formulation produced increased levels of IL-6 from the induced PBMCs compared to A1+ MDP only, but the secretion level was not significant. GLA+MDP+ vac.Ag formulation also increased IL-6 secretion in an equivalent manner [Fig.39(b)].

After viral challenge using the Influenza A Victoria virus (IAV), incubation of PBMCs with 1µg/mL A1 + 1µg/mL MDP (p<0.01), 500ng/mL GLA (p<0.01) and 500ng/mL GLA + 1µg/mL MDP (p<0.001) formulations resulted in significant increases in IL-6 secretion compared to the NT group. A1 + MDP also increased the IL-6 level significantly related to 1µg/mL A1 (p<0.01) alone [Fig39(c)] Vac.Ag with A1 and A1+ MDP significantly increased the levels of IL-6 (p<0.05) in response to the influenza virus, compared to the treatment group without vac.Ag. [Fig39(d)].

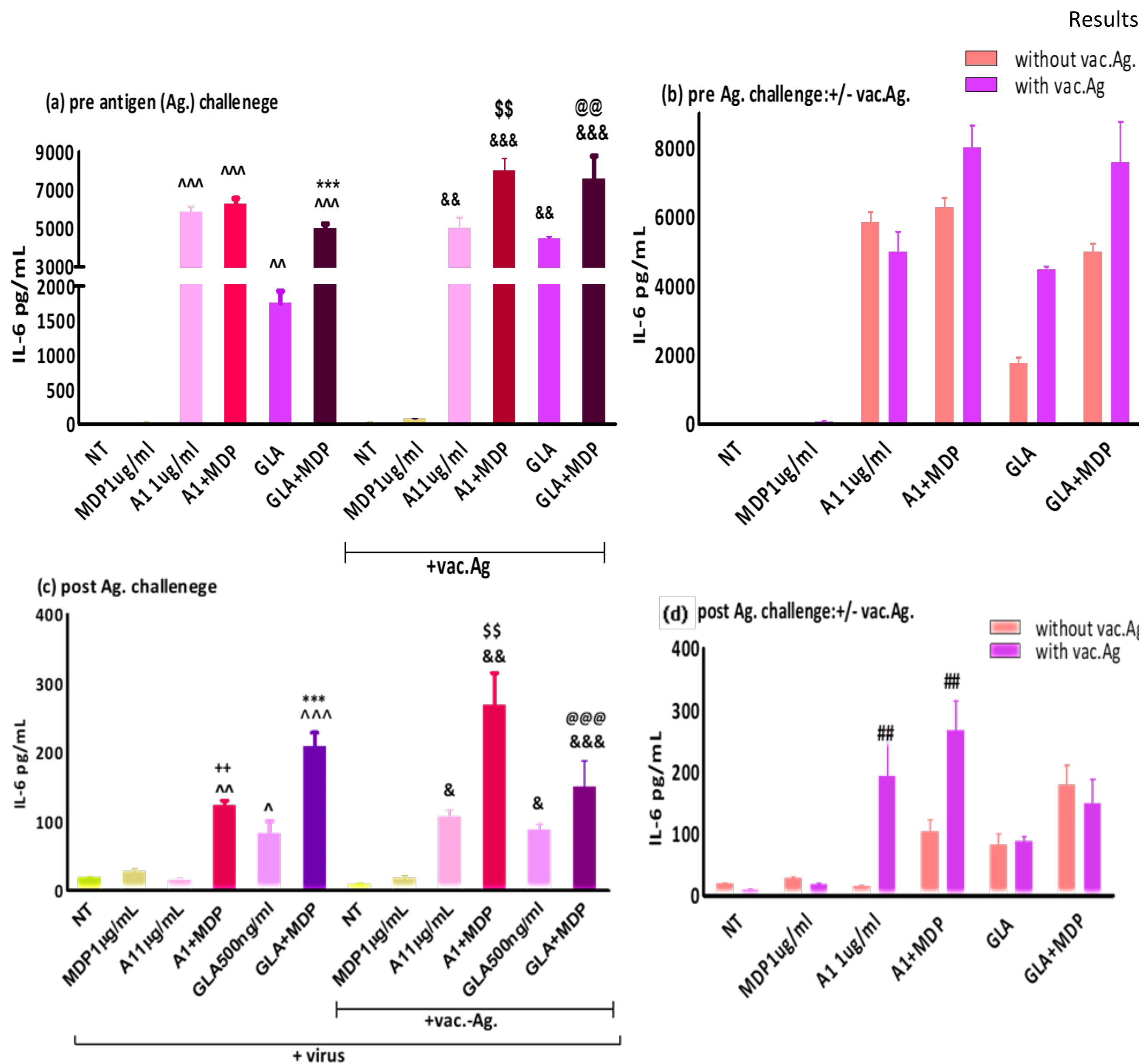

Figure 39 Influenza vaccine (vac.Ag) and +/- virus stimulated enhanced IL-6 secretion by PBMCs
(a) pre-challenge: IL-6 secretion ^^ p<0.01, ^^^p<0.001 compared to NT; *** p<0.01 as compared to GLA 500 ng/mL, In presence of vaccine-antigen 5µg/mL - && p<0.01 , &&& p<0.001 compared to NT(vac.Ag); $$ p<0.01 compared to A1-1µg/mL; @@ p<0.01 as compared to GLA 500ng/mL Statistical significance was determined by one-way ANOVA with a Tukey HSD (**b) pre Ag. Challenge with/without vac.Ag**: IL-6 secretion was not statistically significant. Statistical significance was determined by two-way ANOVA with the Bonferroni posttest **(c)post Ag. Challenge**: IL-6 secretion ^p<0.05, ^^p<0.01, ^^^p<0.001 compared to NT; ++ p<0.01 as compared to A1-1µg/mL. In presence of vaccine-antigen 5µg/mL & p<0.05, && p<0.01, &&& p<0.001 compared to NT(vac.Ag); $$ p<0.01 compared to A1-1µg/mL, @@@ p<0.001 as compared to GLA 500ng/mL**;(d)) post antigen challenge with/without vac.Ag5µg/mL**; ##p<0.01 compared to A1-1µg/mL and A1-1µg/mL+ MDP 1µg/mL; Results are presented as ±SEM and n=3

3.4.4 A1-adjuvant formulation was a potent suppressor for IL-10 secretion in the presence of vaccine antigen in human PBMCs ex vivo antigen challenge

To investigate the adjuvant effect of A1 or GLA on the IL-10 response to vac.Ag., we examined how the levels of IFN-γ relative to IL-10 were altered in the response to influenza virus; the addition of 1µg/mL A1 to vac.Ag. did not alter this response. There was only significant up-regulation of IL-10 secretion in PBMCs stimulated with 1µg/mL A1 + 1µg/mL MDP ($p<0.01$), compared with 1µg/mL A1 alone. The stimulation with 500ng/mL GLA + 1µg/mL MDP ($p< 0.05$) was significantly increased compared to 500ng/mL GLA [Fig.40(a)]). Surprisingly, the most dramatic effects of the 1µg/mL A1 +/- 1µg/mL MDP was the significant reduction in the IL-10($p<0.0001$) response after the addition of 5µg/mL vac.Ag. Similarly, 500 ng/mL GLA + 1µg/mL MDP mediated IL-10 secretion ($p<0.0001$) was suppressed significantly in the presence of 5µg/mL vac.Ag prior to antigen challenge. PBMCs that had been stimulated with 1µg/mL A1 / 500ng/mL GLA-mediated vaccine formulation, prior to virus challenge, followed by the addition of influenza virus resulted in a markedly reduction in IL-10 levels [Fig.40(b)]

After viral challenge using the Influenza A Victoria virus, there was no significant changes in IL-10 secretion relative to NT group and 5µg/mL. vac.Ag alone. There were no significant differences in the basal level of IL-10 secretion from PBMCS when compared to all groups [Fig.40(d)].

