DEVELOPING AN *IN VITRO* MODEL TO STUDY TRAINED IMMUNITY

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science (MSc) in Biology

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THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	DEVELOPING AN IN VITRO MODEL TO STUDY TRAINED IMMUNITY
Name of Candidate Nom du candidat	White, Mallory
Degree Diplôme	Master of Science
Department/Program Département/Programme	Biology
Date of Defence Date de la soutenance	March 29, 2021

APPROVED/APPROUVÉ

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ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

Novel discoveries have proven that the innate immune system has the capacity to adapt a memory response, termed "trained immunity", providing broad non-specific protection against pathogens. To date, trained immunity has been studied *in vivo*, and *ex vivo* using human peripheral blood mononuclear cells (PBMCs) and bone marrow derived macrophages (BMDMs) from mice. However, we aim to develop and characterize an *in vitro* cell model to study trained immunity. J774A.1 macrophages and THP-1 monocytes were evaluated as murine and human models, respectively, to study trained immunity *in vitro*. THP-1 monocytes were differentiated from monocytes to macrophages with 72-hour phorbol 12-myristate-13-acetate (PMA) stimulation. Cells were trained for 24 hours with muramyl dipeptide (MDP), lipopolysaccharide (LPS), glucopyranosyl lipid adjuvant (GLA), or a novel delivery system containing cationic mannosylated liposome (CL) containing muramyl dipeptide (MDP) and glucopyranosyl lipid adjuvant (GLA). After a 2 days' rest, cells were restimulated with MDP, LPS, Dukoral or influenza virus for 24 hours. Subsequently, trained immunity was evaluated in terms of tumor necrosis factor α (TNFα) and interleukin-10 (IL-10) production, and cell surface expression of complement domain 14 (CD14) and complement domain 16 (CD16). We demonstrated that both J774A.1 cells and PMA differentiated THP-1 cells had a trained immunity cytokine profile, as defined by an increase of TNFα but not IL-10, following restimulation with various non-specific pathogens. Next, we developed a delivery system comprised of MDP and GLA entrapped in cationic liposome (CL). The delivery system significantly increased TNFα production following non-specific restimulation in both the murine and human *in vitro* models, indicating the ability to train the cells. By way of flow cytometry, we discovered the delivery system increased the expression of CD14 and decreased the expression of CD16 on PMA differentiated THP-1 cells.

To determine the method of training induced by the delivery system, we used two cell signaling inhibitors, CLI-095 and Gefitinib, and deduced that the delivery system was inducing training partially through toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), respectively. Finally, complement domain 3/ complement domain 4 (CD3/CD28) activated Jurkat T cells co-cultured with trained THP-1 macrophages produced significantly higher levels of interleukin 2 (IL-2) upon subsequent non-specific restimulation, suggesting trained innate immune cells have the potential to influence adaptive immune responses. On the whole this research has contributed to the development of an *in vitro* bioassay model that will allow scientists in all fields of immunology to further explore the phenomenon of trained immunity. Additionally, these *in vitro* models can act as tools to demonstrate the potential of trained immunity as a novel therapeutic strategy. At last, our delivery system proved to be a promising trained immunity stimulant that warrants further investigations.

Key Words: Trained Immunity, Innate Immunity, J774A.1, THP-1, Jurkat, TLR-4, NOD2, Vaccines, Adjuvant

Acknowledgements

I would like to thank my supervisor, Dr. Hoang-Thanh Le for his guidance and support throughout this process. Your patience and understanding have been unwavering, and for that I am grateful. I want to thank you for giving me the opportunity to further my scientific career while also supporting me through medical school.

I would also like to thank the members of my committee, Dr. Gagnon and Dr. Simard. I appreciate all the advice you have given me.

A huge thank you to my lab mates/mentors (Nya Fraleigh, Dr. Jordan Lewicky, and Alexandrine Martel) as well as fellow graduate students (Amanda Boraman and Jovial Praava). Thank you for teaching me the skills I needed to complete this degree and for your friendship throughout this journey.

Finally, I would like to thank my friends and family for supporting me throughout this challenging endeavour.

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List of Chemicals

1% Penicillin/streptomycin (HyClone, Logan, UT)

10X Phosphate-buffered saline; PBS (Fisher, Fredrick, MD)

Bovine serum albumin; BSA Grade V (Fisher, Fredrick, MD)

Cluster of differentiation 11b; CD11b PECy7 (BD, Franklin Lakes, NJ)

Cluster of differentiation 14; CD14 PE (Invitrogen, Fredrick, MD)

Cluster of differentiation 16; CD16 PECy5 (Invitrogen, Fredrick, MD)

Cluster of differentiation 206; CD206 FITC (BD, Franklin Lakes, NJ)

Cluster of differentiation 28.2; CD28.2 (Invitrogen, Fredrick, MD)

Cluster of differentiation 3; CD3 OKT3 (Invitrogen, Fredrick, MD)

Cluster of differentiation 80; CD80 PECy5 (Invitrogen, Fredrick, MD)

CLI-095 (InvivoGen, San Diego, CA)

Dulbecco's modified eagles' medium; DMEM High-Glucose (HyClone, Logan, UT)

Dukoral (Sanofi Pasteur, Cambridge, MA)

Each dose of vaccine suspension (3 ml) contains:

- A total of 1 x 10^{11} *Vibrio cholerae* of the following strains:

  V. cholerae O1 Inaba, classical strain (heat inactivated), 22.5 x 10^{10} vibrios

  V. cholerae O1 Inaba, El Tor strain (formalin inactivated), 2.5 x 10^{10} vibrios

  V. cholerae O1 Ogawa, classical strain (heat inactivated), 2.5 x ^{10} vibrios

  V. cholerae O1 Ogawa, classical strain (formalin inactivated), 2.5 x 10^{10} vibrios

- Recombinant cholera toxin B subunit (rCTB), 1 mg

Fetal bovine serum; FBS (Gibco, Grand Island, NY)

Fixation buffer (Biolegend, San Diego, CA)

Gefitinib (InvivoGen, San Diego, CA)

Glucopyranosyl lipid adjuvant; GLA (Avanti Polar Lipid, Alabama, USA)

Hanks' balanced salt solution; HBSS (Sigma, St Louis, MO)

Influenza Virus (Influenza H3N2/Lee 40, Charles River)

Lipopolysaccharide; LPS (E coli 0111:B4 Code: L4391, Sigma, St Louis, MO)

Monocyte-colony stimulating factor; M-CSF (Invitrogen, Fredrick, MD)

Muramyl dipeptide; MDP (Sigma, St Louis, MO)

Phorbol 12-myristate-13-acetate; PMA (Sigma, St Louis, MO)

Roswell Park Memorial Institute; RPMI 1640 (HyClone, Logan, UT)

Tetrahydrofuran; THF (Fisher Scientific, Fairlawn NJ)

TrypLE (Gibco, Grand Island, NY)

Tween20 (Sigma, St Louis, MO)

1. INTRODUCTION

1.1 The Immune System

The immune system is the body's defense mechanism to infectious pathogens and aberrant host cells. There are two arms of immunity, the innate and the adaptive immune system (Medzhitov, 2007). The innate immune system is responsible for reacting rapidly and non-specifically to invading pathogens, whereas the adaptive immune system reacts in a slower antigen-specific manner conferring memory upon subsequent infection. The innate immune system includes chemical and physical barriers such as the skin, it is also comprised of antigen-presenting cells (APCs)—dendritic cells (DCs) and macrophages—as well as effector cells—natural killer (NK) cells and granulocytes (Banchereau & Steinman, 1998; Kariyawasam & Robinson, 2006; Langermans, Hazenbos, & van Furth, 1994; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). Macrophages and DCs typically reside in tissues and phagocytose pathogens at the site of infection (Banchereau & Steinman, 1998; Langermans et al., 1994). DCs then act as messengers, which travel to lymph nodes to present antigens to lymphocytes (Banchereau & Steinman, 1998). NK cells recognize infected cells and induce apoptosis through a variety of mechanisms including Fas and Fas ligand (Fas-L), cytokines, cytotoxic enzymes and antibody-dependant cell- mediated cytotoxicity (ADCC) (Vivier et al., 2008). Finally, granulocytes such as neutrophils, eosinophils, basophils, and mast cells release toxic materials (nitric oxide, toxic-oxygen species, acid hydrolases, and lysozymes) and perform phagocytosis to kill pathogens (Kariyawasam & Robinson, 2006). Together these components of the innate immune system combat pathogens within the critical first few hours of infection (Medzhitov, 2007).

The adaptive immune system is composed of lymphocytes—B and T cells—which reside in the lymph nodes awaiting activation by APCs (Medzhitov, 2007). As previously mentioned, DCs are the link between the innate and adaptive immune system, traveling to lymph nodes to activate T helper (CD4+) cells (Banchereau & Steinman, 1998). Following APC-T follicular helper cell activation, cytokines—primarily IL-6—are released to induce B cell production of antibodies (humoral immunity) and T cells to become cytotoxic (cellular immunity) in approximately 4-7 days (Reinhardt, Kang, Liang, & Locksley, 2006; Schatz, Oettinger, & Schlissel, 1992). In general, humoral immunity is responsible for clearing extracellular pathogens, such as bacteria, with antibodies (Mesin, Ersching, & Victora, 2016). Various antibodies act to neutralize pathogens, opsonize pathogens, or activate the complement system. Neutralizing antibodies bind to and block surface structures of microbes or infectious particles, rendering them non-pathogenic (Brioen, Dekegel, & Boeyé, 1983). Additionally, antibodies can attach to pathogens, tagging, or "opsonizing" them to be recognized by APCs for further processing (Casadevall & Pirofski, 2003). Finally, antibodies can activate the classical complement system – a proteolytic enzyme cascade leading to a membrane attack complex (MAC), which lyses the membranes of pathogens (Dunkelberger & Song, 2010)

Conversely, cellular immunity clears intracellular pathogens such as viruses (Zhang & Bevan, 2011; Zhu, Yamane, & Paul, 2010). Cytotoxic (CD8+) T cells target infected cells, releasing perforins and granzymes, which penetrate and lyse infected cells, respectively (Andersen, Schrama, Thor Straten, & Becker, 2006). In the course of lymphocyte activation memory B and T cells are generated, which reside in the lymphoid organs ready to react to an infection with the same antigen for many years, and are the basis of adaptive immunity (Hamann et al., 1997; McHeyzer-Williams, Okitsu, Wang, & McHeyzer-Williams, 2012).

Cytokines are a broad category of proteins that are produced by immune cells, endothelial cells, fibroblasts, and stromal cells (Turner, Nedjai, Hurst, & Pennington, 2014). They act through cell surface receptors and subsequent signaling cascades to alter cell function. Ultimately, each cytokine acts in a unique way to coordinate and tailor immune responses (Table 1). Table 1 represents a small sampling of the cytokines that are part of the innate and adaptive immune responses, relevant to our research. Cytokines link the innate and adaptive immune systems together, and balance the recruitment of humoral and cellular immunity based on specific pathogens encountered.

Table 1. Cytokines and their respective function

Adapted from (Linlin Chen et al., 2018; Turner et al., 2014)

Cytokine	Function
IL-1β	Primarily produced by macrophages. Inflammatory action induces activation of APCs, stimulates secretion of other cytokines and acts on the central nervous system as a pyrogen.
IL-2	Produced by T cells. Promotes T cell proliferation and differentiation of CD4+ to CD8+ T cells, enhancing cytotoxicity. Additionally, IL-2 acts on B cells to enhance antibody production.
IL-6	Produced primarily by macrophages. Inflammatory action induces proliferation of neutrophils and IL-6 acts on B cells to induce antibody production.
IL-10	Produced by APCs and T cells. Immune suppression; decreases antigen presentation and cytokine production by APCs and down regulates CD8+ T cells.
IL-12	Produced by APCs and B cells and T cells. Inflammatory action; promotes differentiation of T cells, and activates CD8+ T cells and NK cells.
TNFα	Primarily produced by macrophages. Inflammatory action; activates APCs.

* Interleukin (IL)

* Tumor necrosis factor (TNF)

1.2 Trained Immunity

Over the past decade, researchers have demonstrated that the innate immune system has a memory capacity of its own, termed "trained immunity" (Netea, Quintin, & Van Der Meer, 2011). Following a primary infection, innate immune cells undergo epigenetic and metabolic changes, resulting in increased responsiveness when they reencounter a pathogen (Saeed et al., 2014). In opposition to adaptive immune memory, trained immunity is transient and non-specific (Heintzman et al., 2009).

Early studies of trained immunity were done with β-glucan, a polysaccharide component of *Candida albicans'* cell wall. β-glucan is also a major component of the Bacillus Calmette-Guerin (BCG) live attenuated vaccine against *Mycobacterium tuberculosis* (Quintin et al., 2012). It was noted that both the BCG vaccine and β-glucan induced protection against secondary infections *in vivo* (Quintin et al., 2012). That is, following BCG vaccination, recombination activating gene 1 (RAG-1) deficient mice lacking B and T cells were able to survive a restimulation with a lethal dose of *C. albicans* (Quintin et al., 2012), suggesting innate immune-mediated memory. This led to the study of other bacterial and fungal pattern associated molecular patterns (PAMPs) as stimulants of trained immunity (Table 2).

Table 2. Agents and their mechanism of inducing trained immunity

Adapted from (Netea et al., 2016)

Innate Immune Cell	Trained Immunity Stimuli	Receptor/Signaling Cascade	Epigenetic Modifications	References
Monocytes/ Macrophages	LPS MPL S100A4	TLR4/ATF7	H3K4me1 H3K4me3 H2K27ac H3K9me2	(Foster, Hargreaves, & Medzhitov, 2007) (Ostuni et al., 2013) (Yoshida et al., 2015) (Neidhart et al., 2019) (Fensterheim et al., 2018)
Monocytes/ Macrophages	β-glucan C. albicans BCG	Dectin-1/Akt/mTOR- HIF1α NOD2	H3K4me1 H3K4me3 H3K27ac H3K9me2	(Quintin et al., 2012) (Saeed et al., 2014) (Cheng et al., 2014) (Yoshida et al., 2015) (Kleinnijenhuis et al., 2012)
Monocytes/ Macrophages	MDP CL429	NOD2 TLR2+NOD2	H3K4me3 H3K9me2 H3K27ac	(Ifrim et al., 2014) (Santecchia et al., 2019) (Kleinnijenhuis et al., 2012)
Monocytes/ Macrophages	Chitin	FIBCD1	Histone methylation	(Rizzetto et al., 2016)
Monocytes/ Macrophages	oxLDL	TLR/mTOR- HIF1α	H3K4me3	(Bekkering et al., 2014)
NK cells	CMV	SYK PLZF	Genome wide hypermethylation	(Lee et al., 2015) (Schlums et al., 2015)

The basal metabolic rate of resting immune cells is typically low, relying on glucose metabolism through oxidative phosphorylation (OXPHOS) (Pearce & Pearce, 2013). OXPHOS is the process in which a molecule of glucose, in the presence of oxygen, is broken down through glycolysis, the citric acid cycle, and electron transport chain to produce 32 molecules of energy in the form of adenosine triphosphate (ATP) (Vats et al., 2006). However, when immune cells are activated there is an increase in glucose uptake and cells switch from OXPHOS to aerobic glycolysis (Vats et al., 2006). Aerobic glycolysis is the process in which glucose is converted to lactate through glycolysis and lactic acid fermentation to produce 2 ATP. The benefit of aerobic glycolysis is the production of other essential precursors for the production of lipids, nucleic acids, and proteins as the cell's demands are elevated (Vats et al., 2006). It has been shown that a switch from OXPHOS to aerobic glycolysis is a metabolic hallmark of trained immunity (Cheng et al., 2014). In particular, cells trained with β-glucan acted through its receptor dectin-1 to activate the protein kinase B (Atk)- mammalian target of rapamycin (mTOR)/hypoxia-inducible factor 1α (HIF1α) pathway (Cheng et al., 2014). mTOR-HIF1α then modulates the switch in cellular metabolism from OXPHOS to aerobic glycolysis (Cheng et al., 2014).

In addition to metabolic adaptations, epigenetic modifications are another pilar of trained immunity. Epigenetics refers to a cell's ability to modify the expression level of genes and their products, without directly modifying the sequence of DNA base-pairs (Lu Chen et al., 2016). These modifications often occur on the proteins—histones—that wrap DNA into chromatin (Lu Chen et al., 2016) and therefore play an important role in controlling gene expression by condensing (repressing) or exposing DNA for transcription (Lu Chen et al., 2016). Many of the aforementioned metabolic processes are intimately linked with epigenetic changes because histone-modifying enzymes require their substrates, particularly, the itaconate pathway which

generates succinate and fumarate (Domínguez-Andrés et al., 2019). Generally, increased methylation of histone H3 at lysine 4 (H3K4) has been associated with increased transcription of proinflammatory cytokine genes (Wen, Dou, Hogaboam, & Kunkel, 2008). With respect to trained immunity, it has been proven that immune cells modify their histone acetylation and methylation patterns in response to training stimuli. For example, β-glucan trained macrophages monomethylate H3K4 (H3K4me1), trimethylate H3K4 (H3K4me3), acetylate H2 at lysine 27 (H2K27ac) and dimethylate H3 at lysine 9 (H3K9me2) (Saeed et al., 2014). These epigenetic modifications led to the production of significantly higher levels of TNFα and IL-6 compared to non-trained controls (Saeed et al., 2014). Similarly, in macrophages, MDP and CL429 stimulate nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and Toll like receptor 2 (TLR2), respectively to activate nuclear factor-κβ (NF-κβ) (Santecchia et al., 2019). Subsequently, NF-κβ signaling leads to H3K4me3, H3K9me2, and H3K27ac (Santecchia et al., 2019). These epigenetic modifications lead to increased surface expression of complement domain (CD) 11b, CD14, CD16 and TLR4 compared to non-trained controls (Santecchia et al., 2019). Additionally, MDP-trained macrophages with H3K4me3, H3K9me2 and H3K27ac produced significantly higher levels of TNFα and IL-1β compared to non-trained controls (Kleinnijenhuis et al., 2012). In another study, training macrophages with lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), and S100A4—TLR4 agonists—have activated mitogen-activated protein kinase (MAPK), which in turn phosphorylated activating transcription factor 7 (ATF7) (Neidhart et al., 2019). Phosphorylation of AFT7 leads to the release of AFT7 from chromatin and a decrease in repressive H3K9me2 patterns (Yoshida et al., 2015). Chitin, a major component of fungal cell walls, is another stimulant of trained immunity (Rizzetto et al., 2016). Studies have indicated chitin mediated training takes place through the Fibrinogen C domain-

containing protein 1 (FIBCD1) receptor to induce histone methylation in promoter regions of IL-6 and TNFα, increasing their expression (Rizzetto et al., 2016). Oxidized low-density lipoprotein (oxLDL) is another molecule researchers have investigated as a stimulant of trained immunity in macrophages. OxLDL is of interest as it contributes to macrophage-mediated atherosclerotic plaque formation, a common pathologic condition (Di Pietro, Formoso, & Pandolfi, 2016). Research indicates oxLDL stimulates the previously mentioned mTOR-HIF1α and induces H3K4me3 (Bekkering et al., 2014). Finally, NK cells have also demonstrated the properties of trained immunity. Cytomegalovirus (CMV)-induced NK decrease expression of spleen tyrosine kinase (SYK) transcription factor and promyelocytic leukemia zinc finger (PLZF) to induce genome-wide hypermethylation (Lee et al., 2015; Schlums et al., 2015).

On the whole, cells that have undergone training show a distinct trained immunity profile including metabolic and epigenetic modifications which translate into a unique phenotype. That is trained immune cells metabolically switch from OXPHOS to aerobic glycolysis to meet energetic demands. Additionally, trained immune cells epigenetically adapt; methylating H3K4 and H3K9 and acetylating H3K27. Together these changes increase proinflammatory cytokine expression (IL-1β, IL-6, and TNFα) and increase surface expression of CD11b, CD14, CD16, and TLR4.

1.3 Trained Immunity Restoring Balance

The immune system is an intricate, complex system, and while most of the time homeostasis is maintained there are many conditions in which many things can go awry. There are multiple states where immune regulation or balance is out of order, leading to an under or overactive immune system. Some people are born with poorly functioning immune systems in a

condition known as primary immunodeficiency. Severe combined immunodeficiency (SCID) is an example of a primary immunodeficiency where T and/or B cells are non-functional (Gennery & Cant, 2001). There are also many conditions that can weaken the immune system, leading to secondary or acquired immunodeficiency. Medications such as chemotherapy for cancer or anti-rejection medications following organ transplant can hinder immune function (Lane, 1990). Likewise, lifestyle factors such as old age, smoking tobacco, drinking alcohol or poor nutrition can lead to secondary immunodeficiency (Lane, 1990). Finally, pathogens such as human immunodefiency virus (HIV) can lead to acquired immunodeficiency syndrome (AIDS) (Schuman, Orellana, Friedman, & Teich, 1987). On the other hand, the immune system can be overactive. One example of this is allergies, such as asthma or eczema (atopic dermatitis) in which the immune system over reacts to substances in the environment that are usually harmless (Fonacier, Dreskin, & Leung, 2010; Lötvall et al., 2011). Moreover, the immune system can aberrantly attack self-tissues in a phenomenon known as autoimmunity. Common autoimmune diseases are type one diabetes (T1D), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Neidhart et al., 2019; Plagnol et al., 2011; Salinas, Braza, Brouard, Tak, & Baeten, 2013; Tan et al., 1982). In T1D, CD8+ cells attack pancreatic β cells responsible for making insulin to control blood sugar levels (Plagnol et al., 2011). RA pathology involves rheumatoid factor (RF) auto-antibodies that attack cartilage in joints (Neidhart et al., 2019). Likewise, auto-antibodies are produced attacking tissues body-wide in people with SLE (Tan et al., 1982).

Various studies indicated that trained immunity underlies some immune mediated diseases. For example, atherosclerosis involves the accumulation of fats (oxLDL) in blood that led to aberrant inflammatory macrophage involvement. Eventually, atherosclerosis can lead to

plaques in vessels, ultimately blocking blood flow (Di Pietro et al., 2016). Researchers have shown oxLDL induces trained immunity in atherosclerotic macrophages (Zhong, Yang, Feng, & Yu, 2020). In addition, S100A4 a common protein in the plasma of RA patients was found to induce trained immunity triggering of proinflammatory cytokines production in patients with RA (Neidhart et al., 2019).

Our hope is that trained immunity research will help to further understand its role in disease. Another goal is to use trained immunity to therapeutically tailor immune responses. Perhaps trained immunity could be used to restore cancer patients' immune system following the immunosuppressive effects of chemotherapy. Likewise, trained immunity could be evaluated as a vaccine adjuvant to enhance the immunogenicity of vaccines for immunocompromised populations, such as the elderly. That is, adjuvants that induce trained immunity could be included in vaccine formulations to train antigen presenting cells to react more robustly to the administered antigen, leading to an enhanced adaptive immune response. The development of immune training epigenetic modulators is also a promising therapeutic avenue, since epigenetic modifications are dynamic and reversible. On the other hand, understanding the process of trained immunity could lead to therapies that control hyper-inflammation. For example, inhibiting trained immunity may be a promising therapy for individuals with atherosclerosis, RA or SLE.

1.4 Trained Immunity Based Vaccines

Immunostimulants are a broad category of compounds that have the ability to stimulate any component of the immune system. Specific immunostimulants induce an immune response against a particular antigen (Ag), whereas non-specific immunostimulants promote a broad

response that lacks Ag specificity. Vaccines are an example of clinically relevant, specific immunostimulants, which often include an adjuvant—nonspecific immunostimulant—component to enhance and adjust the immune response. Adjuvants provide the benefit of vaccine dose sparing; lower doses with higher efficacy (Reed, Orr, & Fox, 2013). Additionally, adjuvants broaden immune responses and reduce the number of booster immunizations required by patients (Reed et al., 2013). Adjuvants developed to date include mineral salts, emulsions, cytokines, polymers, microbial products, saponins, and detergents. (Morel et al., 2011; O'Hagan et al., 1991; Romero Méndez, Shi, HogenEsch, & Hem, 2007). Despite the recent discoveries and understanding of trained immunity, there has been minimal research focusing on the implications of trained immunity with respect to adjuvants and vaccine development.

The principle of trained immunity-based vaccines is that inducers of trained immunity could be used as adjuvants, acting through pattern recognition receptors (PRRs) to induce the metabolic and epigenetic changes which will lead to changes in cytokine production and surface marker expression. This trained immunity phenotype will promote a short-term (weeks-months) memory response to nonspecific pathogens (Kleinnijenhuis et al., 2012; O'Leary, Goodarzi, Drayton, & von Andrian, 2006). That is, activated or "trained" immune cells can enhance adaptive responses to "by stander" antigens that were not components of the vaccine formulation. Additionally, we speculate that trained innate immune cells will influence the adaptive immune response via cytokines, linking the two arms of immunity together. Once adaptive immunity is stimulated by antigens in the vaccine formulation, a long-term specific response will be established. Taken together, the goal is that trained immunity will provide nonspecific protection and help promote better long-term adaptive immune responses to specific pathogens (Figure 1) (Covián et al., 2019). In all, generating a vaccine with broad protection is

an appealing approach for vulnerable populations who are prone to multiple coinfection of recurrent infections with antigenically drifting pathogens.

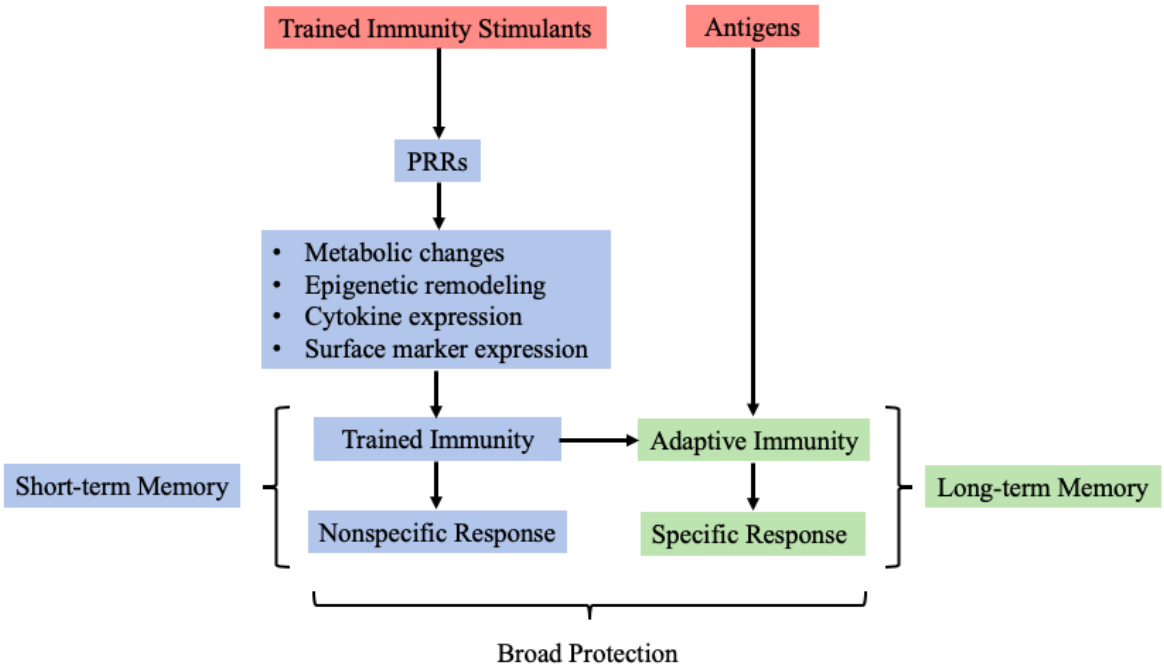


Figure 1. Trained immunity-based vaccines.

Figure modified from (Sánchez-Ramón et al., 2018)

1.5 Developing a Trained Immunity Based Adjuvant

Various agents (Table 2) have been shown to induce trained immunity. Two of particular interest are MDP and LPS.

MDP is an immunoreactive PAMP comprised of N-acetlyglutamic acid linked to an L-alanine-D-isoglutamine dipeptide (Figure 2) (Girardin et al., 2003). MDP is a component of peptidoglycan, which is in both Gram-negative and Gram-positive bacterial cell walls. MDP was recognized as the minimal active component of Freund's complete adjuvant (FCA), which is comprised of heat-inactivated *Mycobacterium tuberculosis*, and while too toxic for human use, is used as an adjuvant in experimental animals (Ellouz, Adam, Ciorbaru, & Lederer, 1974). MDP

potentiates its activity by binding to the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) PRR in the cytoplasm of macrophages, DCs, peripheral blood mononuclear cells (PBMCs) and Paneth cells (Gutierrez et al., 2002; K. S. Kobayashi et al., 2005; Lauro, D'Ambrosio, Bahnson, & Grimes, 2017; Ogura et al., 2001). Upon binding MDP, NOD2 undergoes a conformational change allowing the caspase recruitment domains (CARDs) to dimerize and signal through receptor-interacting protein kinase 2 (RIP2/RIPK2/RICK), inducing the NF-κB and MAPK signaling cascades (Girardin et al., 2003). NF-κB and MAPKs transcriptionally activate proinflammatory cytokines IL-6, IL-12, proIL-1β and TNFα (Girardin et al., 2003). NF-κB and RIP2 additionally activate the inflammasome, composed of a PRR, pro-caspase-1 and apoptosis-associated speck-like protein (ASC) (Latz, Xiao, & Stutz, 2013). Activation of the inflammasome promotes activation of caspase-1, which cleaves proIL-1β into mature IL-1β (K. Kobayashi et al., 2002; K. S. Kobayashi et al., 2005; Murillo, Morré, & Peña, 2003). The proinflammatory cytokines induced by MDP can recruit, and promote the proliferation and differentiation of lymphocytes to tailor immune responses (Table 1). TNFα and IL-1β activate APCs, IL-6 recruits neutrophils and promotes B cell antibody production and IL-12 activates CD8+ T cells and neutrophils. On the whole, MDP is able to modulate the innate immune system as well as humoral and cellular immunity. Thus, it is of much interest to evaluate MDP's ability to induce trained immunity *in vitro*.

LPS, a PAMP present on the outer membrane of Gram-negative bacteria, is recognized by TLR4, a subclass of PRRs expressed by immune cells (Muroi, Ohnishi, & Tanamoto, 2002). LPS is made of three components, including an O side chain, a core oligosaccharide, and lipid A. Lipid A is recognized and interacts with TLR4 via lymphocyte Ag 96 (MD-2) (Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990). The LPS-MD-2/TLR4 complex then initiates TLR4

dimerization and the binding of MyD88 adaptor-like protein (Mal) to MyD88 via the Toll-interleukin-1 receptor (TIR) domain (Hailman et al., 1994). MyD88 then activates the NF-κB signaling cascade (Lu, Yeh, & Ohashi, 2008). LPS toxicity inhibits its clinical relevance, therefore many lipid A derivatives have been isolated or synthesized to decrease toxicity and maintain immunogenicity (Mata-Haro et al., 2007). MPL, is a nontoxic lipid A derivative of LPS derived from *Salmonella minnesota* (Baldridge & Crane, 1999), whereas glucopyranosyl lipid A (GLA; Figure 3) is a purely synthetic lipid A (Caroff, Karibian, Cavaillon, & Haeffner-Cavaillon, 2002). Since both LPS and MPL have proven to induce trained immunity (Table 2), we speculate GLA might as well, given the shared signalling cascade. It has been shown that MDP and LPS have a synergistic effect on proinflammatory cytokine production (Traub, von Aulock, Hartung, & Hermann, 2006). When administered with LPS, MDP increases the amount of NF-κB and also activates MAPKs, resulting in synergistic transcriptional activation of proinflammatory cytokines (Fritz et al., 2005). Therefore, evaluating the combined training effects of MDP and GLA is of interest to us.

The bioavailability of peptides is often a limiting factor to therapeutic potential (Ahmad, Amin, Ismail, & Buang, 2016; Diao & Meibohm, 2013). Peptides are poorly absorbed, rapidly degraded by proteases and quickly eliminated from the body (Ahmad et al., 2016; Diao & Meibohm, 2013). Researchers have investigated liposomal entrapment as a method of extending the half-life of peptides, protecting them from degradation (Ryu & Park, 2010; Tu, Hao, Kharidia, Meng, & Liang, 2007; Zhou et al., 2017). The amphipathic nature of liposomes is an appealing property with respect to entrapment because it allows the incorporation of both hydrophobic and hydrophilic molecules (Sercombe et al., 2015). Furthermore, the amphipathic liposomes allow delivery beyond phospholipid bilayers (Sercombe et al., 2015). The work done

by Dr. Hoang-Thanh Le's lab at HSNRI showed the stability of MDP is preserved by liposomal entrapment (Lewicky et al., 2018). The liposome used by Lewicky et al. was formulated according to previous research outlining the assembly of glycolipids (Goyard et al., 2016). That is, when entrapped in a liposome the degradation of MDP in rat plasma is delayed 24 hours longer than free MDP (Lewicky et al., 2018). Another advantage of liposomal carriers is that they can be formulated to act on the immune system (Zhou et al., 2017). For example, manosylated (Figure 4) liposomes target mannose receptors on APCs (Costa, Sarmento, & Seabra, 2018; Wijagkanalan et al., 2008). Additionally, studies have shown positive charges enhance the uptake of liposomes by APCs (Nakanishi et al., 1999). We are interested in formulating a delivery system (DS) comprised of MDP and GLA entrapped within a cationic manosylated liposome (CL, Figure 5). The goal of the liposome is to stabilize MDP and deliver MDP and GLA together to capitalize on their synergistic effects. We believe that the cationic mannosylated liposomes will deliver the stably entrapped training stimuli, MDP and GLA, directly to APCs and simultaneously train them through the NOD2 and TLR4, respectively

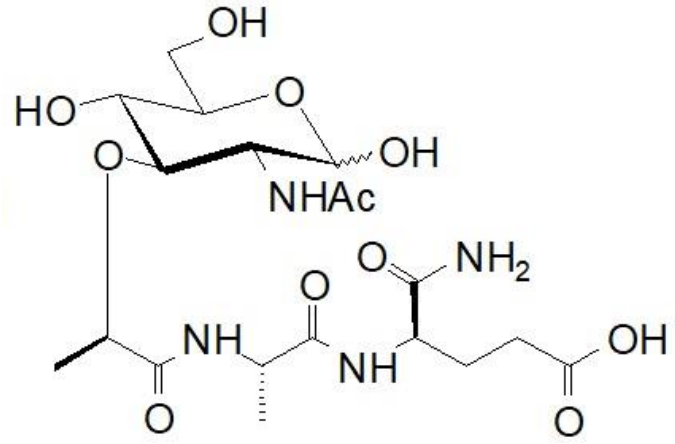


Figure 2. Muramyl dipeptide.