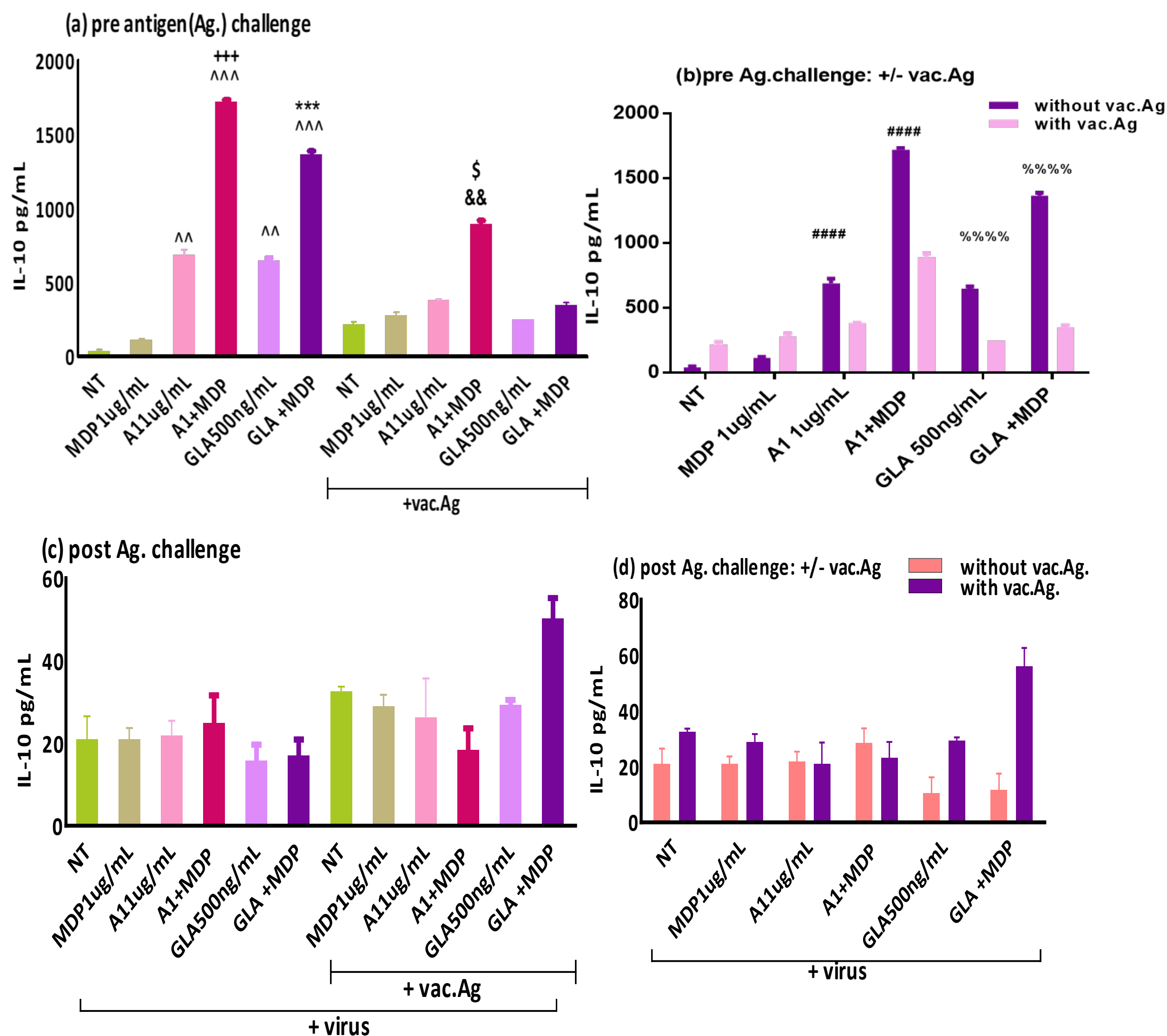

Figure 40 Influenza vaccine (vac.Ag) and +/- virus stimulated suppressed IL-10 secretion by PBMCs (a)pre Ag.challenge:IL-10 secretion ^^ $p<0.01$, ^^^$p<0.001$ compared to NT; +++ $p<0.001$ as compared to A1-1µg/mL; *** $p<0.01$ as compared to GLA 500ng/mL In presence of vaccine-antigen 5µg/mL - && $p<0.01$,&&& $p<0.001$ compared to NT(vac.Ag);$ $p<0.05$ compared to A1-1µg/mL Statistical significance was determined by one ANOVA with a Tukey HSD **(b)pre Ag. challenge-with/without vac.Ag.5µg/mL**: IL-10 secretion #### $p<0.0001$ compared to A1-1µg/mL with A1-1µg/mL+ vac.Ag.; #### $p<0.0001$ compared to A1-1µg/mL+ MDP 1µg/mL with A1-1µg/mL+ MDP 1µg/mL+ vac.Ag; %%%% $p<0.0001$ as compared to GLA 500ng/mL + MDP1µg/mL with GLA 500ng/ml + MDP1µg/mL+ vac.Ag. Statistical. significance was determined by two-way ANOVA with the Bonferroni posttest **(c) post Ag.challenge:** no significant IL-10 secretion **(d) post Ag.challenge cells -with/without vac.Ag.5µg/mL:** No significant secretion. Results are presented as ±SEM and n=3.

3.4.5 A1-adjuvant formulation was a potent stimulator for IL-1β secretion in human PBMCs - ex vivo antigen challenge

IL-1β secretion from PBMCs 1µg/mL A1 + 1µg/mL MDP ($p<0.001$) was significantly increased compared to the NT group. Also, following 5µg/mL vac.Ag stimulation, PBMCs had significantly higher secretion of IL-1, A1 + MDP ($p<0.01$) and 500ng/mL GLA + MDP ($p<0.05$) compared to NT (vac.Ag). There was only significant up-regulation of IL-1β secretion following 1µg/mL A1 + 1µg/mL MDP stimulation compared to 1µg/mL A1 alone in the presence of 5µg/mL vac.Ag [Fig.41(a)]. PBMCs that had been stimulated with 1µg/mL A1 and 1µg/mL MDP-mediated vaccine formulation resulted in significant increases in IL-1 β secretion compared to without vac.Ag group [Fig.41(b)]

After viral challenge, combined stimulation of Influenza A Victoria virus with 1µg/mL A1 + 1µg/mL MDP ($p<.001$) and 500ng/mL GLA + 1µg/mL MDP ($p<0.05$)resulted in significantly higher level of IL-1β compared to single agonists [Fig.42(c)]. Also, after the addition of vac.Ag,

incubation of PBMCs with 1µg/mL A1 + 1µg/mL MDP ($p<0.001$) and 500 ng/mL GLA + 1µg/mL MDP ($p<.001$) led to a pronounced increase in the secretion of IL-1β compared to a single agonist [Fig.41(c)]. Vac.Ag with A1 and A1+ MDP significantly increased the levels of IL-1β ($p<0.0001$) in response to influenza virus [Fig41(d)].

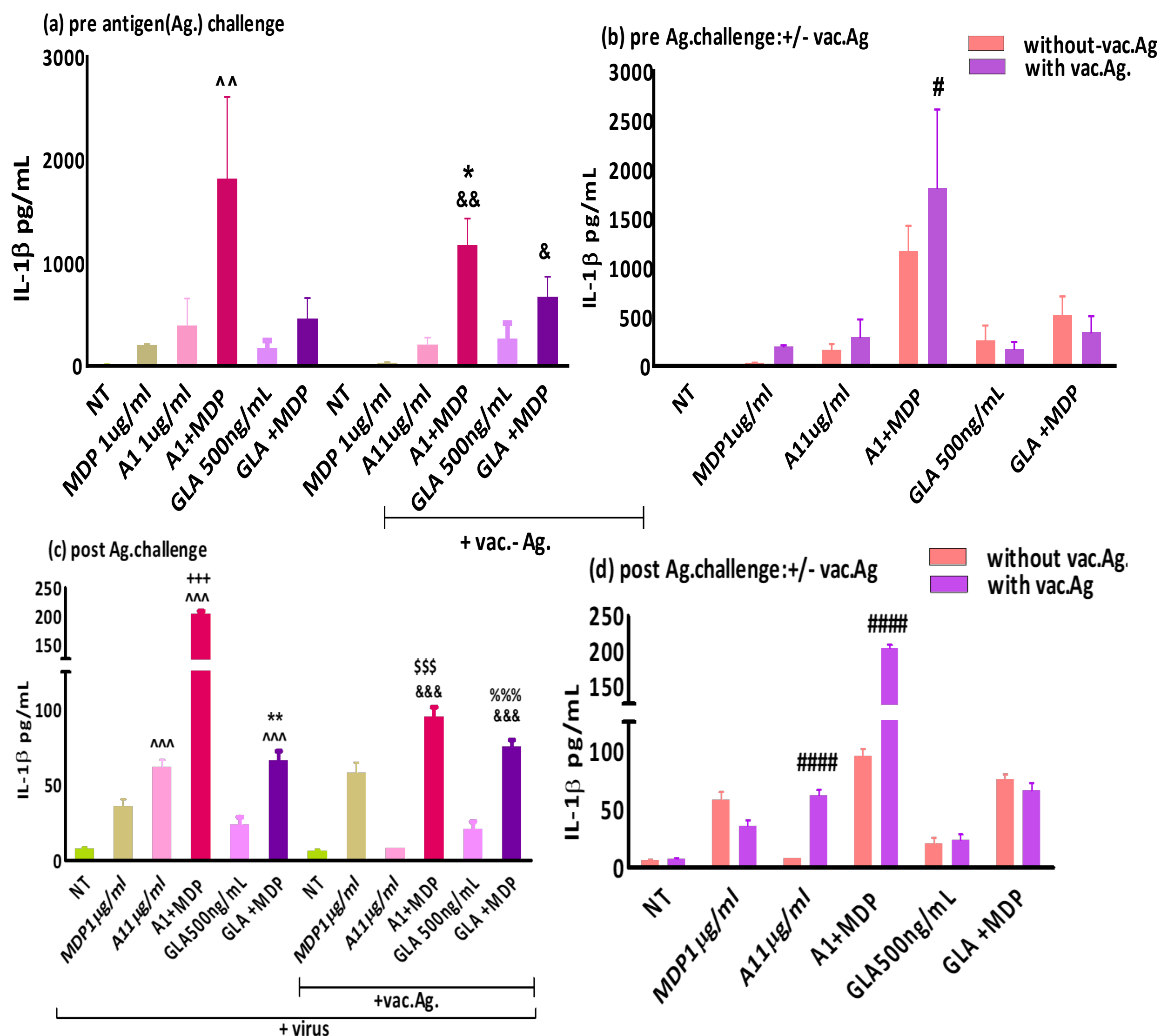

Figure 41 Influenza vaccines and + virus enhanced the secretion of IL-1β by PBMCs
(a) pre-challenge IL-1β secretion ^^p<0.01 compared to NT, in presence of vac.Ag 5ug/mL &p<0.05,&& p<0.001 compared to NT(vac.Ag.);*p<0.05 compared to A1-1µg/mL. Statistical significance was determined by a one-way ANOVA with Tukey's Post Hoc Test.**(b)pre-Ag. challenge-with/without vac.Ag:**IL-1 β secretion # p<0.05as compared to A1-1µg/mL+ MDP 1µg/mL with A1-1µg/mL+ MDP 1µg/mL + vac.Ag. Statistical significance was determined by two-way ANOVA with the Bonferroni posttest **(c) post Ag. challenge:** IL-1β secretion ^^^p<0.01 compared to NT; +++ p<0.01 as compared to A1-1µg/mL.; ** p<0.01 as compared to GLA 500ng/mL. In presence of vac.Ag 5µg/mL. &&& p<0.001 compared to NT (vac.Ag); $$$ p<0.001 compared to A1-1µg/mL.; @@@ p<0.001 compared to GLA 500ng/mL.**(d) post Ag. challenge-with /without vac.Ag:** ####p<0.0001 compared to A1-1µg/mL and A1-1µg/mL + MDP 1µg/mL Results are presented as ±SEM and n=3.

3.4.6 A1 adjuvant formulation was potent stimulator for IL-17 secretion in human PBMCs - antigen challenge- ex vivo

Treatment of human PBMCs with formulations

Treatment of human PBMCs with formulations having PRR agonists, 1µg/mL A1 + 1µg/mL MDP ($p<0.001$), 500ng/mL GLA ($p<0.01$), and 500ng/mL GLA + 1µg/mL MDP ($p<0.001$) resulted in an increased expression of IL-17 compared to NT cells. There was only significant up-regulation of IL-17 secretion in PBMCs stimulated with 1µg/mL A1 + 1µg/mL MDP ($p<0.01$), compared with 1µg/mL A1 alone. [Fig.42(b)].

Whereas, after the Influenza A virus (IAV) challenge, vac.Ag. formulation containing A1 ($p<0.05$), A1 + MDP ($p<0.01$), GLA ($p<0.01$) and GLA + MDP ($p<0.001$) resulted secretion of IL-17 synergistically compared to NT (vac. Ag). Also, A1 + MDP, increased the IL-17($p<0.05$) level significantly related to A1 alone [Fig.42(c)]. The addition of vac.Ag to GLA and GLA + MDP significantly increased the levels of IL-17 ($p<0.0001$) compared to the treatment group without vac.Ag in response to virus [Fig.42(d)].

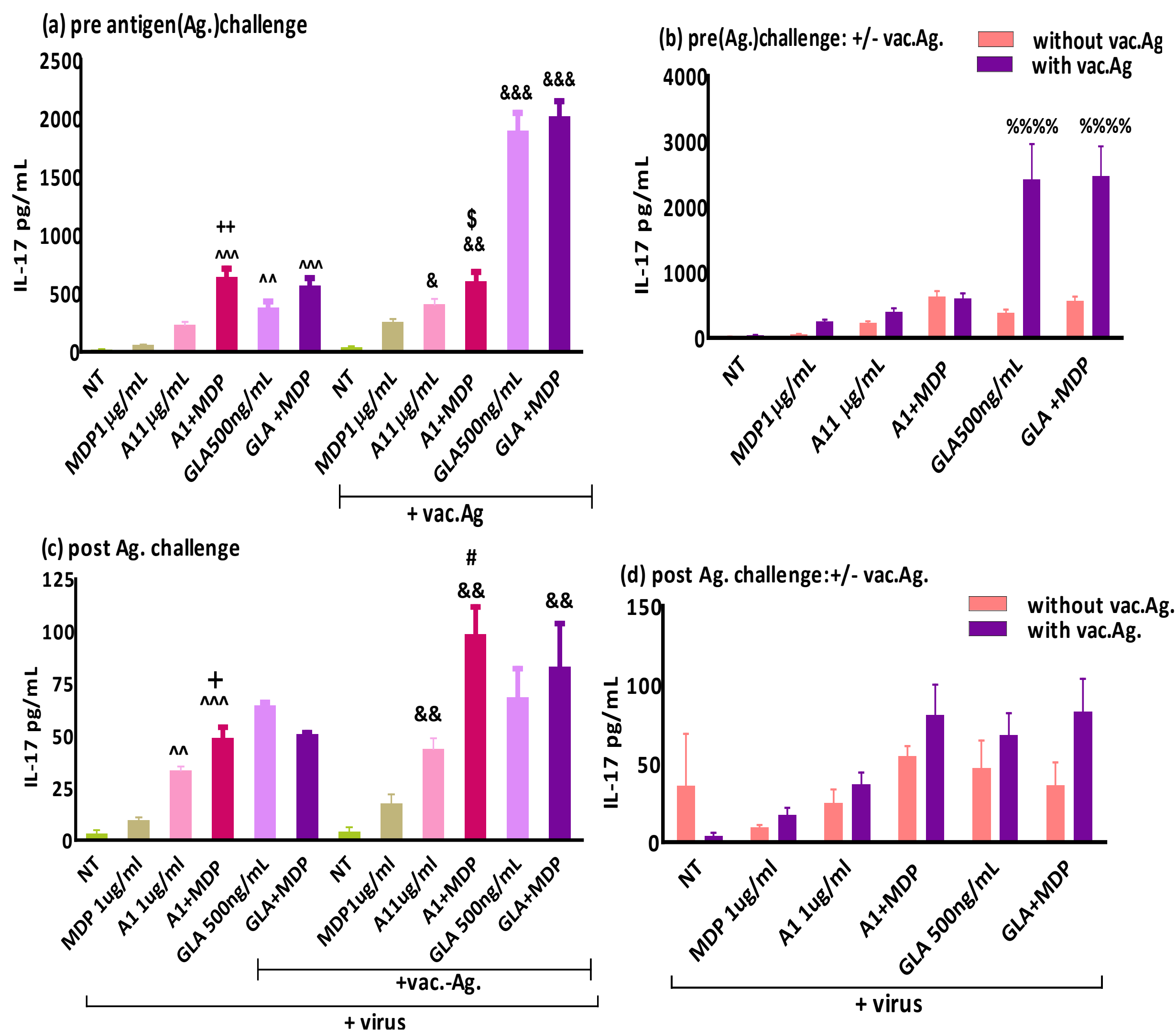

Figure 42 Influenza vaccines (vac.Ag) and virus enhanced the secretion of IL-17 by PBMCs (a)pre- Ag.challenge:IL-17 secretion ^^ p<0.01, ^^p<0.01 compared to NT; ++ p<0.01 as compared to A1 1μg/mL. **In presence of vaccine-antigen 5μg/mL** -& p<0.05, && p<0.01, and &&& p<0.001 compared to NT(vac.Ag);$ p<0.05 compared to A11μg/mL Statistical significance was determined by one ANOVA with a Tukey HSD **(b)pre -Ag. challenge:** IL-17secretion %%%% p<0.0001 as compared to GLA 500ng/mL and GLA 500ng/mL + MDP1μg/mL with GLA 500ng/mL + vac.Ag. and GLA 500ng/mL + MDP1μg/mL+ vac.Ag respectively Statistical significance was determined by two- way ANOVA with Bonferroni posttest. **(c) post-Ag.challenge:** IL-17 secretion ^^p<0.01 ^^^p<0.001 compared to NT; + p<0.05 compared to A1-1μg/mL; && p<0.01, compared to NT(vac.Ag); # p<0.05 as compared to A1 1μg/mL + vac.Ag 5μg/mL. Statistical significance was determined by one ANOVA with a Tukey HSD**. (d) post -Ag.challenge:** No significant IL-17 secretion. Results were presented as ±SEM and n=3.

Thus, the net adjuvant effect is the increase in TLR-mediated secretion of IL-1β, IL-6, and IL-17, which results in suppression of IL-10 in response to an influenza virus challenge and the associated increase in the IFN-γ:IL-10 ratio.

3.4.7 A1-adjuvant formulation was a potent stimulator for the proliferative capacity of antigen specific memory effector CD8+ T cell in human PBMCs - stimulated ex vivo with influenza

Human PBMCs were untreated or treated with either 1µg/mL A1, 1µg/mL MDP, 500ng/mL GLA, and combinations in the presence or absence of flu vaccine, 5g/mL vac.Ag. Pellets were collected after 7 days, and cells were re-stimulated with the virus at 2: 1 MOI for 20 h as an Ag. challenge. Pellets were fixed with formaldehyde and stained with fluorescently tagged antibodies against CD3.

Figure 43(A) shows the percent of CD8+ T cells, CD4+, and T reg cells in the PBMCs. The percent of CD4+ Treg cells upon stimulation with A1 and MDP alone or both in combination in the presence or absence of vac.Ag. was similar to the non-treated group. Most T cells found in PBMCs at this time point exhibited CD8+ T cell phenotype, but a huge reduction in the subset expressing CD4+ T cells, except for the group treated with both A1 and MDP +/- vac.Ag. Importantly, the highest number of CD8+ T cells was obtained from PBMCs containing both agonists. Although, the CD8+ population increased sharply in samples treated with A1 and A1+MDP in the absence of antigen, compared to the non-treated group. Similar effects were observed in cells treated with GLA and GLA + MDP. The number of CD8+ T cells stimulated with both agonists or alone were not different from the following *ex-vivo* restimulation of PBMCs in the presence of both agonists. However, this indicated that the addition of A1 and MDP can boost the generation of CD8+ T cells shown to be independent of exogenous antigen stimulation, which could still aid in eliminating the viral infection.