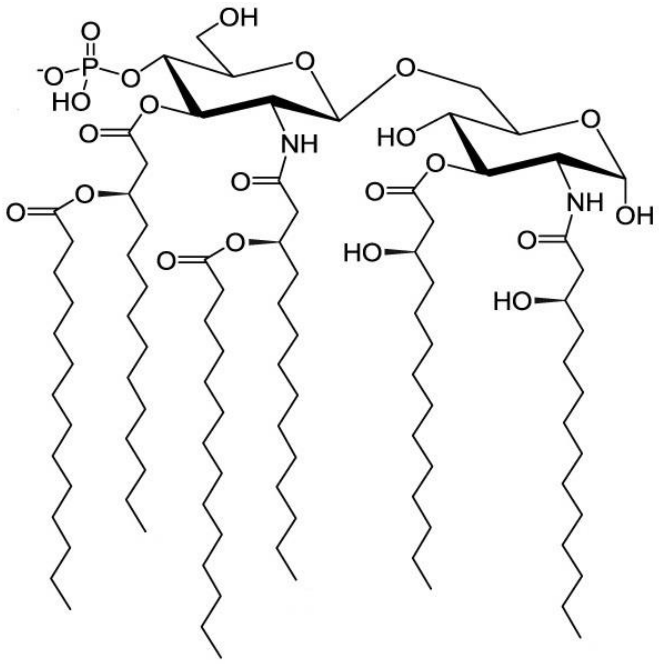


Figure 3. Glucopyranosyl Lipid A.

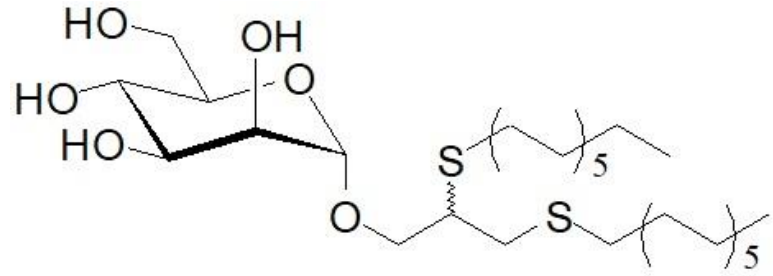


Figure 4. Mannose Lipid (ML).

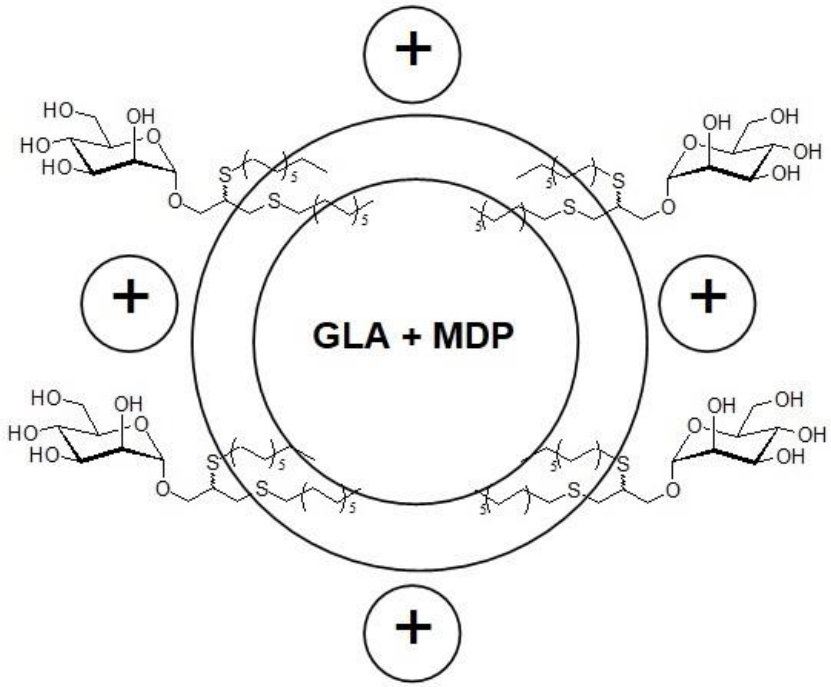


Figure 5. Delivery system (DS); MDP and GLA in a cationic mannose lipid liposome (CL).

1.6 Models Used to Study Trained Immunity

To date trained immunity has been studied using human PBMCs and primary cells from mice such as bone marrow derived macrophages (BMDMs). Studies using PBMCs use a variety of methods to extract and purify monocytes from whole PMBC populations (Domínguez-Andrés et al., 2019; Santecchia et al., 2019). One method used to isolate monocytes from PBMCs is a Percoll gradient, which utilizes separation by both size and density (Repnik, Knezevic, & Jeras, 2003). Another method is the use of magnetic cell separation (MACS) technology using nano-sized beads to positively select monocytes from PBMCs (Cheng et al., 2014). Finally, researchers have also used wash off methods, in which monocyte/macrophage are selected for experimentation by seeding PBMCs in plates, allowing them to settle and then washing off suspended cells, leaving adherent monocytes/macrophages behind on the plate (Quintin et al.,

2012). The benefits of using primary cells from humans for immunological research are numerous. For one, PBMCs contain a mosaic of peripheral immune cells; lymphocytes (70-90%), monocytes (10-20%) and dendritic cells (1-2%) (Autissier, Soulas, Burdo, & Williams, 2010). Among the lymphocyte population 70-85% are T cells, 5-10% are B cells and 5-20% are NK cells (Autissier et al., 2010). PBMCs can be used whole to model an interactive immune response or cell subsets can be isolated for experimentation, as previously mentioned. Another benefit of PBMCs, is they can be easily obtained from human blood (Pourahmad & Salimi, 2015). However, PBMCs can be costly as a trained nurse or phlebotomist must extract blood samples (Fuss, Kanof, Smith, & Zola, 2009). Additionally, a variety of ethical concerns are raised when using human samples in research. While there are many benefits of using PBMCs, there are some drawbacks with respect to trained immunity focused research. For example, macrophages are a major focus of trained immunity research and PBMCs are only comprised of approximately 10-20% monocytes, thus requiring larger quantities of PBMCs. These monocytes can then be differentiated *in vitro* to macrophages using monocyte colony stimulating factor (M-CSF) or LPS and interferon gamma (IFNγ) (Zarif et al., 2016). Alternatively the monocytes can be differentiated *in vitro* to DCs using granulocyte-monocyte colony stimulating factor (GM-CSF) (Zarif et al., 2016). Additionally, PBMCs are sensitive to variables such as temperature and method/time of extraction in a lab setting that may hinder data (Cosentino et al., 2007). Another important aspect is the heterogeneity found in PBMCs between donors, which is both advantageous as it offers a better representation of the population, but it also requires more samples from different donors to obtain statistically relevant results. Finally, while PBMCs mimic an immune responses *ex vivo*, they do not mimic *in vivo* responses involving heterogeneity, environmental exposures, sex and travel through the lymph system.

Bone marrow derived macrophages (BMDMs) are also extensively used in trained immunity related research (Madaan, Verma, Singh, Jain, & Jaggi, 2014). Typically, bone marrow cells are isolated from the femurs of mice and then subjected to macrophage-colony stimulating factor (M-CSF) to differentiate the monocyte population of the bone marrow into macrophages (Ciarlo et al., 2019; Santecchia et al., 2019). When the M-CSF procedure is complete macrophages adhere to plates and the remaining BMDM suspension can be removed (Ciarlo et al., 2019; Santecchia et al., 2019). Finally, trained immunity has been studied *in vivo*, with mice (Covián et al., 2019; Garcia-Valtanen, Guzman-Genuino, Williams, Hayball, & Diener, 2017; Quintin et al., 2012). *In vivo* training protocols involve intraperitoneal (IP) injection-based training and restimulation, followed by collection of blood to analyze immune responses (Covián et al., 2019; Garcia-Valtanen et al., 2017; Quintin et al., 2012). The mouse (*Mus musculus*) has been extensively used as an animal model for mammalian immunological research *in vivo*, for many reasons. Primarily, mice share many characteristics with humans on physiological and pathological levels. In particular, mice share 90% gene homology with humans (Waterston et al., 2002). Additionally, mice are small, breed rapidly and are relatively easy to maintain in the laboratory (Viney, Lazarou, & Abolins, 2015). Additionally, common mouse strains are inbred allowing for reproducibility (Viney et al., 2015). However, there are unique challenges when using mice for trained immunity studies. Namely, species specific idiosyncrasies—differences in genetics, lifespan and multiorgan responses—confound research making it more difficult to extrapolate results to humans (Mestas & Hughes, 2004). Additionally, lab husbandry practices greatly influence a mouse's immune response (Mestas & Hughes, 2004). Mice are very sensitive to variables such as temperature, humidity, and sound, which can lead to

stress (Andrews, 1995; Feldmann, Golozoubova, Cannon, & Nedergaard, 2009). This stress can profoundly influence immune responses (Andrews, 1995; Feldmann et al., 2009).

While both PBMCs and mice are excellent models to study trained immunity they each come with their own setbacks outlined above. It is important to acknowledge the challenges these models propose, take them into account when interpreting studies and work to bridge those gaps. Therefore, we aim to develop an *in vitro* model to study trained immunity. Better characterization of an *in vitro* cell model to study trained immunity would provide a sustainable avenue for researchers to elucidate the phenomenon and develop novel therapeutics. The many advantages of *in vitro* cell models include cost efficiency, ease of use, reliability, reproducibility and less ethical concerns (Kaur & Dufour, 2012).

1.7 Cell lines

Three murine monocyte/macrophage cell lines RAW 264.7, J774A.1 and IC-21 are commonly used in immunology focused research (Chamberlain, Godek, Gonzalez-Juarrero, & Grainger, 2009). RAW 264.7 are from an adult male BALB/c mouse with Abelson murine leukemia virus-induced tumor (Taciak et al., 2018). J774A.1 are from an adult female BALB/c mouse reticulum cell sarcoma (Lam, Herant, Dembo, & Heinrich, 2009). Whereas, IC-21 are from an adult male C57BL/6 mouse (Wu-Hsieh & Howard, 1989). Each of the cell lines mentioned are along the differentiation spectrum between monocytes and macrophages, which we will denote as "monocyte/macrophage". RAW 264.7 cells are monocyte/macrophages with a high basal rate of cytokine production (Chamberlain et al., 2009). However, following LPS stimulation RAW 264.7 cells produce very little IL-6 (Chamberlain et al., 2009). Similarly, following stimulation with LPS, IC-21 cells did not produce significantly more TNFα, IL-6, IL-

12 or IL-10 compared to control cells (Chamberlain et al., 2009). Perhaps attributable IL-21 cells' low expression of CD14, involved in responding to LPS in conjunction with TLR4 (Chamberlain et al., 2009). It is for that reason that RAW 264.7 and IL-21 cells were not selected as a cell line for developing a trained immunity model. Finally, J774A.1 cells express a monocyte/macrophage phenotype (Chamberlain et al., 2009). Moreover, it has been shown J774A.1 cells express TLR4 and NOD2, receptors of interest with respect to trained immunity stimulants (Mazaleuskaya, Veltrop, Ikpeze, Martin-Garcia, & Navas-Martin, 2012; Schäffler et al., 2014). These differences in phenotype warrant justification for selection of a cell line for specific research purposes. Thus, all things taken into consideration, J774A.1 will be studied as a potential murine *in vitro* model for trained immunity.

THP-1, U937, HL-60 and PBL-985 are human monocyte cell lines frequently used in immunological research (Chanput, Mes, & Wichers, 2014; Fleck, Romero-Steiner, & Nahm, 2005; Tucker, Lilly, Heck, & Rado, 1987). THP-1 cells have the phenotype of a monocyte, whereas U937 have an intermediated monocyte/macrophage phenotype (Chanput et al., 2014). THP-1 cells originate from the blood of a leukemia patient, therefore are less mature, whereas U937 cells are of tissue origin and more mature (Chanput et al., 2014). Research has shown THP-1 and U937 are differentially inclined towards M1 and M2 phenotypes, respectively (Dintakuri et al., 2017). In 2016 a study was done comparing the response of THP-1, U937 and human macrophages derived from PBMCs following mycobacterial infections. The finding of this study indicate U937 has a lower phagocytic capacity than THP-1 and PBMC derived macrophages (Mendoza-Coronel & Castañón-Arreola, 2016). Furthermore, THP-1 cells functioned more similarly to macrophages derived from PBMCs, producing similar amounts of TNFα following mycobacterial infections (Mendoza-Coronel & Castañón-Arreola, 2016). Since,

mycobacteria BCG is a stimulant of trained immunity these studies suggest that THP-1 cells will be more likely to show a trained immune profile in comparison to U937 cells. Literature also shows that LPS and MDP act synergistically in macrophage differentiated THP-1 cells to enhance proinflammatory immune responses (S. Yang et al., 2001). This prompts us to believe GLA and MDP—training stimuli of interest—may elicit a similar response in this cell line. It is important to note THP-1 cells are a human monocyte cell line and therefore require PMA, vitamin D3 (VitD3) or M-CSF to differentiate them into macrophages (Chanput et al., 2014). Additionally, THP-1 cells can be pushed to become M1 macrophages (inflammatory) when incubated with LPS or interferon gamma (IFNγ) (Chanput et al., 2014). Alternatively, THP-1 cells can be pushed to become M2 macrophages (anti-inflammatory) when incubated with IL-2, IL-4 or IL-10 (Chanput et al., 2014). HL-60 and PBL-985 both have a neutrophil phenotype, since we are seeking a cell line with a monocyte-macrophage phenotype these cells were excluded (Fleck et al., 2005; Tucker et al., 1987)Taken together, the similarity to PBMC macrophages and inflammatory responses to BCG, MDP and LPS make THP-1 cells the best candidate to be evaluated as a human model to study trained immunity *in vitro*.

Finally, Jurkat cells are a human-derived T lymphocyte cell line used in immunology research, particularly acute T cell leukemia and human immunodeficiency virus (HIV) research (Abraham & Weiss, 2004; Schneider, Schwenk, & Bornkamm, 1977; Takeuchi, McClure, & Pizzato, 2008). Furthermore, Jurkat cells are well established model used to study T cell signaling (Abraham & Weiss, 2004; Schneider, Schwenk, & Bornkamm, 1977). Therefore, we are interested in using Jurkat T cells to evaluate the link between innate trained immunity and the adaptive immune system.

1.8 Hypothesis and Objectives

While the majority of research has been focused on using *ex vivo* and *in vivo* models to evaluate trained immunity, we are interested in developing an *in vitro* model to study trained immunity. We specifically chose the murine J774A.1 and human THP-1 cell lines as *in vitro* models because of their well characterized immunology and speculated trained immunity potential.

Trained immunity will be evaluated by analyzing cytokine output and cell surface marker expression by the cells *in vitro*. Studies show that when stimulated with various agents (Table 2) TNFα production increases compared to non-trained cells. Furthermore, TNFα is a proinflammatory cytokine primarily produced by macrophages (Table 1), which is the immune cell of interest in our studies. Therefore, TNFα responses will be evaluated. Conversely, IL-10 is an anti-inflammatory cytokine produced to counteract proinflammatory immune responses to achieve homeostasis (Linlin Chen et al., 2018). While IL-10 has not been a major focus in trained immunity-based research, we thought it would be critical to evaluate trained immune responses in terms of pro and anti-inflammatory cytokine production. Elevated expression of cell surface markers CD14, CD16 and CD11b have been associated with trained immunity. Therefore, flow cytometry will be used to track changes in expression of these surface markers. Finally, epigenetic modification is another way to verify trained immunity. Markers such as H3K4 methylation and H3K9/H3K27 acetylation have been associated with trained immunity. While our study does not plan to focus on epigenetics, we acknowledge it as an important player in the trained immunity cell profile, and would like to explore it in future studies.

We aim to utilize the delivery system comprised of MDP and GLA entrapped within a cationic manosylated liposome (Figure 5) to target immune cells and simultaneously train them

through the NOD2 and TLR4 signaling cascades. Finally, we will evaluate the link between trained immunity and adaptive immunity.

All in all, we had 4 main objectives:

1. Develop murine and human *in vitro* models to study trained immunity
2. Use these *in vitro* models to screen for trained immunity stimulants
3. Develop a NOD2-TLR4 delivery system to induce trained immunity
4. Evaluate the link between delivery system mediated trained immunity and adaptive immunity

We hypothesize that both J774A.1 cells and THP-1 cells will show a trained immunity profile following stimulation with various adjuvant/delivery system formulations. Additionally, we hypothesize that delivery system will induce trained immunity. Finally, we speculate that trained innate immune cells will influence the adaptive immune response via cytokines, linking the two arms of immunity together.

2. MATERIALS & METHODS

2.1 Cell culture

2.1.1 J774A.1 cell line and subculturing

J774A.1 (ATCC® TIB-67™, Manassas, VA) cells are a reticulum cell sarcoma tumor derived monocyte-macrophage cell line, isolated from female BALB/c mice. J774A.1 cells were cultured in 75 cm^2 treated tissue-culture polystyrene (TCPS) flasks (Thermo Fisher Scientific, Rochester NY) in DMEM media supplemented with 10% heat inactivated (HI) FBS and 1% penicillin-streptomycin. Cells were incubated in the presence of 5% CO_2 at 37 °C. For inactivation, FBS was incubated in a water bath at 56 °C for 30 minutes. Cells were grown to confluency, dislodged with a cell scraper, collected and centrifuged at 1000 rpm for 5 minutes. The cell pellet was then resuspended in media and the cells re-seeded for subculture. J774A.1 cells were used for experiments between passage number 5-25.

2.1.2 THP-1 cell line and subculturing

THP-1 (ATCC® TIB-202™) cells are an acute monocytic leukemia derived monocyte cell line, isolated from a 1-year-old male. THP-1 cells were cultured in 25 cm^2 non-treated TCPS flasks in RPMI-1640 medium with 10% HI-FBS and 1% penicillin-streptomycin. Cells were seeded at a density of 5 x 10^5 cells/mL and incubated in the presence of 5% CO_2 at 37 °C. Cells were maintained between the recommended densities according to ATCC, collected and centrifuged at 400 x g for 10 minutes. The cell pellets were then resuspended, counted using trypan blue and a haemocytometer and re-seeded for subculture. THP-1 cells were used for experiments between passage number 5-25.