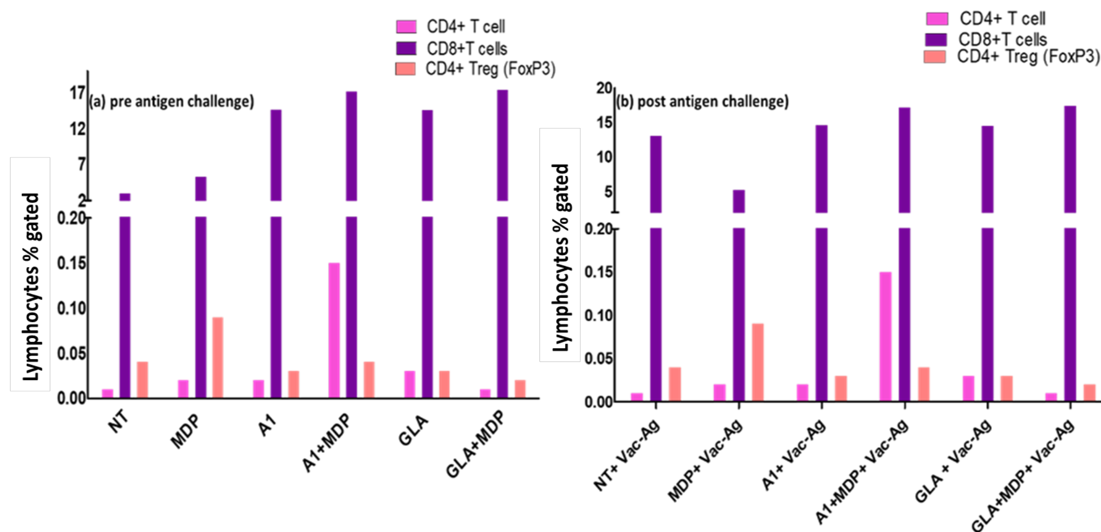

Figure 43 Influenza specific memory CD8+ T cells augmented by the addition of adjuvant-vac.Ag formulation

(a) without vaccine-antigen: Human PBMCs from healthy adults have been stimulated with influenza A virus for 20 h without vac.ag, then examined by flow cytometry. The graph showed the percentage of CD8+ T, CD4 + T, and T reg cells. Combined agonist, A1, and MDP, compared to single agonist, showed a slightly higher portion of effector CD8+ memory T cells. However, CD4+ T cells almost diminished (b) with vaccine-antigens: almost similar T subset T proliferation expression patterns were seen. Results are presented as ±SEM and n=1.

4 Discussion

The main purpose of the experiments in this thesis was to evaluate the efficacy of a potential adjuvant labeled A1, as an integrated part of vaccine formulation. These studies used different cell lines in order to determine whether A1 would be a suitable TLR4 agonist for enhancing "adjuvanticity" in activating a successful antigen-specific response in the immune system. Our experiments were based on *in vitro* and *ex vivo* culture systems, which allowed us to compare the ability to stimulate various cell lines from different ancestor cells. In addition, these experiments allowed us to observe the potency of the A1 adjuvant in a purified cell system minus the systemic physiological complications. The potency of an adjuvant is often described in terms of the intrinsic immune factors, such as inflammatory cytokines, it induces. In addition to cytokines, other parameters, including transcription factors and effector T cell proliferation, were assessed.

This study has several strengths, including using different cell lines under various conditions (drug ratio and time points) and the presence of control groups. These optimal environmental conditions helped to assess the production of different biomarkers. An important caution for considering our findings is that the current study used a small sample size (n) =3. Future work will expand on this by increasingsample sizes.

4.1 The synergism between TLR4 and NOD2 receptors for secretion of multiple biomarkers *in vitro*

It is well established that the interaction between TLR4 agonists and the TLR4 receptors produces proinflammatory cytokines, leading to polarizing Th1 responses (149). MDP has previously been tested

as an adjuvant and has been shown to induce cytokine secretion by macrophages(150). In order to evaluate the immune response following treatment with LPS with or without MDP, we analyzed the secretion of pro- as well as anti-inflammatory cytokines.

First, we tried to optimize the doses of LPS and MDP where synergism could be detected. Previous data suggested that the effects of LPS on T cell polarization were lost when high concentrations of LPS (>100 ng/mL) were used(150). MDP enhances the activity of LPS at 1-50 ng/mL. Also, MDP alone exhibited a weak ability to induce cytokines but proved to be a powerful inducer even at low concentrations of 0.1 µg/ml when combined with LPS(151). The relative LPS/MDP dose ratios were chosen (LPS: MDP = 10 ng/mL:1 ug/mL = 1:100 and 10 ng/ml:10 µg/ml = 1:1000)(152) (153).

The selection of optimal time points was important for all experiments where immune activations in response to TLR4 activation would be investigated. Many studies suggested that macrophage-derived cytokines can be detected in the conditioned medium as early as 1-4 h after LPS stimulation. For example, a TNFα surge occurs after only 1 h of LPS treatment (161). Therefore, 6h, 12h, 24h, and 48h time points were chosen to track the cytokine secretion. Also, different cytokines showed different optimal time points (125).

LPS is known to induce proinflammatory cytokines(154), which we also observed when macrophages (Mϕs) were stimulated with LPS. Interestingly, the highest secretions of TNFα, IL-6, IL-12p40, and IL-1β were seen after coadministration of MDP and LPS, which proves the prominent result of synergy in cytokine secretion (except for IL-10) (Fig. 18). Similarly, an early investigation from Wang *et al.* demonstrated the synergistic release of TNFα and IL-6, but not of IL-10 following LPS and MDP stimulation of murine *in vitro* cell lines(151).

An adjuvant co-delivered with antigens is expected to promote adaptive immune responses, where modulation of the innate immunity as a key mechanism of activity. In this study, adjuvant responses at different time points following treatment were evaluated by cytokine secretion. LPS-induced TNFα secretion peaked at 6 h, and after that, it started declining gradually. These findings were consistent with the literature (131). On the other hand, IL-6 secretion increased over time. The induction of IL-10 following stimulation with LPS or MDP alone or with both was not significantly different from nontreated cells, and it also increased with time. In the case of IL-12p40 and IL-1β, significant amounts of cytokines secretion were observed at 24 h. In addition, the amounts of TNFα and IL-6 were significant at 24 h and also showed prominent synergism. Therefore, based on the data, we have chosen 24 h as our optimal time point.

We, and others, reported that the TLR4 and NOD2 signaling pathways appear to interact or 'synergize,' resulting in amplified cytokine responses upon coadministration of TLR4 agonists and MDP(150). LPS and other bacterial products, such as lipoteichoic acid (a component of gram-positive bacteria), have been shown to induce the secretion of IL-1α, IL-1β, IL-6, and TNFα in human monocyte-derived derived DCs (155),(156).

Overall, cytokine secretion was more enhanced by LPS combined with MDP formulations compared to LPS alone, which also reflected TLR4 signaling. IL-6, TNFα, and IL-1β are the cytokines associated with acute phase responses when present at elevated levels in the blood. The key to polarization into the Th1 phenotype is IL-12. T cells exposed to IL-12 during this time differentiate to become Th1 cells. Each of these cytokines plays an important role in the induction of adaptive immunity, with IL-6 particularly important to T cell activation(157).

4.2 Evaluation of a novel adjuvant system

The current vaccine-adjuvant design is dominated by agonists of TLR4 due to the incredibly unique character of TLR4 in inducing both the pro-inflammatory MyD88 and the conserved TRIF pathways (158). However, minor changes within the structure of the agonists will dictate which pathway is favored. TLR4 agonist, A1, is less potent compared to LPS. Researchers suggested that A1 promoted the immunostimulatory effects in a TLR4-dependent manner(54). Therefore, determining whether A1 could act as an adjuvant was of interest. Measuring cytokine secretion was an attractive option for understanding how adjuvants mediate their biological effects and would help identify the best adjuvants to target vaccines in a rational manner(116). To establish an optimal method for examining the immune-modulating mediators and their potential for targeting in inflammatory disease, we established a cytokine secretion model using immortal murine monocyte/macrophages (Mϕs) J774A.1 and dendritic cells (DCs) JAWSII. TLR4 agonist, A1, is less potent compared to LPS. Based on that, we have selected a dose of A1 at 1 μg/mL.

4.2.1 TLR4 agonist, A1, increased cell viability / was not cytotoxic

The results of MTT assays to assess the viability of Mϕs (J774A.1) and DCs (JAWSII) treated with A1 at 1 μg/mL and MDP at 1 μg/mL showed that the treatments did not exhibit any significant cytotoxic effect on for both cell lines *in vitro*. Moreover, thus, were used at these concentrations for all subsequent experiments. This effect on proliferation was detected as early as 6 h and increased over 48 h post-treatment. At longer exposures, A1 (1μg/mL) and MDP (1μg/mL) collectively enhanced the viabilities of both cell lines (Fig.19, Fig.21). The synthetic TLR4 agonist, GLA (at 500ng/mL), has been previously investigated and showed excellent safety profiles in higher species (159). In summary, these findings showed that A1 (at 1μg/mL) did not show any cytotoxicity.

4.2.2 The synthetic lipid A analog, A1, and the NOD2 agonist, MDP, stimulated J774A.1 cell to produce more proinflammatory mediators

In order to demonstrate the immune potency of the novel TLR4 agonist, A1, in combination with MDP, we utilized the J774A.1 cells to examine changes in cytokine secretion. After 24 h, co-application of 1μg/mL A1 and 1μg/mL MDP were able to induce significant levels of the proinflammatory cytokines TNFα, IL-6, IL-12p40, and IL-1β (Fig.20), which showed that these compounds could act together to enhance innate immune responses. In contrast, the induction of IL-10 was decreased significantly compared to the nontreated group following the addition of A1 and MDP. TNFα is associated with acute-phase Mϕs activation and phagocytosis, IL-12p40 promotes DCs migration and cross-presentation, and IL-1β is associated with pyrogenic inflammation, all of which thereby induce the secretion of CD8+ T cells.

An interesting finding from our result was that MDP, alone was not a stimulant of IL-12p40. However, the data showed that MDP synergistically assisted A1 to promote IL-12p40 responses while decreasing the levels of IL-10 ($p<0.05$) [Fig.20(c), (d)]. This observation suggested that these Mϕs might have divergent effects on their ability to produce this cytokine(160). In line with our findings, other scientists have reported enhanced levels of IL-12 following stimulation by TLR4 agonists in the presence of MDP(161).

It was already suggested that several mechanisms underly the adjuvant effect of GLA. Coler *et al*. found that GLA could induce the expression of Th1 bias mediators like IL-12 and TNFα by murine and human DCs and Mϕs and provide protective immune responses(162). The results demonstrated that A1 and GLA showed similar trends in activating proinflammatory cytokine secretion (Fig.20). Also, A1 suppressed IL-10 secretion more than GLA, which proved that A1 could be a better effective stimulant than GLA. Ma *et al*. reported that IL-12p40 and IL-10 act antagonistically by suppressing each other(120).

These studies of adjuvant function in the earliest innate immune responses were promising as they demonstrated that synergism occurs quickly, which could lead to stronger downstream responses(163).

4.2.3 Treatment with A1 enhanced the secretion of proinflammatory cytokines in response to MDP in JAWSII DCs

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4.2.3 Treatment with A1 enhanced the secretion of proinflammatory cytokines in response to MDP in JAWSII DCs

Next, the capacity of the NOD2 agonist, MDP at 1µg/mL in combination with A1 at 1µg/mL to stimulate immature JAWSII DCs was examined. In summary, treatment of JAWSII cells with A1 and MDP significantly induced IL-6 and IL-1β secretion compared to A1 alone (Fig.22), which was in line with our previous experiments with J774A.1 cells (except TNFα and IL-12p40). There are conflicting reports in the literature regarding the secretion of IL-12 from JAWSII DCs. Jiang *et al*. reported a large amount of IL-12 secretion from JAWSII DCs when stimulated with LPS(164). In contrast, Jorgensen *et al*.(165) and Zapala *et al.*(166) reported low levels of IL-12 secretion which supported our findings. MPLA treatment was also shown to have weak activity relative to LPS (167). Ismaili *et al.* found minimal IL-12 secretion by human dendritic cells responding to MPLA relative to LPS(168). Since A1 was a synthetic Lipid A analog, like MPLA, this might explain why A1 treatment promoted low levels of IL-12 secretion from DCs.

Low-level expression of IL-10 was found after stimulation of JAWSII DCs with either A1 or GLA alone or in combination with MDP (data not shown). JAWSII DCs are p53 knockout cells. Morelli A.E. *et al*. suggested that p53**-** might be involved in the undefined biochemical pathway that underlies the inhibition ofIL-10(169). p53 is a transcription factor that can induce cell growth arrest, apoptosis, and

cell senescence. So, cells lacking p53 would be the opposite of senescent and would be overly sensitive. They also showed that the expression of IL-4, IL-5, IL-10, and IFNα were absent in JAWSII DCs, which was confirmed by FACs analysis(169).

It is well established that combined stimulation with TLR4 and NOD2 agonists have synergistic effects on cytokine secretion, which are involved in Th1 polarization (TNFα, IL-1β, and IL-12p40) and Th2 polarization (IL-6, IL-10)(152,170). *In vitro* studies of Mϕs and DCs have shown a similar trend regarding releasing inflammatory mediators (Fig.20 and Fig.22). Especially an innate proinflammatory cytokine, TNFα works with IL-12p40 and IL-1β to regulate the early innate immune responses. IL-12p40 works as a chemoattractant to recruit Mϕs towards infection sites and enhances DCs activation. In addition, TNFα is a critical factor related to LPS-dependent APC activation. In a study using TNFα knockout mice, researchers showed that the maturation of DCs depends on TNFα for the activation of different T helper cells subsets(171).

4.3 Evaluation of the effects of A1 on heterologous murine primary cell lines- *ex vivo*

4.3.1 Cytokine responses to A1 and MDP were detected in mouse splenocytes

The costimulatory effects of TLR4 and NOD2 agonists were also observed in responses of the *ex vivo* naïve splenocytes of mice, suggesting that the addition of TLR4 and NOD2 could act together to enhance innate immune responses. However, IL-12p40 was upregulated to a lesser degree compared to other proinflammatory cytokine secretion[(Fig.23(c)]. The induction of IL-10 upon stimulation with A1 and MDP alone or both in combinations, in general, is on par or suppressed compared to the NT group. Also, IL-10 secretion was reduced in the presence of A1 and MDP, and IL-12 secretion was increased.

Similar to our findings, other researchers have reported enhanced levels of these proinflammatory cytokines following stimulation by TLR4 agonists in the presence of MDP by micesplenocytes(172).

These preliminary data suggest that the A1 and MDP can alter cytokines, resulting in enhanced immune responses that could be better for polarizing T cells towards a Th1 phenotype. While the outputs between the immortalized and heterogeneous cultures were different, the trends were the same, which is more relevant.

4.3.2 Murine primary cell lines respond strongly to adjuvant-vaccine-antigen formulations

TLR4 agonists are known to induce proinflammatory cytokines in primary cells, similar to what we saw when BMDCS/ BMMϕs were stimulated with the A1 (1µg/mL) adjuvant. Dowling et al. showed similar levels of secretion of the cytokines IL-6, IL-10, IL-12p40, and TNFα from BMDCs following LPS stimulation, as shown in our studies looking at the effects of A1 on BMDCs (173). IL-1β secretion, in response to LPS, has also been reported by Dearman et al., which was consistent with our findings (174). Our results agree with Agrawal et al., who showed that TLR4 agonists stimulated higher levels of TNFα, IL-6, and IFNα in monocyte-derived DCs (175).

The TNFα, IL-6, and IL-12p40 (172) cytokines have been reported to be secreted in substantial amounts. Although IL-1β secretion by BMDCs was not significant (Fig.28), it was from BMMϕs (Fig.33). GLA stimulation resulted in a similar pattern of secretion to A1 stimulation. Several studies showed that MDP acted synergistically with LPS in the presence of vaccine antigens on activated BMDCs/ immortal cells to amplify the secretion of inflammatory cytokines and their surface marker expression (125).

The induction of IL-10 upon stimulation with A1 and MDP was significantly enhanced. Also, IL-10 secretion was increased in the presence of combined application of A1 and MDP; at the same time, IL-12p40 secretion was increased by both cell lines (Fig.26, Fig.31). This data proved the dual role of

NOD2 agonists, which is in line with the research from *Butler et al.*(176). Their studies demonstrated that stimulation of DCs with LPS in the presence of MDP constantly resulted in a significant enhancement of inflammatory cytokines with an associated upregulation of IL-10. In many cases, APCs induce IL-10 and proinflammatory cytokines, negatively regulating this initial proinflammatory cytokine secretion. Prolonged activities of proinflammatory cytokines may lead to detrimental pathological damages. The relative balance between the secretion of IL-12p40[Fig.31(140050pg/mL)] with respect to IL-10[Fig 32(167pg/mL)] from BMMϕs and IL-12p40[Fig.26 (11370pg/mL)] with respect to IL-10[Fig 27(478pg/mL)] from BMDCs indicated a predominant proinflammatory role of these agonists. In addition, Tukhvatulin et al. evaluated the effect of simultaneous stimulation of TLR4 and NOD2 receptors on downstream signaling pathways. They found that both PRR agonists within vaccine formulations enhanced the expression of cytokines like IL-1β, IL-12, TNFα, and IL-10, which were statistically significant. These findings, including our results, led us to hypothesize that NOD2 activity might have a different effect on DCs and Mϕs in their ability to polarize T helper cells. It was previously shown that simultaneous activation of TLR4 (MPLA induces Th1 response predominantly) and NOD2 (MDP which induces Th2 response) with intracellular vaccine antigens, upregulated IL-12, and TNFα, responsible for Th1 polarization and IL-6 cytokines associated with Th2 polarization, altogether which proved a better and balanced Th1/Th2 immunity by priming naïve Th cells toward specific Th1 phenotypes to activate cytotoxic CD8+ Th cells.