2.1.3 Jurkat cell line and subculturing

Jurkat (ATCC® TIB-152™) cells are a male-isolated acute T cell leukemia derived T lymphocyte cell line. Jurkat cells were cultured in 25 cm^2 non-treated TCPS flasks in RPMI-1640 medium supplemented with 10% HI-FBS and 1% penicillin-streptomycin. Cells were seeded at a density of 5 x 10^5 cells/mL and incubated in the presence of 5% CO_2 at 37 °C. Cells were maintained between the recommended densities according to ATCC, collected and centrifuged at 400 x g for 10 minutes. The cell pellets were then resuspended, counted using trypan blue and a haemocytometer and re-seeded for subculture. Jurkat cells were used for experiments between passage number 5-25.

2.2 Training and restimulation agents

2.2.1 Delivery system formulation

MDP (Fig. 2) and GLA (Fig. 3) aliquots, both in water, were combined with a stock concentration of ML (Figure 4), in tert-Butanol. The resulting solution—DS (Fig. 5); MDP and GLA entrapped in the cationic mannose liposome—was vortexed thoroughly, and then diluted to a final concentration of 5 μg/mL based on the particle (Lewicky et al., 2018). ML was provided by collaborator Rene Roy from University de Quebec au Montreal (UQAM), QC, Canada. Preparation of the mannose lipid for entrapment followed previously published methods (Goyard et al., 2016). DS training concentration along with other training agents and their respective concentrations are outlined in Table 3. Restimulation agents and their concentrations are outlined in Table 4.

Table 3. Training Agents

Training Agent	Concentration
Non-trained; NT (media)	
LPS	5 ng/mL
GLA	5 ng/mL
MDP	10 μg/mL
CL	5 μg/mL
CL+GLA	5 μg/mL* (5 μg/mL 5 ng/mL)
CL+MDP	5 μg/mL* (5 μg/mL, 10 μg/mL)
DS (CL+MDP+GLA)	5 μg/mL* (5 μg/mL, 10 μg/mL, 5 ng/mL)

***** Concentration based on the particle.

Table 4. Restimulation Agents

Restimulation Agent	Concentration
Dukoral	1 x 10^3 vibrios
LPS	10 ng/mL
MDP	10 μg/mL
Influenza virus (H3N2)	Multiplicity of infection (MOI) 2

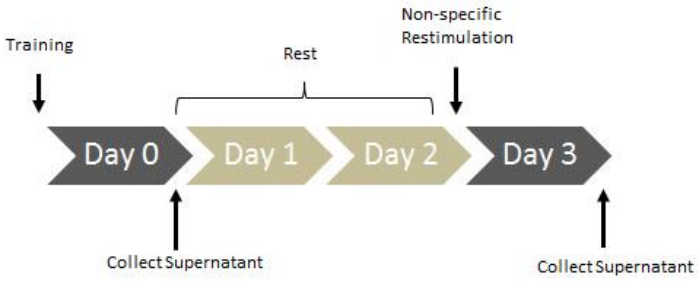
2.3 *In vitro* studies

2.3.1 J774A.1 and THP-1 training optimization

J774A.1 cells were harvested at confluency, counted and reseeded at a density of 5 x 10^5 cells/mL in 75 cm^2 treated TCPS flasks for training. THP-1 cells were harvested at a density of 1 x 10^6 cells/mL, counted and reseeded at a density of 5 x 10^5 cells/mL in 25 cm^2 non-treated TCPS flask for training. J774A.1 cells and THP-1 cells were left untrained, or trained with LPS

or MDP (Table 3). Following a 24-hour incubation (5% CO_2, 37 °C) supernatants were collected, cells were washed with 1X PBS and re-seeded in flasks with media to rest for either 2 days (Fig. 6A) or 5 days (Fig. 6B). On day 3 (Fig. 6A) and day 6 (Fig. 6B), supernatants were collected, and cells were harvested and seeded in 24-well tissue-culture treated plates (Thermo Fisher Scientific, Rochester NY) at a density of 5 x 10^5 cells/mL of media and left untreated or were non-specifically restimulated with LPS (Table 4), in triplicate, for 24 hours (5% CO_2, 37 °C). Following the 24-hour incubation, cell-free supernatants were collected, stored at -20 °C and later analyzed by ELISA (section 2.5.1) to quantify TNFα and IL-10 cytokine production.

A.



B.

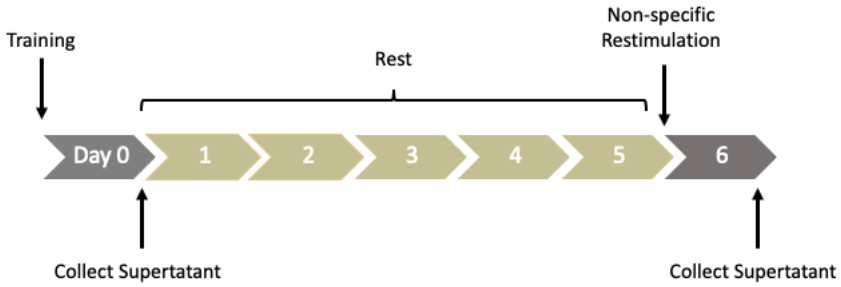


Figure 6. J774A.1 and THP-1 training optimization.

(**A**) 3-day training and restimulation protocol. (**B**) 6-day training and restimulation protocol.

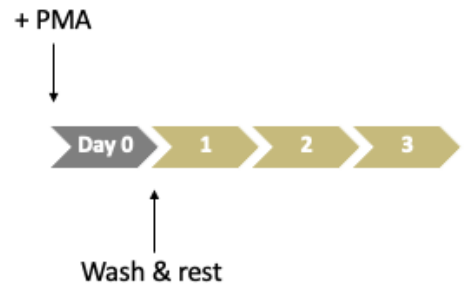
2.3.2 J774A.1 and THP-1 monocyte training and restimulation

The 3-day training/restimulation protocol was used (Fig. 6A), J774A.1 cells were trained using the stimuli outlined in Table 3. On day 3, cells were harvested and seeded in 24-well tissue-culture plates at a density of 5 x 10^5 cells/mL media and left untreated or non-specifically restimulated (Table 4), in triplicate, for 24 hours (5% CO_2, 37 °C). Following the 24-hour incubation, cell-free supernatants were collected, stored at -20 °C and later analyzed by ELISA (section 2.5.1) to quantify TNFα and IL-10 cytokine production.

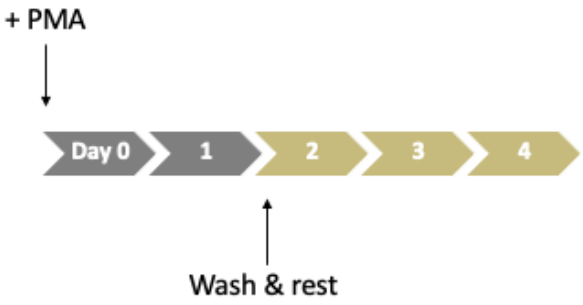
2.3.3 THP-1 monocyte to macrophage differentiation

THP-1 cells were harvested and seeded as previously described with the addition of 100 ng/mL phorbol myristate acetate (PMA) (Daigneault, Preston, Marriott, Whyte, & Dockrell, 2010; Mendoza-Coronel & Castañón-Arreola, 2016; Starr, Bauler, Malik-Kale, & Steele-Mortimer, 2018). THP-1 cells were PMA-differentiated for 24, 48, or 72 hours (5% CO_2, 37 °C) (Fig. 7). Following their respective differentiation and 3-day rest period, THP-1 macrophages were harvested (Fig. 7) and prepared for analysis via flow cytometry (section 2.5.2, Table 5).

A



B



C

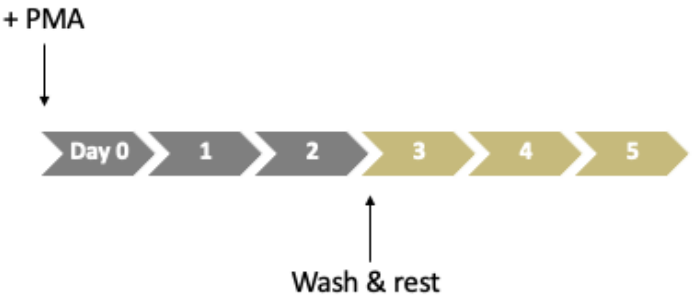


Figure 7. THP-1(**A**) 24 hour, (**B**) 48 hour, and (**C**) 72-hour PMA differentiation protocol.

2.3.4 THP-1 macrophage training and restimulation

THP-1 cells were differentiated according to the 72-hour PMA-differentiation and 3-day rest protocol, previously outlines in section 2.4.3. Next, PMA differentiated THP-1 cells were treated with their respective training stimuli (Table 3) in 24-well tissue-culture plates at a density of 5 x 10^5 cells/mL in triplicate. Cells were incubated for 24 hours (5% CO_2, 37 °C), supernatants were collected, cells were washed with 1X PBS and supplemented with media to rest for 2 days. On day 3, trained THP-1 macrophages were left untreated or non-specifically restimulated (Table 4), in triplicate, for 24 hours (5% CO_2, 37 °C). Following the 24-hour incubation, cell-free supernatants were collected, stored at -20 °C and later analyzed by ELISA (section 2.5.1) to quantify TNFα and IL-10 cytokine production (Fig. 8).

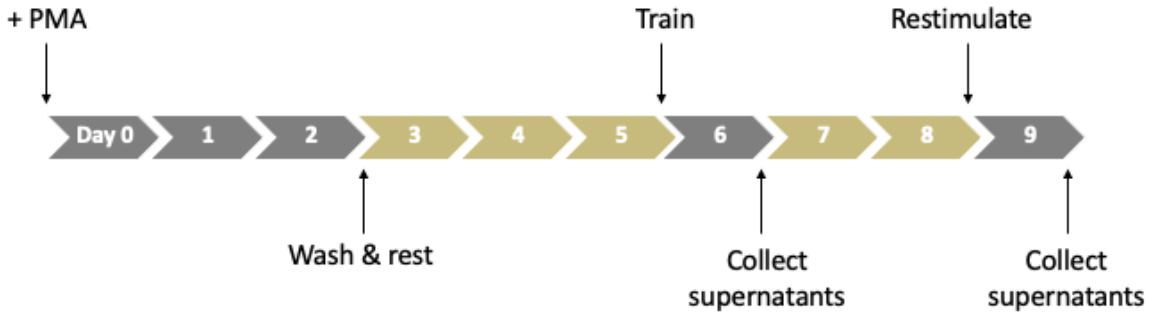


Figure 8. THP-1 PMA differentiation and training timeline.

2.3.5 THP-1 macrophages inhibited with CLI-095 and/or Gefitinib

PMA differentiated THP-1 macrophages were seeded in 24-well tissue-culture treated plates at a density of 5 x 10^5 cells/mL and incubated with 20 ng/mL CLI-095 for 6 hours and/or incubated with 500 nM Gefitinib (InvivoGen, San Diego, CA) for 1 hour in the presence of 5% CO_2 at 37 °C (Fig. 9). As a control, a group of THP-1 cells were seeded in media and left uninhibited. Following inhibition, the THP-1 macrophages were delivery system trained (Table

3) and restimulated with Dukoral (Table 4) according to the optimized 3-day training/restimulation protocol (Fig. 6A).

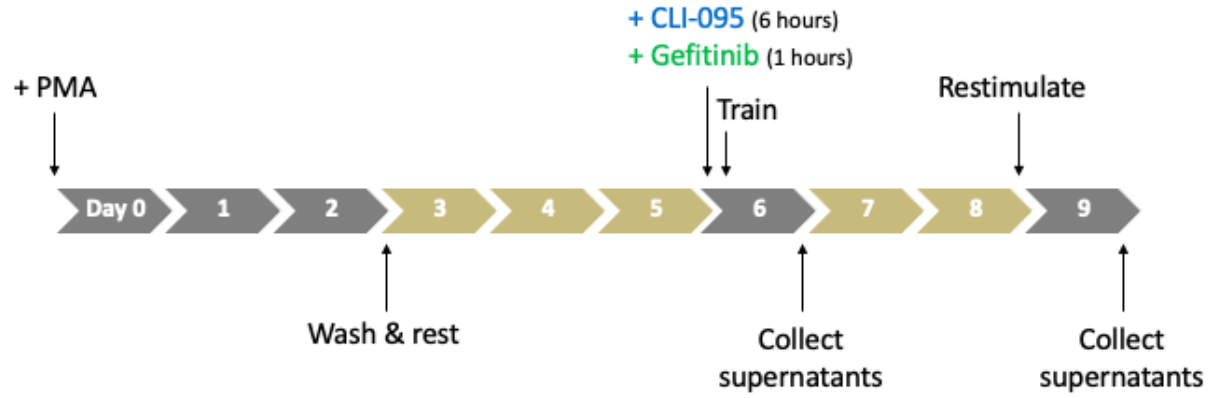


Figure 9. THP-1 PMA differentiation, CLI-095/Gefitinib inhibition and training timeline.

2.3.6 Jurkat activation (CD3 and CD28 costimulation)

96-well flat-bottom high binding assay plates (Corning 9018) were incubated with 50 µL of 5 µg/mL adherent anti-CD3e (OKT3) at 37 °C for 2 hours. For the control, 50 µL 1X PBS was added to the wells. The plates were then washed and rinsed two times with 200 µL 1X PBS. Jurkat cells were then harvested at a density of 1 x 10^6 cells/mL, collected and resuspended to 1 x 10^5 cells/mL in RPMI-1640 medium supplemented with 10% HI-FBS and 1% penicillin-streptomycin. Sequentially, 100 µL of the cell suspension was added to each well for a final cell concentration of 1 x 10^4 cells/well. Finally, 50 µL of 2 µg/mL soluble anti-CD28 was added to the wells and plates were incubated in the presence of 5% CO_2 at 37 °C for 48 hours. Following stimulation, cells were left to rest in media for 48 hours before starting the co-culture experiments.

2.3.7 Trained THP-1 macrophage and Jurkat co-culture

The PMA differentiated THP-1 macrophages were delivery system trained (Table 3), in triplicate, for 24 hours (5% CO_2, 37 °C), supernatants were collected, and cells were washed with 1X PBS (Fig. 10). Next, CD3/CD28 activated Jurkat T cells (1 x 10^6 cells/mL) were added to PMA differentiated THP-1 macrophages (5 x 10^5 cells/mL) in the 24-well tissue-culture treated plates, supplemented with media and left to rest for 48 hours (day 7-8; Fig.10). On day 9, co-cultured cells were left untreated or non-specifically restimulated with Dukerol (Table 4, Fig. 10) for 24 hours (5% CO_2, 37 °C). Following the 24-hour incubation, cell-free supernatants were collected, stored at -20 °C and later analyzed by ELISA (section 2.5.1) to quantify IL-2 cytokine production. (Fig. 10).

Figure 10. THP-1 PMA differentiation, training and Jurkat cell co-culture timeline.

2.4 *Ex vivo* studies

2.4.1 Animal Husbandry

Six to eight-week-old female BALB/c mice were purchased from Charles River (OC, Canada). Mice were housed in Innocage® cages at the Animal Care Facility at Laurentian University where they were fed specialized rodent feed and watered via Aquavive® 250 mL water bottles. Mice were feed and watered *ad libtum*. The temperature in the animal care room

was maintained at 21± 2 °C and a humidity of 55 ± 5%, parameters were recorded daily. All protocols were approved by the Animal Care Committee at Laurentian University and the Biosafety Committee at HSNRI. Mice were sacrificed under isoflurane followed by a cardiac exsanguination and cutting of the diaphragm prior to BMDM isolation.

2.4.2 BMDM isolation, differentiation, training and restimulation

Hind legs were obtained from 3 female BALB/c mice and excess tissue was removed to isolate the tibia and femur of each leg. The epiphyses of each bone were then trimmed off and the marrow was flushed out using HBSS and a 1-mL insulin syringe. The bone marrow cell suspension was then washed and centrifuged 3 times at 250 x g for 8 minutes. The cell suspension was then counted using trypan blue and a haemocytometer and resuspended to achieve a concentration 10 x 10^6 cells/mL concentration. The cells were resuspended in RPMI-1640 supplemented with 8% HI-FBS, 1% penicillin-streptomycin and 20 ng/mL M-CSF. To differentiate the bone marrow cells to macrophages, 4 x 10^6 cells were added to each petri dish containing 10 mL of the aforementioned M-CSF supplemented media. The cells were then incubated for 3 days (5% CO_2 at 37 °C) and then another 10 mL of fresh M-CSF media was added for an additional 3 days (Fig. 11). The BMDM isolation and differentiation protocol was adapted from (Madaan et al., 2014). Following differentiation, the BMDMs dislodged with a cell scraper, collected and centrifuged at 250 x g for 8 minutes. The cell pellet was then resuspended in media and the cells were seeded in 24-multiwell tissue culture plates at a density of 5 x 10^5 cells/mL and trained with their respective stimuli (Table 3) for 24 hours, in triplicate (Fig. 11). Supernatants were collected after the 24 hours of training, the cells washed and rested in fresh media for 5 days. On day 6, the BMDMs were non-specifically restimulated (Table 4) for 24

hours. Cell-free supernatants were collected, stored at -20 °C and later analyzed by ELISA (section 2.5.1) to quantify TNFα and IL-10 cytokine production.

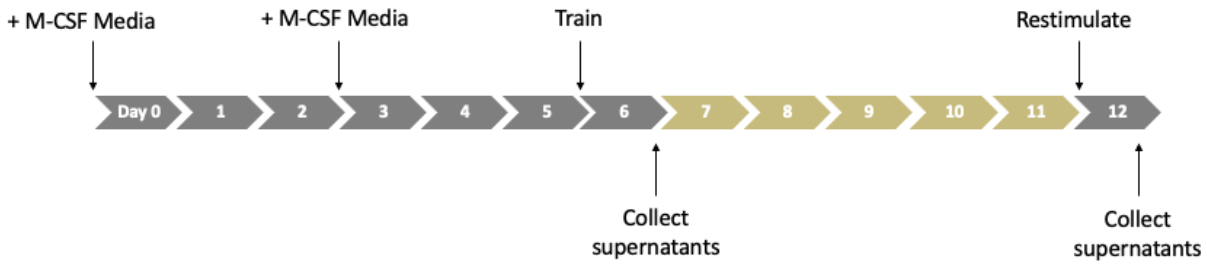


Figure 11. BMDM extraction and training timeline.

2.4.3 PBMC Training

PBMCs from one donor were given to our lab by Dr. Hoyun Lee's Lab at HSNRI, from a biobanked depository of healthy human PBMCs from Canadian Blood Services. PBMCs were taken out of liquid nitrogen and the 1 mL cryo-vial of PBMCs was rapidly thawed at 37 °C and added to 9 mL of RMPI-1640 supplemented with 10% HI-FBS, 1% penicillin-streptomycin. Next, the suspension was centrifuged at 300 g for 8 minutes, the supernatant was discarded, and cells were resuspended in media for counting. PBMCs were counted and seeded in 24-well tissue culture plates at a density of 1 x 10^6 cells/mL in triplicate and left to rest for 24 hours in the presence of 5% CO_2 at 37 °C. Following the rest period, PBMCs were trained and restimulated according to the 3-day training/restimulation protocol outlined (Fig. 6A).

2.5 Quantification methods

2.5.1 ELISAs (quantification of cytokine production)

Supernatants were thawed from -20 °C storage, centrifuged and analyzed for TNFα and IL-10 using the respective ELISA kits (R&D Systems). The assay was performed according to the instructions provided by the manufacturer. The mouse and human TNFα limits of detection were 31.3 pg/mL and 15.6 pg/mL, respectively. Additionally, the mouse and human IL-10 limits of detection were 15.6 pg/mL and 7.8 pg/mL, respectively. Washing steps were performed with ELx405 Select Deep Well Microplate Washer (Biotek, Winooski VT). The optical density was measured at 450 nm and a correction of 570 nm using the Synergy H4 Multi-mode Hybrid Microplate Reader (Biotek, Winooski VT).

2.5.2 Flow Cytometry

To harvest PMA differentiated THP-1 macrophages , 24-well tissue culture treated plates were shook on an orbital plate shaker set to 200 rpm for 15 minutes with 500 μL TrypLE per well. Next, 1 mL of media was added to each well to deactivate the TrypLE and cells were vigorously pipetted and collected into microcentrifuge tubes. Tubes were spun at 2000 rpm and washed two times with 1X PBS. Controls were left to incubate in 100 μL BSA solution (1% BSA in 1X PBS) and stained cells were incubated in the BSA solution with their respective antibodies (Table 5) for 20 minutes in the dark; the BSA solution acted as a block. Antibody concentrations were determined according to B & D Biosciences recommendations. Cells were then washed two times with 1X PBS and resuspended in BSA solution for analysis by flow cytometry. If the cells were being analyzed at a later time (within 24 hours), they were fixed in equal volumes of BSA solution and Biolegend fixing solution in the dark for 30 minutes, washed

two times with 1X PBS and stored at 4 °C. Data was acquired on Cytometrics FC-500 flow cytometer (Beckman Coulter, Fullerton CA) using CXP Analysis Software, in which 10,000 events were recorded. Debris and dead cells were excluded by gating forward scatter (FSC) and side scatter (SSC). Additionally, fluorescence minus one (FMO) controls were run in order to set gates for multicolour flow cytometry panels.

Table 5. Flow cytometry antibodies

Antigen	Fluorochrome	Vendor	Catalog Number
CD11b	PECy7	BioLegend (BD, Franklin Lakes, NJ)	301321
CD14	PE	Invitrogen (Fredrick, MD)	2048980
CD16	PECy5	Invitrogen (Fredrick, MD)	1978142

Antibodies were used at a concentration of 5 μL/test in 100 μL volume.

2.6 Statistical Analysis

All statistical analyses were done using Graph Pad Prism 5. Multiwise group analyses were performed using a one-way ANOVA with Tukey's HSD. We choose to perform a one-way ANOVA instead of a two-way ANOVA to analyze our data because we were comparing the mean cytokine production of trained celled to their non-trained controls within their respective restimulation groups, rather than between restimulation groups. Data was considered significant when $P \leq 0.05$.

3. RESULTS

3.1 Training & restimulation optimization protocol

3.1.0 J774A.1 and THP-1 cytokine production following 3 vs. 6-day non-specific restimulation

To optimize the training and restimulation protocol, J774A.1 and THP-1 cells were trained and subsequently restimulated at 3 days or 6 days. When J774A.1 (Fig. 12A) and THP-1 (Fig. 12B) were restimulated with LPS, both the MDP and LPS trained cells produced significantly more TNFα compared to the non-trained (NT) control cells. Additionally, the training response was much larger when the cells were restimulated 3 days after training opposed to 6 days after training.

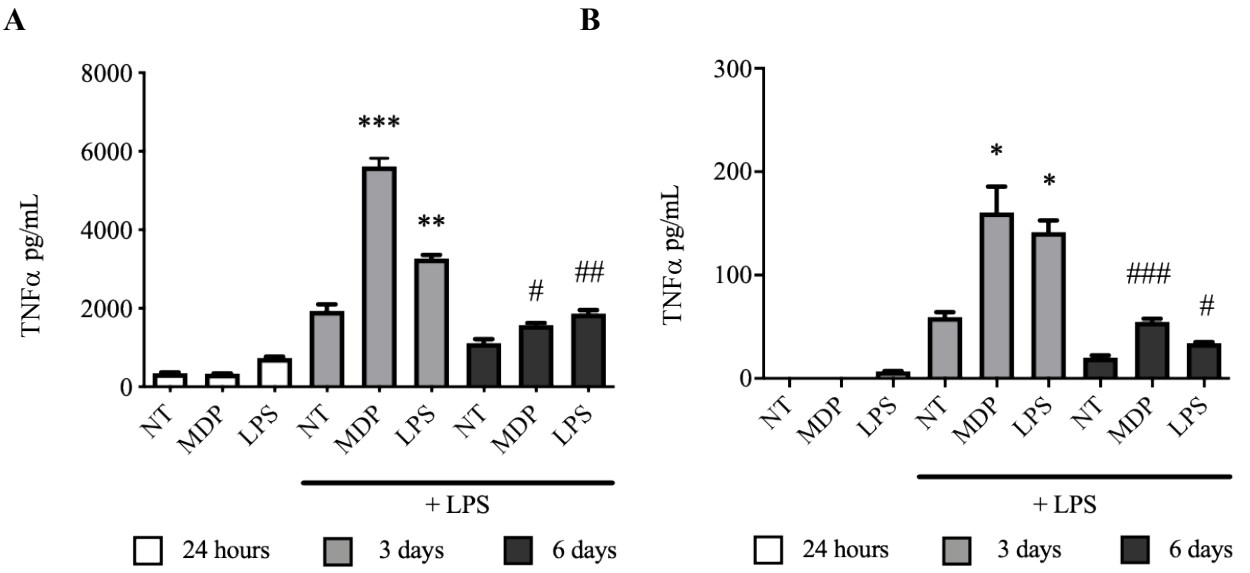


Figure 12. TNFα production by trained and non-trained (**A**) J774A.1 and (**B**) THP-1 cells following 3 day or 6-day non-specific restimulation.

J774A.1 cells and THP-1 cells were left untrained (NT), or trained with 5 ng/mL LPS or 10 μg/mL MDP for 24 hours (n = 3). Cells were washed and left to rest in media for 2 days or 5 days. After their respective rest periods, on day 3 and 6, trained and untrained J774A.1 and THP-1 cells were seeded in 24-multi-well plates at a density of 5 x 10^5 cells/mL and restimulated with LPS (10 ng/ml) for 24 hours (n = 3). Supernatants collected and analyzed by ELISA to quantify cytokine production. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ as compared to 3-day NT group. #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$ as compared to 6-day NT group.

3.2 J774A.1 cells as a murine *in vitro* model for trained immunity

3.2.1 Cytokine production by trained and non-trained J774A.1 cells

Data shows that J774A.1 cells displayed a trained immunity cytokine profile. When restimulated with Dukoral, MDP or an influenza virus, J774A.1 cells trained with the delivery system produced significantly more TNFα compared to the NT cells (Fig. 13A). Interestingly, the J774A.1 cells trained with delivery system also had less IL-10 production following restimulation compared to the cells trained with GLA or MDP alone (Fig. 13B).

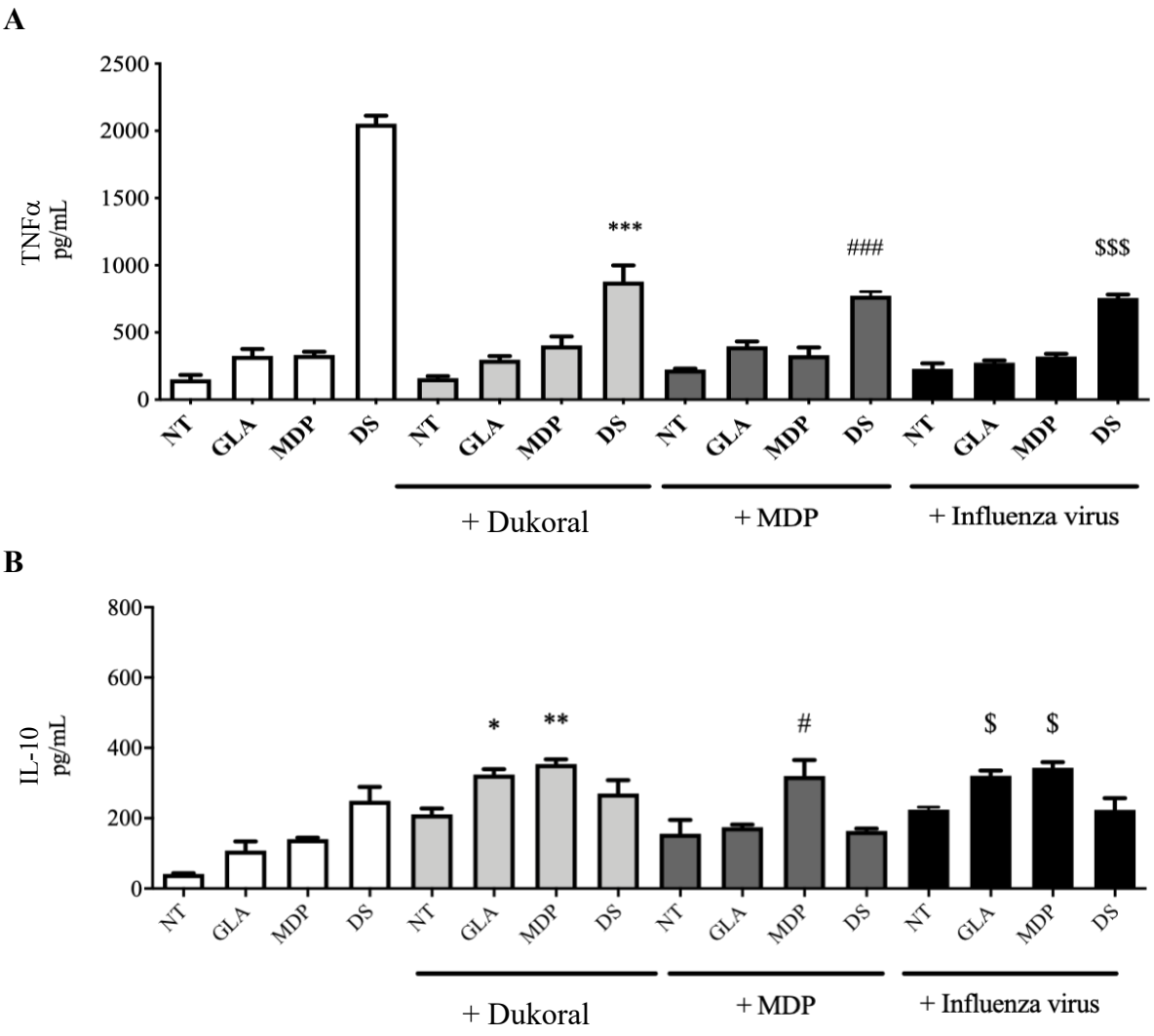


Figure 13. Cytokine production by trained and non-trained J774A.1 cells.

J774A.1 cells were seeded in 75 cm^2 tissue-culture treated flasks at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 ng/mL GLA, 10 μg/mL MDP or 5 μg/mL delivery system (DS; CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in DMEM for 2 days. On day 3, trained and untrained cells were seeded in 24-multi-well plates at a density of 5 x 10^5 cells/mL and restimulated with Dukoral (10^3 vibrios), MDP (10 μg/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ as compared to NT + Dukoral. #$p \leq 0.05$, ###$p \leq 0.001$ as compared to NT + MDP. \$$p \leq 0.05$, \$\$\$$p \leq 0.001$ as compared to NT + Influenza virus.

3.2.2 Cytokine production by J774A.1 cells trained with various CL combinations

J774A.1 cells trained with various combinations of the delivery system components show a trained immunity cytokine profile. In particular, J774A.1 cells trained with CL+GLA, CL+MDP and CL+GLA+MDP all produce significantly higher levels of TNFα compared to NT cells following Dukoral restimulation (Fig. 14A). Similarly, CL+MDP and CL+GLA+MDP trained J774A.1 cells also produced significantly higher levels of TNFα compared to NT controls when virally challenged (Fig.14A). It should be noted, CL training alone had no impact on cytokine production following non-specific restimulation (Fig 14A, B). Following restimulation with Dukoral and influenza virus, CL+GLA+MDP and CL+MDP trained J774A.1 cells, respectively, produced significantly higher levels of IL-10 compared to NT controls (Fig. 14B).

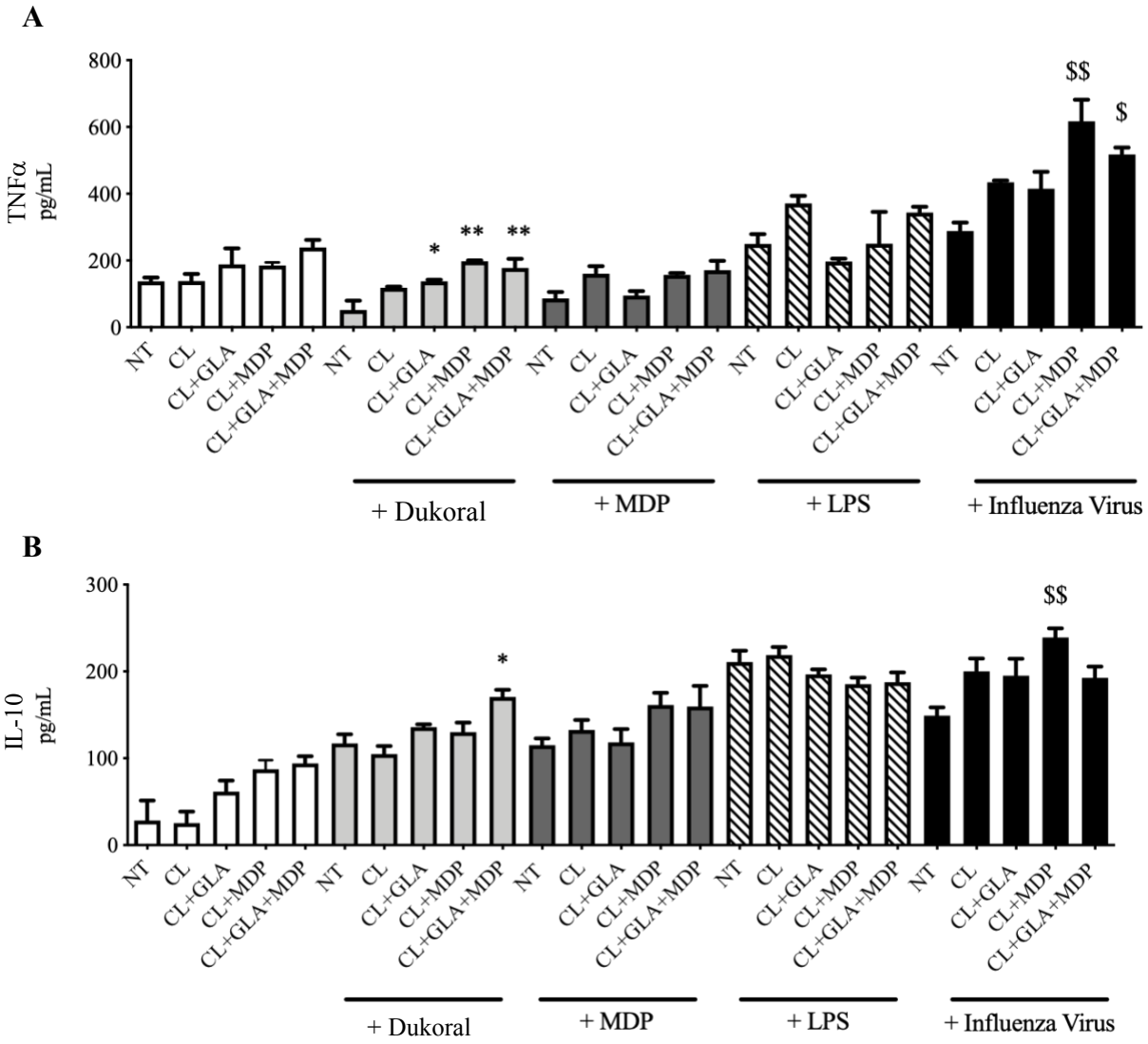


Figure 14. Cytokine production by J774A.1 cells trained with various CL combinations.