Also, generation and activation of BMMϕs in *in vitro* were influenced by many factors of local tissue environments, such as adding M-CSF (M2 stimuli) might promote M2-like polarized BMMϕs. This M2 type phenotype induced secretion of IL-12 low, IL-1βlow, and IL-10 high. Also, dislodging the

adherent cells from the tissue culture vessels with cell scrappers could be stressful on the health status of BMMϕs which might impact the overall outcome of cytokine secretion.

Lehman *et al.* and others previously showed in human patients and in data from mice models that IL-12p40 is important for mounting a cellular Th1-driven immune response for immunity against intracellular pathogens, which is essential for protection against intracellular pathogens(177,178).). Here, we showed that there was a trend of enhanced levels of IL-12p40 secretion by both BMDCs and BMMϕs in response to A1+ MDP-vac.Ag conjugate compared to any other treatment groups [Fig.26, Fig.31]. Researchers found that MPLA can induce Th1 immunity, characterized by T-cell secreting IFNγ and TNFα over Th2, for a target intracellular pathogen in BMDCs from mice(179). Additionally, a direct antiviral role against IAV has been demonstrated(179).

Our data on the effects of the influenza vaccine are in good agreement with the study on the effects of the whole-cell influenza vaccine on DCs, which also demonstrates the induction of DC surface marker expression and IL-12secretion (180). In addition, our data showed that A1 and MDP activated BMMϕs and BMDCs produced more proinflammatory cytokines when combined with the vac.Ag. and suppressed IL-10 secretion with an associated increase in the IL-12p40: IL-10 ratio (Fig.26, Fig.31). In agreement with our findings, a recent study by Kim *et al.* showed cross-regulation between TLR4 and NOD2 receptors; NOD2 was able to balance the homeostasis of the immune system by inhibiting IL-12 synthesis while TLR4 signaling intensifies or synergistically produces IL-12 when the TLR signaling intensity alone was low inIL-12 secretion(102). Their findings are consistent with our results and suggest that the host environment can be kept in homeostasis by the ability of NOD2 to suppress the over-secretion of IL-12p40. This could be the reason that after the addition of vac.Ag to A1 and MDP,

we did not see any marked enhancement in the level of IL-12p40 secretion from both BMMϕs and BMDCs.

In summary, the pattern of induction and the overall magnitude of cytokine secretion among the immortalized, primary, and heterogeneous cell cultures were different. However, the trends were the same, which is more relevant. Surprisingly, the levels of cytokines from stimulated BMDCs were similar to those observed in BMMϕs. These preliminary data suggested that the co-application of A1 and MDP could alter cytokine secretion and enhance immune responses, which could be better for polarizing naive T cells towards a Th1 phenotype.

GLA has already been publicized to be well-tolerated in human subjects(181). Given the similarities between GLA and A1, I hypothesize that A1 might act in a similar fashion. Our data demonstrated that A1 has also been shown to positively enhance the proinflammatory cytokine secretion by Mϕs and DCs, similar to GLA. The tolerizing ability of A1 had been attributed to the induction of IL-10 by BMDCs and BMMϕs as well. Conversely, inhibition of IL-10 secretion by JAWSII DCs was also thought to be involved. We can speculate that A1 might act at multiple points to exert its adjuvant effect; further studies were needed to investigate the mechanisms underlying the adjuvant effect of A1 precisely.

4.3.3 Coadministration of A1 and MDP induced greater ERK phosphorylation than a single adjuvant in JAWS II and BMDCs

It is well recognized that TLR4 ligand-stimulated DCs activates the ERK1/2, JNK, and p38 MAPK pathways(173). Tukhvatulin *et al.* and Gorskaya *et al.* have extended their findings with TLR4 by simultaneously stimulating the cells with a NOD2 receptor agonist. They found that it synergistically

enhanced the phosphorylation of NFkB and ERK1/2 compared to cells treated with a TLR4 agonist alone. Their results also showed that this synergistic stimulation correlated with enhanced cytokine synthesis and secretion in murine DCs/ BMDCs, and human THP1/PBMCs *in vitro/in vivo* (172),(182). The proposed mechanism is that activated NOD2 and TLR4 can recruit “bridging adapters” such as Mal/MyD88 and TRAM/TRIF and activates two distinct signaling pathways that impact ERK and NF-kB signaling (183).

In this study, the relative band intensities of phosphorylated ERK, relative to the total ERK1/2 control, showed that ERK was phosphorylated and activated in both DCs and primary mouse BMDCs treated with A1 but not treatment with MDP (Fig.34 and 35). This shows that treatment with A1 can promote ERK phosphorylation even in the absence of MDP. Combined A1 and MDP stimulation showed minor upregulation of ERK1/2 phosphorylation. However, these data did not show a clear synergistic relationship between TLR4 and NOD2 agonists since treatment with A1 and MDP did not synergistically increase ERK1/2 phosphorylation above A1 alone. It should be noted that these studies result from a preliminary experiment using a posy-treatment timepoint of 30 min. and a single high concentration of each of the TLR4 and NOD2 agonists. Further experiments are required to properly assess the potential synergistic actions of A1 and MDP on ERK pathway activation.

The MAPK pathways are known for their overlapping signaling network, which makes it hard to decide the ultimate outcome(124). We have shown that combined treatment of GLA and MDP on JAWSII cells and BDMCs increased ERK phosphorylation to levels similar to GLA alone (Fig.34 and 35). However, MDP alone could not activate ERK phosphorylation of BMDCs (Fig.35), as was seen with JAWSII DCs. To confirm whether the inhibition of TLR4 signaling suppressed the phosphorylation of ERK1/2, we examined the effects of CLI-095 on signaling induced by treatment with A1 as a TLR4-specific

ligand in this study. CLI-095 is known to block the signaling mediated by TLR4. This inhibitory molecule can block the different steps of TLR4 activation and the signaling cascade. GLA, a well-characterized TLR4 agonist, was used as a control to confirm that CLI-095 inhibits the TLR4 receptor. They showed that the levels of ERK1/2 phosphorylation for all the treatments were decreased (Fig.36), which suggested that ERK1/2 phosphorylation depended on TLR4 activation. However, we could not state it definitely as there was no positive control to prove it. There is a piece of evidence that blocking the ERK pathway can suppress the secretion of TNFα, IL-6, and IL-12 by TLR4 ligand-induced DCs(173).

Our data could not demonstrate the cooperation between TLR4 and NOD2 in stimulating the ERK signaling pathway. Because the ERK phosphorylation between A1 and A+MDP was almost similar. Overall, the bands from our western blot experiments were not very clear.

ERK was known to regulate numerous essential cell functions, including cell survival, development and differentiation, and the regulation of immune responses(184). Because ERK1/2 signaling is necessary for effective formation of DC-T cell bridge and the following downstream signaling(146). Of interest, subsequent findings have recently demonstrated that ERK inhibitors decreased cell surface markers of DCs maturation. On the other hand, BMDCs treated with NOD2 and TLR4 agonists up-regulated and transcribed the mRNA for IL-6, IL-1α, IL-1β, TNFα, and IL-12p40. After spontaneous differentiation of these cells, higher levels of IL-6, IL-1β, IL-12p40, and TNF-α transcripts/proteins were found, and higher levels of secreted cytokines were found by enzyme-linked immunosorbent assays (ELISA) (155,185). Again, our Western Blot assays have provided a preliminary look into the phosphorylation of ERK1/2. Future experiments that increase the n value and improve the overall quality of the Western Blots will be required for quantitative analysis.

4.4 Treatment of PBMCs with a vaccine-adjuvant formulation enhances the release of cytokines

The effects of vaccine-adjuvant treatment on PBMCs were examined to find a novel approach to re-stimulate the immune response for memory T cell activation by an A1 adjuvant formulation in the presence of vaccine-antigen.

The data proved that MDP acted in cooperation with A1 just as it did with GLA in the formation of proinflammatory cytokines after 72 h incubation of human PBMCs (Fig.37). Similarly, studies with human primary myeloid cell subsets revealed that MDP acted cooperatively with TLR4 agonist to produce proinflammatory cytokines such as IL-1β, IL-6, TNFα, and considerably amplify the secretion of the anti-inflammatory cytokine IL-10(125). The elevation of IL-10 was observed, which was similar to our data from previous experiments with murine primary cell lines. In general, there will be an early increase in proinflammatory cytokines and then an increase in IL-10 later in the inflammatory response. Therefore, the 72 h timepoint observation suggested that simultaneous stimulation with A1 and MDP would likely promote a mixed Th1/Th2- mediated immune response on human PBMCs. Similarly, early investigations from Wang *et al*. and Tada *et al*. demonstrated the synergistic release of IFNγ, IL-12, and IL-6 following LPS and MDP stimulation from whole human blood and human DCs (152),(151). Our results demonstrate that A1 effectively induced IFNγ and eventually promoted a Th1 bias cytokine secretion against the Influenza vaccine. antigen(vac.Ag.). Because IFNγ is known for its immunostimulatory and immunomodulatory effects by increasing phagocytosis, oxidative burst, and intracellular killing of microbes within the innate and adaptive immunity(116). These responses were also noticeable against subsequent challenges with the relevant pathogen Influenza A virus (IAV).