J774A.1 cells were seeded in 75cm^2 tissue-culture treated flasks at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 μg/mL CL, 5 μg/mL CL+ GLA, 5 μg/mL CL+MDP or 5 μg/mL delivery system (DS; CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in DMEM for 2 days. On day 3, trained and untrained cells were seeded in 24-multi-well plates at a density of 5 x 10^5 cells/mL and restimulated with Dukoral (10^3 vibrios), MDP (10 μg/mL), LPS (10 ng/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$, ** $p \leq 0.01$, as compared to NT + Dukoral. \$$p \leq 0.05$, \$\$ $p \leq 0.01$, as compared to NT + Influenza virus.

3.2.3 Cytokine production by trained and non-trained BMDMs

BMDMs displayed a trained immunity cytokine profile *in vitro*. When restimulated with Dukoral, MDP and influenza virus, BMDMs trained with the delivery system produced significantly more TNFα compared to the NT cells after non-specific restimulation (Fig. 15A). GLA trained BMDMs also produced significantly higher levels of TNFα following restimulation with Dukoral and LPS compared to NT controls (Fig. 15A). Whereas, MDP trained BMDMs produced significantly higher levels of TNFα following restimulation with MDP compared to NT controls (Fig. 15A). It should be noted that the proportion of TNFα generated by MDP restimulated BMDMs was much higher compared to the other restimulation agents. The BMDMs trained with GLA and delivery system also had significantly higher IL-10 production following restimulation compared to NT cells (Fig. 15B).

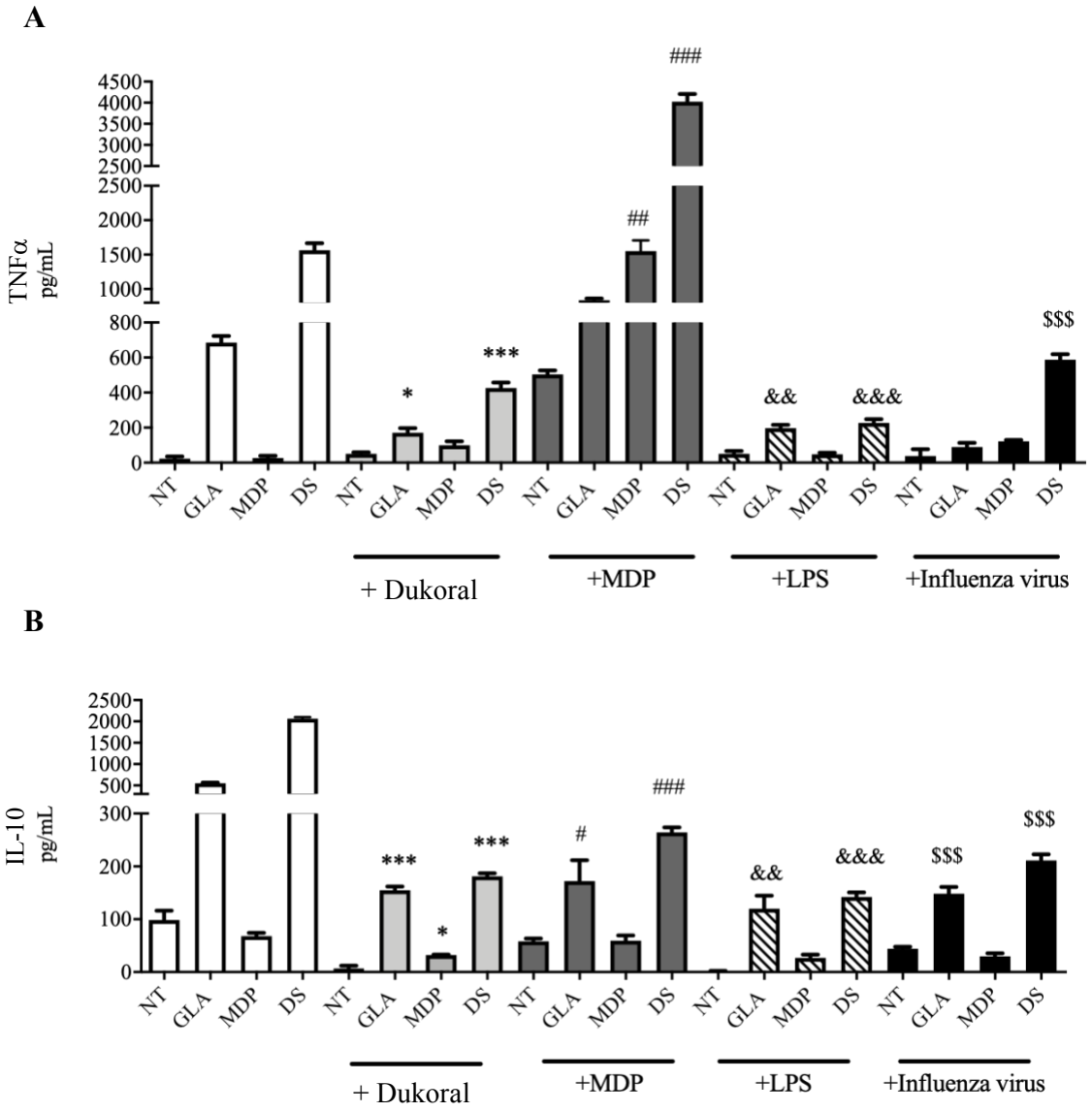


Figure 15. Cytokine production by trained and non-trained BMDMs.

BMDMs were seeded in 24-well plates at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 ng/mL GLA, 10 μg/mL MDP or 5 μg/mL delivery system (CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in media for 6 days. On day 7, trained and untrained cells were restimulated with Dukoral (10^3 vibrios), MDP (10 μg/mL), LPS (10 ng/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$, ***$p \leq 0.001$ as compared to NT + Dukoral. #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$ as compared to NT + MDP. &&$p \leq 0.01$, &&&$p \leq 0.001$ as compared to NT + LPS. $$$$p \leq 0.001$ as compared to NT + Influenza virus.

3.3 THP-1 cells as a human *in vitro* model for trained immunity

3.3.1 Cytokine production by trained and non-trained THP-1 cells

THP-1 cells trained with GLA, MDP and the delivery system only showed a trained immunity cytokine profile—significantly higher TNFα production compared to NT cells—when restimulated with LPS (Fig. 16A). Furthermore, THP-1 cells trained with delivery system had significantly higher IL-10 production following MDP restimulation compared to NT cells (Fig. 16B).

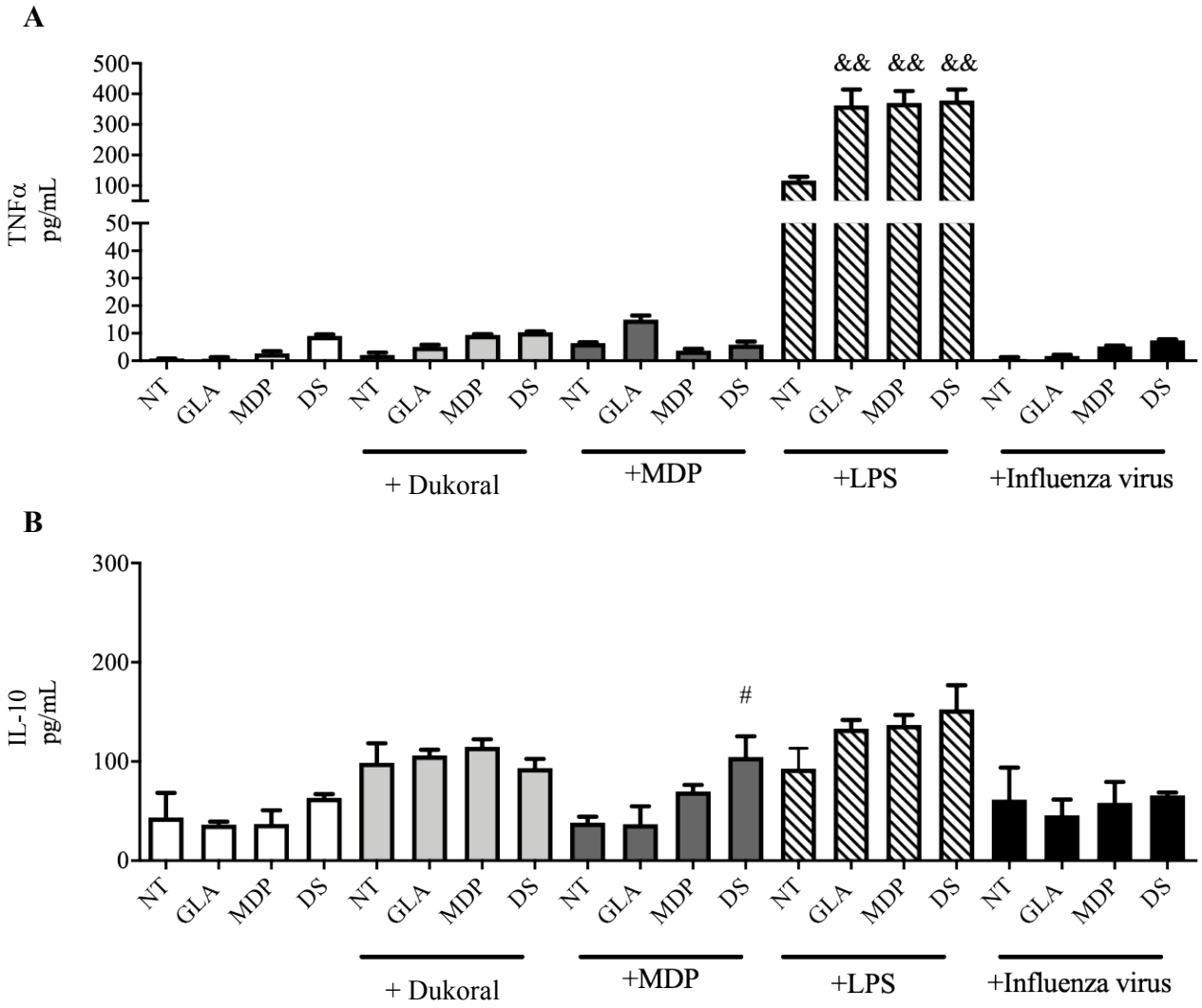


Figure 16. Cytokine production by trained and non-trained THP-1 cells.

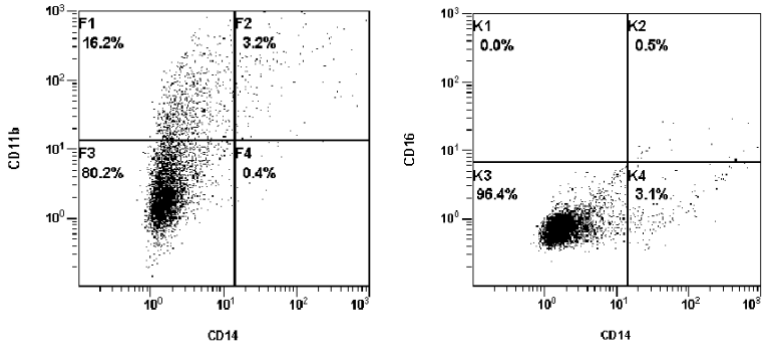
THP-1 cells were seeded in 25 cm^2 non-treated tissue-culture non-treated flasks at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 ng/mL GLA, 10 µg/mL MDP or 5 µg/mL delivery system (DS; CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, trained and untrained cells were seeded in 24-multi-well plates at a density of 5 x 10^5 cells/mL and restimulated with Dukoral (10^3 vibrios), MDP (10 µg/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. #$p \leq 0.05$ as compared to NT + MDP. &&$p \leq 0.01$as compared to NT + LPS.

3.3.2 PMA differentiated THP-1 macrophage expression of various surface markers.

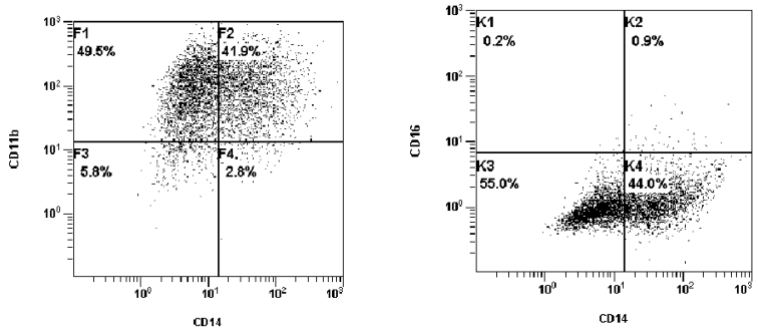
Surface markers, CD11b, CD14 and CD16 were used to track PMA mediated differentiation of THP-1 monocytes to macrophages. CD11b is a standard marker used to illustrate monocyte to macrophage maturation and differentiation. Whereas CD14 is a marker of macrophage activation. At last, CD16 is present on different subtypes of macrophages. The expression of CD11b increased over time with PMA incubation from 91.4% at 24 hours, 94.5% at 48 hours, and 97.1% at 72 hours (Fig. 17, Table 6). Likewise, THP-1 cells expressed 44.7%, 66.1% and 91.4% CD14 when incubated with PMA for 24, 48 and 72 hours, respectively (Fig. 17, Table 6). Following a similar trend THP-1 cells expressed 1.1%, 3.7% and 21.5% CD16 when incubated with PMA for 24, 48 and 72 hours, respectively (Fig. 17, Table 6).

Data shows both $CD14^{+}CD11b^{+}$ and $CD14^{+}CD16^{+}$ THP-1 cells increased over time with PMA incubation. Non-differentiated THP-1 expressed 3.2% $CD14^{+}CD11b^{+}$ (Fig. 17, Table 6). Comparatively, THP-1 cells expressed 41.9%, 63.4% and 89.2% $CD14^{+}CD11b^{+}$ when incubated with PMA for 24, 48 and 72 hours, respectively (Fig. 17, Table 6). $CD14^{+}CD16^{+}$ THP-1 cell expression increased from 0.5% for non-differentiated cells to 21.3% when differentiated with PMA for 72 hours (Fig. 17, Table 6).

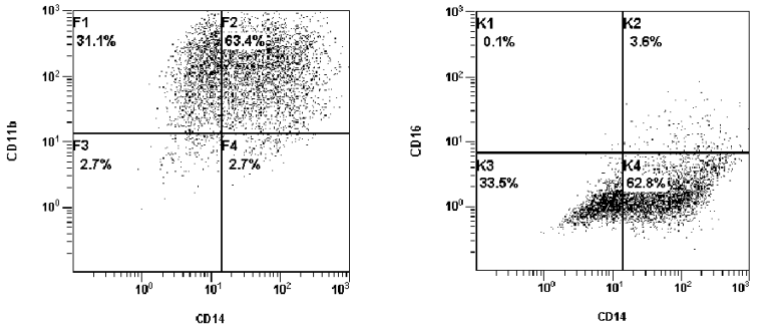
A. Non-treated THP-1 cells



B. 24-hour PMA differentiated THP-1 cells



C. 48-hour PMA differentiated THP-1 cells



D. 72-hour PMA differentiated THP-1 cells

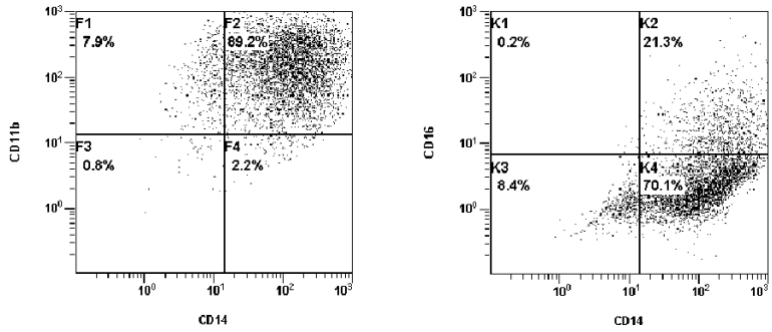


Figure 17. PMA differentiated THP-1 macrophage phenotype.

Representative flow cytometry analysis of THP-1 cells non-treated (**A**) or differentiated with PMA for 24 (**B**), 48 (**C**) or 72 (**D**) hours.

Table 6. PMA differentiated THP-1 macrophage expression of various surface markers.

Marker	THP-1	THP-1 + PMA 24h	THP-1 + PMA 48h	THP-1 + PMA 72h
$CD11b^+$	19.4	91.4	94.5	97.1
$CD14^+$	3.6	44.7	66.1	91.4
$CD16^+$	0.5	1.1	3.7	21.5
$CD14^+CD11b^+$	3.2	41.9	63.4	89.2
$CD14^+CD16^+$	0.5	0.9	3.6	21.3

Values represent percentage of positive cells. "h" denotes time in hours.

3.3.3 Cytokine production by trained and non-trained PMA differentiated THP-1 cells

When restimulated with Dukoral, MDP and influenza virus, PMA differentiated THP-1 cells trained with delivery system produced significantly more TNFα compared to the NT cells (Fig. 18A). PMA differentiated THP-1 cells trained with MDP and GLA also produced significantly more TNFα compared to the NT cells when virally restimulated (Fig. 18A). However, MDP, GLA and delivery system trained PMA differentiated THP-1 cells did not produce more TNFα compared to the NT cells following LPS restimulation (Fig. 18A). Delivery system trained PMA differentiated THP-1 cells were the only group to produce significantly more IL-10 compared to the NT control when restimulated with Dukoral (Fig. 18B).