We observed that these synergistic inductions of cytokine were increased to values several-fold higher at 7 days after treatments than were obtained at 72 h. Treatment of human PBMCs with the combination of A1 and MDP with the flu vaccine after 7 days resulted in significant increases in the secretion of proinflammatory cytokines such as IFNγ, a known APCs modulator, along with IL-1β, IL-17, and IL-6. Here, we proved the ability of A1 with MDP to induce polarizing Th1 responses. These results were also supported experimentally by the study of Tada *et al.,* which showed the combinatory activation of human DCs through the TLR4 and NOD2 pathways significantly enhanced Th1 lineage immunity by demonstrating that this synergy promoted high IL-12 induction and that the IL-12 thus generated, promoted IFNγ secretion by T helper cells(152). Researchers showed that GLA, combined with split-virus vaccines (SVV), significantly increased IFNγ secretion in PBMCs, and promoted Th1 bias immune responses to influenza virus by suppressing the IL-10 (118).

It was acknowledged that synthetic lipid A analogs such as MPLA retain a TRIF-biased TLR4 stimulation(187) and are also activated by some MyD88-associated inflammatory (IL-12) and anti-inflammatory cytokines (IL-10) (127). Siegemund *et al.* showed that NOD2 and TLR4 agonists synergistically enhance IL-12 while the level of IL-10 was increased in DCs(186). The low toxicity of MPLA and LPS could be the reason of high IL-10 expression(52). In addition, coadministration of MPLA and Respiratory syncytial virus (RSV) vaccine-induced IL-10 expression that was able to reduce the inflammatory reaction. This hypothesis was supported by our data with the synthetic lipid A analog, A1, which stimulated PBMCs to enhance the secretion of IL-10 (Th2 bias) and other proinflammatory cytokines at the same time (Fig.37).

Schultze V*. et al*. and others showed that TLR4 and flu vaccine-antigens, as well as GLA , promoted a protective immune response where IFNγ and IL-1β induced a Th1 biased environment and

delayed-type hypersensitivity responses also promoted a low level of IL-10 response(189,190)which in line with our data. IL-12 induces Th1 responses by stimulating the secretion of IFNγ whereas IL-10, known as a human cytokine synthesis inhibitory factor (CSIF), suppresses them by inducing a delayed hyperresponsive response with the assistance of antigen-presenting cells (APCs)(188). The initial rise in the IL-10 level was reduced significantly after the addition of flu vac.Ag. to A1 or GLA +/- MDP [Fig.40(b)].

To determine if the adjuvant formulation could alter the adaptive immune response, we utilized a subsequent *ex vivo* challenge using the IAV virus model on human PBMCs. After the viral challenge with the IAV virus, there were marked increases in the levels of IFNγ[Fig.38(c)], IL-6[Fig.39(c)], IL-1β[Fig.41(c)], and IL-17[Fig.42(c)] secretion. In the case of IL-10, treatment with A1 and MDP showed a lack of response to live-virus [Fig.40(c)]. The reduction in IL-10 resulted in significantly higher ratios of IFNγ: IL-10 in A1-1µg/mL +MDP1µg/mL /vac—ag-treated PBMCs. Similarly, the IFNγ:IL-10 ratio was increased by GLA500ng/mL +MDP1µg/mL /vac.Ag. in PBMCs.

Overall, we demonstrated in PBMCs that the A1-MDP-vac.Ag. cocktail, could augment the IFNγ: IL-10 ratio (Th1/Th2) in response to the influenza virus challenge. A similar finding showed that DCs activated by virus-infection, mainly through TLR4 (golden standard)((173), secreted Th1 polarizing cytokines. In addition, our findings agree with those of Frey *et al.,* who showed that AF04, a TLR4 agonist combined with the Cytomegalo virus-granzyme B (CMV-g B) vaccine, elicited Th1/Th2 responses in a mouse model and following *in vitro* virus challenge demonstrated strong induction of IFNγ(191). In line with our data, several publications have reported results regarding MPLA(192),(55) and GLA (193–195) with a similar outcome.

The FACS analysis in Fig 43 showed most of the cells were CD8+ Th cells; there were very few Treg and CD4+Th cells. However, the low relative number of CD4+Th cells was less consistent. However, the effects of the CD4+Th cells at any stage of the memory responses, particularly for *in vivo/ex vivo* models of influenza infections, are still controversial*.* One possible hypothesis is that the influenza virus-stimulated APCs are not contingent on the CD4+Th cells for the primary immune response but it relies on the CD8+ Th cells/CTL (196). Therefore, the subsequent effects helped to develop functional CD8+Th cells, which replaced CD4+ Th cell activation(196). Moreover, Saurwein-Teissl *et al.* and Hayashi *et al.* showed that influenza virus-infected PBMCs generated strong CD8+ Th cells and the CD4+ Th cells secretion was diminished(180,197). Their explanation for the obtained data was that a subpopulation of CD8+ T/APCs cells stimulated with influenza virus express the Fas ligand (FasL) and killed the CD4+Th cells(198). In addition, several studies showed that a CTL population without CD4+ Th cells was common in mice. Buller *et al.* and Rahemtulla *et al.* showed extensive proliferation of cytotoxic T cells with a huge depletion of CD4+ Th cells following *in vivo* influenza infection (199,200). Further, Lauder et al. also showed that wild-type mice vaccinated with the influenza vaccine induced very few CD4+ Th cells but significantly induced IFNγ and IL-1β compared to the control group (201). In contrast, other studies showed that in *in vivo* cultures, CD4+ Th cells were required during influenza A virus infection(136,202).

The data showed that the *ex-vivo* -stimulated PBMCs model was not ideal for looking at CD4+ Th cell responses under the investigated conditions. For example, the time required for functional CD8+ Th cells development contrasts with CD4+ Th cells development, which is generally detected within 5-7 days of stimulated culture containing the primary APCs (e.g., DCs). Furthermore, it is important to state that the volunteer-donated blood had not been exposed to recent influenza virus infection. So, the lack of CD4+ Th cells after the antigen challenge might be due to the absence of a “memory pool” of CD4+ Th

cells. Thus, I could not recover sufficient CD4+ Th cells to perform any cellular assays. A possible reason for the discrepancy in our findings could be the result of an unexpected staining artifact (excess fluorescently labeled antibodies added to antigen). However, it is important to note that the flow cytometry experiment was conducted in a different research facility. These issues should be resolved in future studies in order to provide a complete picture of the effect of CD4+ Th cells responses for antigen-specific immune responses.

GLA coadministered with an antigen, such as (Tuberculosis B -vaccine), was shown to induce Th1 responses associated with an innate cytokine response and released antigen-specific immune responses *in vitro/ in vivo*(203–205). A1 proved to be a potential adjuvant that strongly induced the Th1 recruiting cytokine, IL-12p40, during the early immune response. Later, the A1-vaccine formulation provided evidence of enhanced effector memory T cells activity by producing an increased level of IFNγ with no change in IL-10 levels, supporting the finding that A1 treatment could shift the adaptive immune responses towards the Th1 direction. Similar trends were seen in our study in the presence of the IAV virus.

Moreover, many studies showed that IL-1β, IL-6, and IL-12 were synergistically induced upon stimulation with TLR4 and NOD2 ligands in addition to antigens of different types of cells (151,170,206). As mentioned in the introduction, the IL-6, IL-12p40, and IL-1β cytokines are associated with acute phase responses and are master regulators in activating adaptive immunity, especially for IL-6, which promotes Th-17 differentiation, IL-1β promotes pyrogenic inflammation and IL-12p40, and IL-17 which correlates with IFNγ induction via CD4+ Th cells activation. IL-12p40 enhances the Th1-cell response by upregulating the level of IFNγ. IFNγ, in turn, is a major key for the clearance of viral/intracellular infections(207).

Also, IL-1β has a stimulatory effect on CD4+Th cells to promote differentiation into the Th17 cells and Th1 cell lineages. Thus, IL-1β, IL-6, IFNγ, and IL-12p40 activate the Th1 cell-mediated responses observed in our study before and after the antigen challenge.

We have already shown that after viral challenge with the IAV virus, there were significant increases in the levels of IFNγ and IL-6 with no significant changes in IL-10 secretion (Fig 38; Fig 39 and Fig.40). This would suggest the activation of a polarized antigen-specific Th1 T cell response after the challenge. The lack of endogenous IL-6 also decreased the IL-6–induced IL-21 secretion, which is required to maintain optimal T helper B cell function(208). Collectively, IL-6 regulates the transition between innate and adaptive immunity by playing a pivotal role in the restoration, generation, and responses of CD4+ effector T cells. Our data suggested that the adjuvant-vaccine formulations can enhance Th1 responses by activating an innate inflammatory response leading to an enhanced antigen-specific adaptive response against the flu virus. A1 proved to be an effective adjuvant to stimulate human PBMCs to promote a Th1 bias cell response to IAV by suppressing IL-10 secretion and enhancing the IFNγ response to the influenza virus challenge. Together these preliminary data suggest that A1 in the presence of MDP may help generate a new multi-adjuvant formulation that can help topolarizeTh1 responses.

5 Concluding remarks and future directions

Herein, we demonstrated a model to evaluate the potency of an immunoadjuvant, with MDP in the presence of vaccine-antigen, based on innate *in vitro/ex vivo* activity. In summary, this preliminary study showed that the output between the immortalized, primary, and heterogeneous cultures was different. However, the trends were the same, which is more relevant. These outcomes may make A1 an attractive adjuvant.

As this was the first step, future studies, both *in vivo*/pre-clinical, will be required to provide concrete evidence about using optimal compositions of both agonists to induce the desired type of adaptive immunity. My cell-mediated immune responses described here pertain primarily to cytokine secretion following the stimulation of primary heterologous cell lines. However, we did not determine the role of A1-MDP-vac.Ag formulation in cell-mediated immunity within the context of the mucosal immune system. To fully understand the potency of the A1 in vaccine formulation, an immunized animal *(in vivo*) model would be beneficial. Whether this formulation can induce the desired type of T helper subsets or stimulate B cells for humoral responses should be addressed.

Only licensed vaccines that are made of live attenuated pathogens can generate cell-mediated immunity, which is not safe for the immunosuppressed population, such as the yellow fever vaccine(42). Therefore, most current vaccines, even with alum adjuvants, provide limited protection only through a Th2 biased-humoral immunity and do not involve activation of effector T cells.

The next step is to determine whether these results translate to *in vivo* T-cell responses to influenza virus challenge in clinical trials to demonstrate its potential for enhanced protection against influenza in older people. Specially influenza A virus-related impairment in the elderly is well described,

indicated by a lack of the type 1 cytokine (IFNγ) in humans associated with this issue(195),(203). Senescence-related impairment is mainly associated with an age-related decline in cytolytic (CTL) activity of effector CD8+ T cells(195). The immunosenescent population is also associated with a reduction in the numbers of naïve T and B cells, which cannot proliferate when activated. However, memory T and B-cell populations are maintained in the immune system. A subpopulation of T and B cells differentiate into memory cells, which respond quickly in secondary immune response(203). Interestingly, the reversibility of this age-related senescence appears to be possible by activating TLR4 on various APCs by incubation with one of its ligands(209). Although we did not use PBMCs from older adults in our study, we could still speculate on the potency of A1. As our results showed, the function of PBMCs could be persevered by exploiting TLR4 and NOD2 ligands which can restimulate the PBMCs to produce an elevated level of IFNγ and IL-6. The advancement of an aged CD4+ Th-cell response was largely dependent on IL-6 secretion by APCs. A study by Lauder *et al.* showed that IL-6 deficient (IL-6-/-) mice failed to produce influenza-specific memory T cells and that the number of virus-specific CD8+ T cells decreased after antigen challenge(201).

In addition to discovering the magnitude of adaptive immunity of A1 in *in vivo* model, a delivery system (vector) should be added to ensure that both the antigen and the adjuvant are targeted efficiently to APCs to increase the effects of the adjuvant. Lastly, further studies should investigate the effectiveness of A1 in the context of different vaccines or in the presence of different antigens, which will confirm whether the ultimate immune response of A1 occurred independently. Such findings would reconcile that more precise *in-vivo* evidence would increase the possibility of adding A1 as an immunoadjuvant to currently available vaccines to enhance their immunopotency.

The limited immunogenicity of new and recombinant vaccine antigens has increased the importance of adjuvant research in vaccine development, particularly those not associated with undue toxicity. In light of the continued scarcity of successful vaccines with the ability to induce CD8+ cellular immune response, it certainly underscores the growing demand for adjuvanted-vaccines with strong Th1-type cellular response(135). Quantifying the early innate immune responses of the adjuvant-vaccine formulation may complement the evaluation of the desired adaptive immunostimulatory profile to define an adjuvant's proper dose and formulation. Besides, this may benefit the target populations with known deficits in innate responses. Therefore, these observations may have implications as proof of concepts for developing a new class of adjuvant based on combined PRRs stimulation for the 3prevention and treatment of a range of important infectious diseases.

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7 Appendix

Appendix A TLR4 agonists were more effective at inducing cytokine release than TLR 2/4 agonists with MDP

The combination of a TLR2/4 agonist and MDP (10ug/mL) resulted in a significance increase in the secretion of IL-6 ($p \leq 0.01$) compared to treatment with the TLR2/4 agonist, LPS, alone. However, there was no synergism seen for any of the other cytokines. In the case of the TLR4 agonist, we reported more secretion of IL-12p40 and less of IL-10. It is already known that activation of the NOD2 receptor does not act synergistically with activation of TLR2. The TLR2/4 agonist is known to activate a strong Th2-biased humoral immune response as well. Based on this data a TLR4 agonist was chosen for rest of the experiments, since the primary concern was to stimulate a Th1 based humoral immune response which means that a TLR2/4 agonist cannot be used as an adjuvant

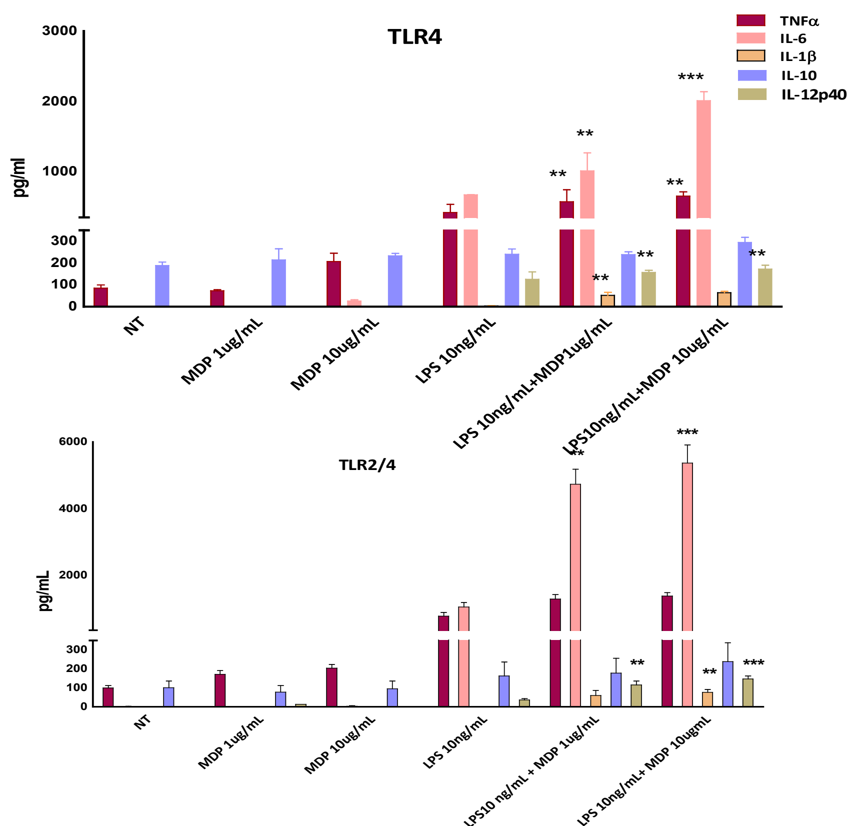

Figure (panel a and b) Comparison between TLR 2/4 and TLR 4 Agonists based on the cytokine secretion. Levels of TNFα, IL-6, IL-10, IL12p40 and IL-1β produced by J774A.1. Cells were seeded at a concentration of 5 x10^5 cells/mL/well in a 24 well plate and left untreated or treated with either 10 ng/mL TLR 2/4 and TLR4 agonist (LPS), MDP 1 and 10 µg/mL or both. Supernatants were collected after 24 hours of treatment. N = 6, ±SEM. representative of two experiments and statistical significance was determined by an ANOVA with a Tukey HSD. *** p<0.001, **p<0.01 compared to the LPS 10 ng/mL treatment group.

Appendix B Media and Buffer

10X Phosphate buffered saline (PBS)

$Na_2HPO_4.2H_2O$	8 mM
KH_2PO_4	1.5 mM
NaCl	137 mM

of 7.4

10 X Tris buffered saline (TBS) pH 7.6

NaCl	1.5M
Trizma Base	0.2M

Dissolve dH_2O pH to 7.6

Lysis buffer

Tris HCL (pH 7.5)	50mM
NaCl	0.5%
Protease Inhibitor CocktaIL	1%
Phosphatase Inhibitor CocktaIL	1%

5X Sample buffer

125 mM Tris	6.25 ml
Tris HCl pH 6.8	1 M
10 % Glycerol	5 ml
2 % Sodium dodecyl sulphate (SDS)	10 ml (10 % (w/v)
Bromophenol Blue	0.01 g

dH_2O	28.75 ml
0.25 M Dithiothreitol (DTT)*	250 µl 1 M DTT

* Added to 1 ml 5X Sample Buffer just before use

Separating gel (10% (v/v))

Acrylamide/Bisacrylamide (30% stock)	33% w/v
Tris-HCl pH8.8	1.5M
SDS	1% w/v
Ammonium persulpate	0.5% w/v
TEMED	0.1% v/v

Dissolved in dH_2O

Stacking gel

Acrylamide/Bisacrylamide (30% stock)	6.5% v/v
Tris-HCl pH6.8	0.5M
SDS	1% w/v
Ammonium persulphate	0.5% w/v
TEMED	0.1% v/v

Dissolved in dH_2O

Running buffer

Tris base	25mM

Glycine	200mM
SDS	17mM
Dissolved in dH_2O	
KCl	2.7 mM
Dissolved in dH_2O to a pH	

Blocking Buffer

(5% non fat dry milk in TBST)

Non fat dry milk	50g
TBST	1 liter

Wash buffer (ph-8.0)

Tris buffered saline (1X)	1liter
Tween 20	0.5%