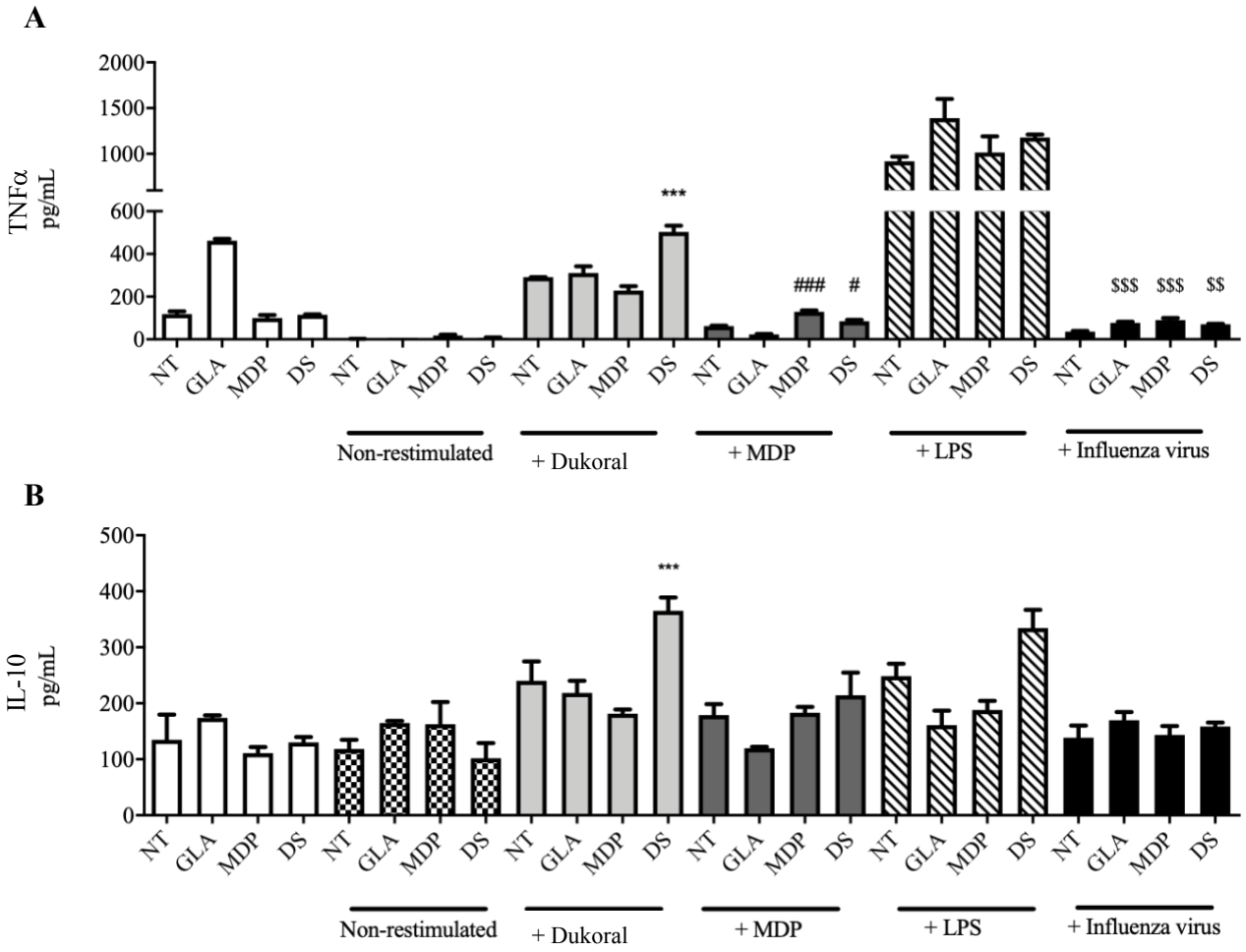


Figure 18. Cytokine production by trained and non-trained PMA differentiated THP-1 cells.

PMA differentiated THP-1 macrophages were seeded in a 24-well plate at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 ng/mL GLA, 10 μg/mL MDP or 5 μg/mL delivery system (DS; CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, trained and untrained cells were non-restimulated or restimulated with Dukoral (10^3 vibrios), MDP (10 μg/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. ***$p \leq 0.001$ as compared to NT + Dukoral. #$p \leq 0.05$, ###$p \leq 0.001$ as compared to NT + MDP. $$ $p \leq 0.01$, $$$$p \leq 0.001$ as compared to NT + Influenza virus.

3.3.4 Cytokine production by PMA differentiated THP-1 cells trained with various CL combinations

PMA differentiated THP-1 cells trained with various different components of the delivery system show a trained immunity cytokine profile. In particular, PMA differentiated THP-1 cells trained with CL+GLA and CL+MDP+GLA (DS) produce significantly higher levels of TNFα compared to NT cells following Dukoral, MDP and influenza virus restimulation (Fig. 19A). Additionally, PMA differentiated THP-1 cells trained with CL+MDP produced significantly higher levels of TNFα following Dukoral restimulation compared to NT cells (Fig. 19A). When left non-restimulated, only CL+GLA trained PMA differentiated THP-1 cells produced a significantly higher level of basal TNFα secretion compared to NT controls (Fig. 19A). Interestingly, levels of IL-10 production were only significantly increased by CL+MDP and CL+GLA+MDP trained cells following Dukoral restimulation (Fig. 19B).

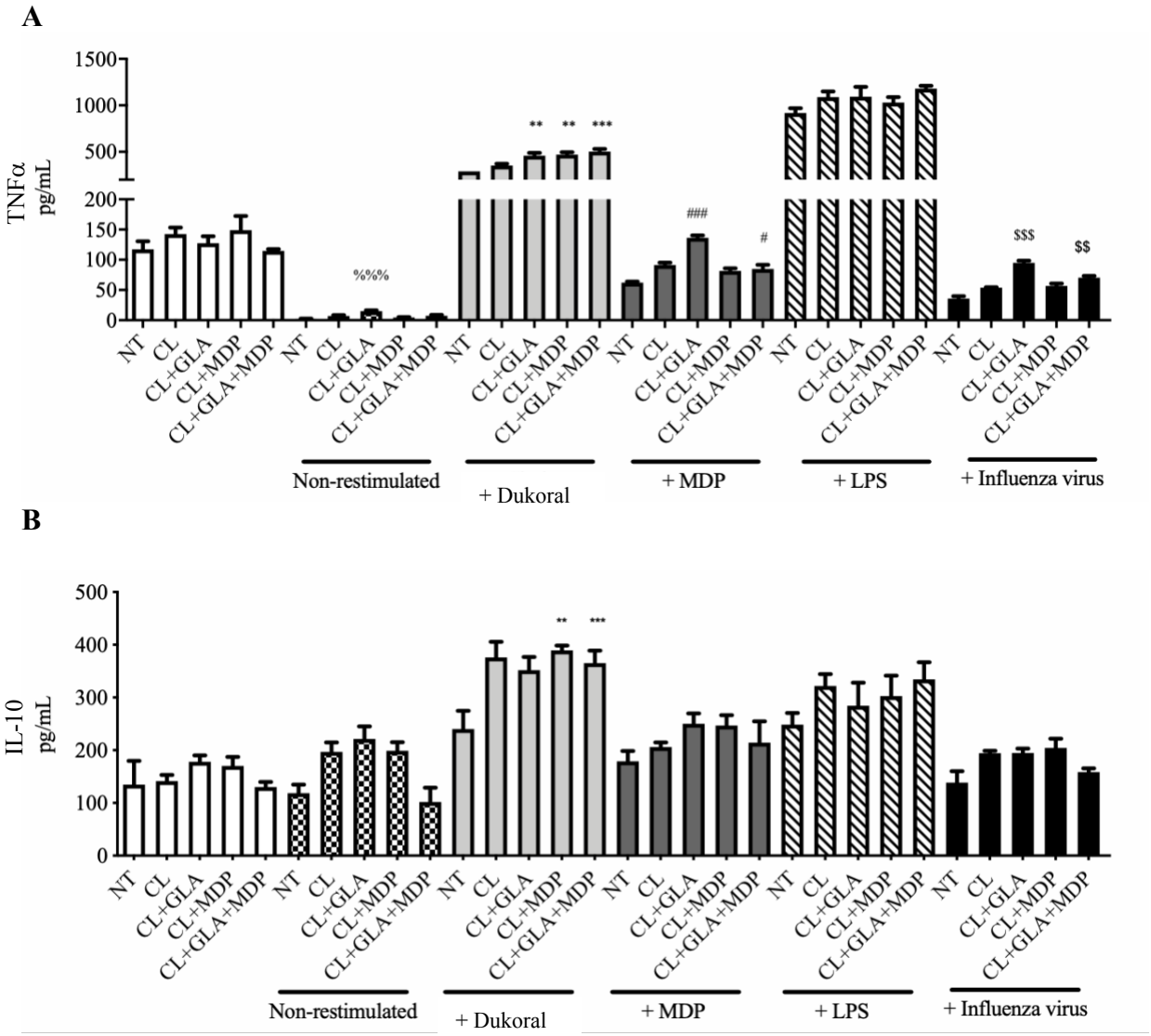


Figure 19. Cytokine production by PMA differentiated THP-1 cells trained with various CL combinations.

PMA differentiated THP-1 macrophages were seeded in 24-well plates at a density of 5 x 10^5 cells/mL and left untrained (NT), or trained with 5 µg/mL CL, 5 µg/mL CL+ GLA, 5 µg/mL CL+MDP or 5 µg/mL delivery system (CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, trained and untrained cells were non-restimulated or restimulated with Dukoral (10^3 vibrios), MDP (10 µg/mL), LPS (10 ng/mL) or influenza virus (MOI 2) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. %%% $p \leq 0.001$ as compared to NT + Non-restimulated, ** $p \leq 0.01$, ***$p \leq 0.001$ as compared to NT + Dukoral. #$p \leq 0.05$, ###$p \leq 0.001$ as compared to NT + MDP. $$ $p \leq 0.01$, $$$$p \leq 0.001$ as compared to NT + Influenza virus.

3.3.5 Cytokine production by trained and non-trained PBMCs

PBMCs displayed a trained immunity cytokine profile *in vitro*. When restimulated with Dukoral, delivery system trained PBMCs produced significantly more TNFα compared to the NT cells (Fig. 20A). TNFα production by the delivery system trained PBMCs was also significantly higher than the NT control even when the cells were not restimulated (Fig. 20A). The production of IL-10 did not significantly change following training and restimulation compared to the NT control (Fig. 20B).

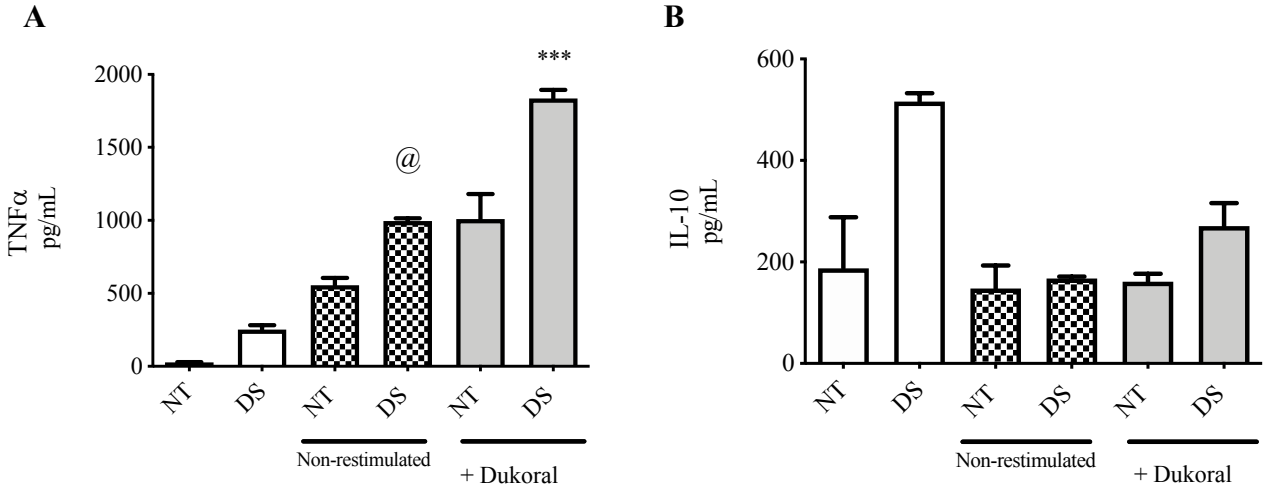


Figure 20. Cytokine production by trained and non-trained PBMCs.

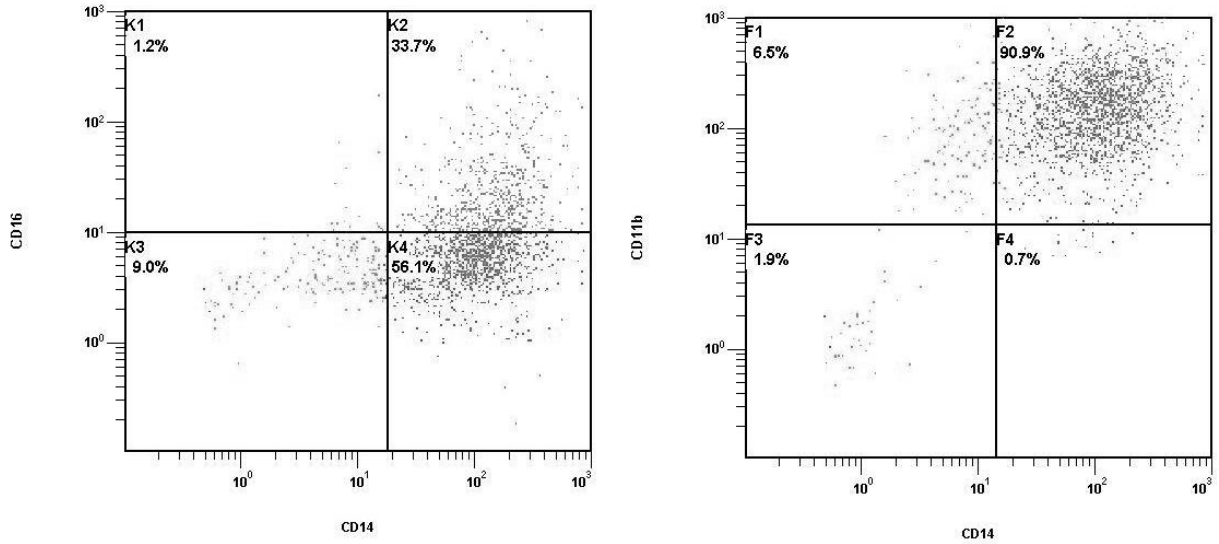
PBMCs were seeded in a 24-well plate at a density of 5 x 10^6 cells/mL and left untrained (NT), or trained with 5 μg/mL delivery system (CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, trained and untrained cells were non-restimulated or restimulated with Dukoral (10^3 vibrios) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. @$p \leq 0.05$, as compared to NT + Non-restimulated. ***$p \leq 0.001$ as compared to NT + Dukoral.

3.4 Delivery system induced trained immunity

3.4.1 PMA differentiated THP-1 cell (THP-1 M0) expression of various surface markers following delivery system training

To determine if delivery system training affects macrophage surface marker expression, CD11b, CD14 and CD16 were analyzed. CD11b expression did not fluctuate when PMA differentiated THP-1 cells were trained with the delivery system (Fig. 21, Table 7). However, following delivery system training there was a 1.9% increase in the number of CD14+ cells, compared to NT cells (Fig. 21, Table 7). Alternatively, following delivery system training there was a 16.2% decrease in the number of CD16+ cells, compared to NT cells (Fig. 21, Table 7).

A. NT PMA differentiated THP-1 cells



B. DS trained PMA differentiated THP-1 cells

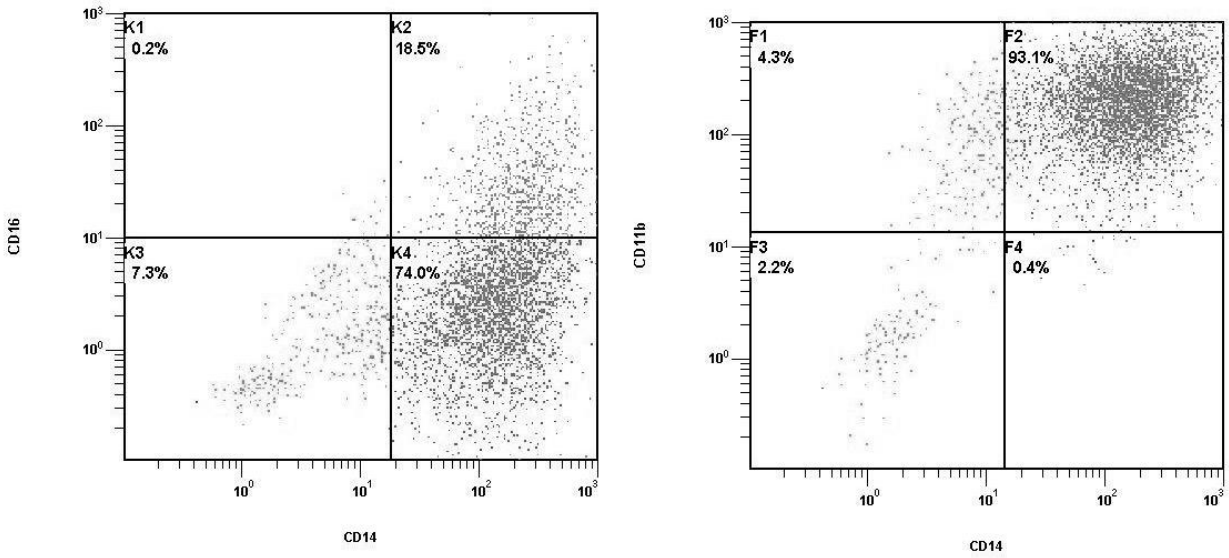


Figure 21. PMA differentiated THP-1 cell surface expression following delivery system training.

Representative flow cytometry analysis of NT (**A**) and delivery system (DS) trained (**B**) PMA differentiated THP-1 cells.

Table 7. PMA differentiated THP-1 cell (THP-1 M0) expression of various surface markers following delivery system training.

Marker	THP-1 M0 NT	THP-1 M0 DS
$CD11b^+$	97.4	97.4
$CD14^+$	91.6	93.5
$CD16^+$	34.9	18.7
$CD14^+CD11b^+$	90.9	93.1
$CD14^+CD16^+$	33.7	18.5

Values represent percentage of positive cells.
72h PMA differentiated THP-1 cells (M0); Non-trained (NT); Delivery system (DS).

3.4.2 Cytokine production by trained and non-trained PMA differentiated THP-1 macrophages inhibited with CLI-095 and/or Gefitinib

When restimulated with Dukoral, PMA differentiated THP-1 cells trained with the delivery system produced significantly more TNFα compared to the NT cells (Fig. 22A). Inhibition with CLI-095 and/or Gefitinib blocks this TNFα response by the PMA differentiated THP-1 cells. Cells incubated with CLI-095 and/or Gefitinib prior to delivery system training had no change in TNFα production following Dukoral restimulation (Fig. 22A). The production of IL-10 did not significantly change following inhibition, training and restimulation compared to the NT controls (Fig. 22B).

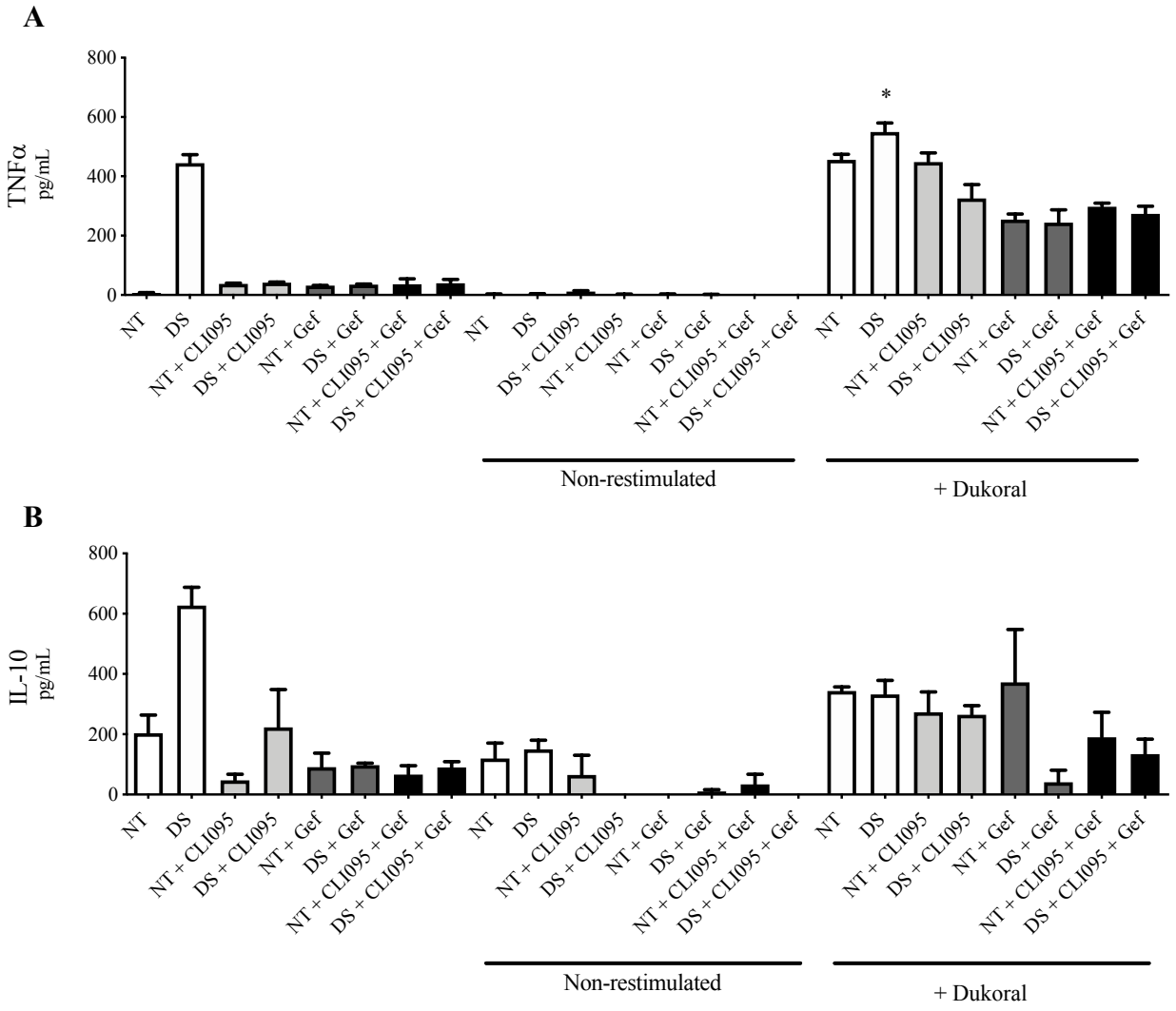


Figure 22. Cytokine production by trained and non-trained PMA differentiated THP-1 macrophages inhibited with CLI-095 and/or Gefitinib.

PMA differentiated THP-1 macrophages were seeded in a 24-well plate at a density of 5 x 10^5 cells/mL and inhibited with 20 ng/mL CLI-095 and/or 500 nM Gefitinib (Gef) for 6 hours and 1 hour, respectively. The cells were then left untrained (NT), or trained with 5 μg/mL delivery system (CL+MDP+GLA) for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, trained and untrained cells were non-restimulated or restimulated with Dukoral (10^3 vibrios) for 24 hours at 37°C, 5% CO_2 (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production, **(A)** TNFα **(B)** IL-10. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$ as compared to NT + Dukoral.

3.4.3 IL-2 production by activated Jurkat T cells co-cultured with trained and non-trained PMA differentiated THP-1 macrophages

Results show that delivery system training had no effect on IL-2 production by PMA differentiated THP-1 cells or activated Jurkat T cells alone (Fig. 23). Co-culturing PMA differentiated THP-1 cells with activated Jurkat T cells increased the production of IL-2, however there was no significant difference between NT and delivery system training (Fig. 23). However, following Dukoral restimulation the co-culture containing delivery system trained THP-1 cells produced significantly more IL-2 compared to the co-culture with untrained THP-1 cells (Fig. 23).

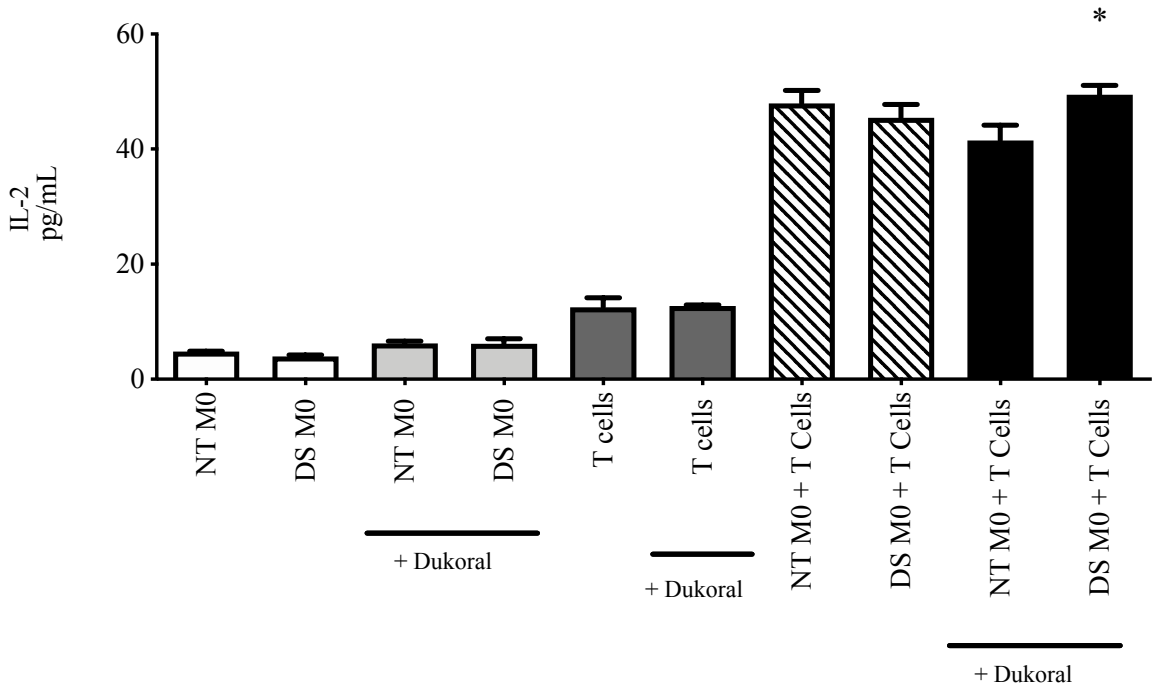


Figure 23. IL-2 production by activated Jurkat T cells co-cultured with trained and non-trained PMA differentiated THP-1 macrophages.

Jurkat T cells activated with 5 μg/ml CD3 and 2 μg/ml CD28 were co-cultured with non-trained delivery system (5 μg/mL) trained PMA differentiated macrophages for 24 hours (n = 3). White bars represent cytokine production following 24 hours of training. Cells were washed and left to rest in RPMI with 10% HI-FBS and 1% penicillin-streptomycin for 2 days. On day 3, cells were non-restimulated or restimulated with Dukoral (10^3 vibrios) for 24 hours (n = 3). After 24 hours, supernatants collected and analyzed by ELISA to quantify cytokine production. Results are presented as ± SEM. A one-way ANOVA followed by Tukey's HSD was performed using Graph Pad Prism 5. *$p \leq 0.05$ as compared to NT M0 + T cells + Dukoral.

4. DISCUSSION

4.1 Training protocol optimization

Our first objective was to develop murine and human *in vitro* models to study trained immunity. J774A.1 and THP-1 monocyte/macrophage—mouse and human cell lines, respectively—were strategically chosen to be evaluated. Previous studies have shown LPS and MDP induce trained immunity in macrophages (Table 2). Therefore, LPS and MDP were used as training agents to optimize our training protocol. The cells were trained and left to rest in media for either 3 or 6 days before restimulation. By this, we sought to determine how long training lasts *in vitro*, allowing us to optimize our *in vitro* training protocol.

Our results indicate that J774A.1 and THP-1 cell lines can be trained *in vitro* by MDP and LPS to respond more robustly—produce more TNFα—following LPS restimulation (Fig. 12). MDP and LPS have proven to induce trained immunity in previous studies (Rusek, Wala, Druszczyńska, & Fol, 2018). However, to the best of our knowledge, J774A.1 and THP-1 cell lines have not been established as models used to study trained immunity. We demonstrate with this data, that J774A.1 and THP-1 cells prove to be a promising model (Fig. 12). Furthermore, J774A.1 and THP-1 cells produced the largest amount of proinflammatory TNFα following restimulation after a 3-day rest period, with a declining response beyond 3 days of rest (Fig. 12). Therefore, we determined that a 3-day rest period was optimal when using these *in vitro* models to study trained immunity.

Studies done on monocytes/macrophages showing trained immunity lasts about 7 days, whereas NK cells show a trained immune profile up to six months (Kleinnijenhuis et al., 2012; O'Leary et al., 2006; Quintin et al., 2012), suggesting that the longevity of trained immunity

depends on cell type. The temporary nature of trained immunity has been attributed to the epigenetic reprogramming that takes place in the innate immune cells (Mourits, Wijkmans, Joosten, & Netea, 2018). A number of studies describe increased H3K4m3 and decreased H3K27ac at the promoter regions of genes encoding proinflammatory cytokines, such as TNFα, which are associated with trained immunity (Netea & Van Crevel, 2014; Quintin et al., 2012; Saeed et al., 2014). Histone modifications are dynamic in nature, having a limited inheritance capacity. Histone methylation and acetylation are largely reversible processes which can be lost as cells divide, perhaps this is the reason why training effects are diminished overtime in the rapidly dividing immortal *in vitro* cell lines used in this study. A study done by Bekkering et al., (2016) showed the induction of tolerance when trained PBMCs were only left to rest for 24 hours before restimulation, whereas training effects were seen when cells were left to rest for 3 or 6 days. The authors attribute the importance of resting time to changes in metabolism (glycolysis), which take place over 3 to 6 days (Bekkering et al., 2016). Metabolic changes are another pilar of trained immunity. That is trained immune cells metabolically switch from OXPHOS to aerobic glycolysis to meet energetic demands (Cheng et al., 2014). This explains why cells need to rest following training to metabolically adapt. Furthermore, metabolic changes fluctuate in order to maintain homeostasis, which may be why the trained effects diminish over time.

4.2 J774A.1 cells as a murine *in vitro* model for trained immunity

In comparison to GLA or MDP training alone, the delivery system trained J774A.1 cells showed a trained immune response following restimulation with Dukoral, MDP or Influenza virus (Fig. 13A). This may be due to a cumulative effect of the stimuli—CL, GLA and MDP—in

the delivery system. Previous studies have shown that LPS and lipid A derivative MPL induce trained immunity (Fensterheim et al., 2018; Foster et al., 2007). Therefore, it is logical that GLA—another lipid A derivative—would induce trained immunity. LPS and its component derivatives, MPL and GLA all signal through the TLR4 cascade (Muroi et al., 2002). Suggesting the TLR4 cascade plays an important role in trained immunity. Additionally, consistent with our results, studies illustrate MDP mediated training through the NOD2 signaling cascade (Gutierrez et al., 2002; Kleinnijenhuis et al., 2012). In the delivery system, both GLA and MDP are able to elicit their effects simultaneously, perhaps leading to the enhanced TNFα production. In fact, a study done by Fritz et al. (2005), show MDP and LPS act synergistically to enhance proinflammatory cytokine production. Finally, the CL component of the delivery system has multimodal effects on APCs. Our CL was formulated to be cationic because research has indicated that positively charged lipids enhance the uptake of liposomes by APCs (Nakanishi et al., 1999). Furthermore, the CL is mannosylated to target the liposomes to mannose receptors, which are highly abundant on the surface of APCs (Keler, Ramakrishna, & Fanger, 2004). All in all, the delivery system has three ways in which it can act on the J774A.1 cells to induce training; first by targeting the CL to J774A.1 cells via cationic charge and mannosylaed lipids, second inducing GLA training through TLR4, and third inducing MDP training through NOD2.

Interestingly, the delivery system trained J774A.1 cells produced less IL-10 production following restimulation compared to the cells trained with MDP or GLA alone (Fig.13B). According to studies done in mice, cationic liposomes decrease the toxicity of PAMPs towards phagocytic cells (Filion & Phillips, 1997), thus the CL might play a role in decreasing the toxicity of MDP and GLA as immune stimulants

Our data demonstrates that CL+MDP and CL+GLA+MDP (DS) trained J774A.1 cells produce significantly more TNFα compared to NT cells when challenged with either Dukoral or Influenza virus (Fig. 14A). These results suggest delivery system training in J774A.1 cells are vastly attributed to the MDP and CL components of the system. It has been well documented in literature that MDP signals though the NOD2 signaling pathway (Gutierrez et al., 2002; K. S. Kobayashi et al., 2005; Lauro et al., 2017; Ogura et al., 2001). Additionally, a recent study indicates cationic liposomes may trigger pro-inflammatory responses through TLR2, in a mechanism resembling bacterial lipopeptide sensing through the hydrophobic pocket of TLR2 (Pizzuto et al., 2017). Furthermore, research suggests co-stimulation of NOD2 and TLR2 in J774A.1 cells promotes enhanced activation of the immune system compared to stimulation of each receptor alone (Schäffler et al., 2014). In this respect, we speculate MDP and CL training could be synergistically enhancing NOD2 and TLR2 signaling cascades, preparing the J774A.1 cells to react more vigorously to Dukoral and influenza restimulation. Additionally, it has been noted that TLR2 is essential for immune responses to Dukoral. Outer membrane protein-U (OmpU) is the major PAMP present in Dukoral, which signals though TLR2 (Khan, Sharma, & Mukhopadhaya, 2015). In fact, it has been documented that TLR2 plays a predominant role in generating immune responses to the Dukoral vaccine, perhaps explaining the increased responsiveness of MDP+CL trained J774A.1 cells to Dukoral (J. S. Yang et al., 2015).

It should be emphasized, the trained J774A.1 cells responded most robustly to restimulation with complex pathogens, Dukoral and influenza virus. Dukoral contains a vast array of bacterial PAMPs, as the vaccine contains four inactivated *Vibrio cholerae* strains and recombinant cholera toxin B subunit. In contrast, influenza virus expresses an array of viral

PAMPs. Both restimulation agents are very different with respect to immunostimulatory composition, yet the cells have been trained to respond robustly to both.

BMDMs trained *in vitro* follow similar trends to the J774A.1 cells with respect cytokine profiles following training and restimulation (Fig. 15). In particular, the delivery system proved to be the most successful training agent in the BMDMs akin to J774A.1 cells (Fig. 15A). In literature, BMDMs have been used as a model to study trained immunity (Santecchia et al., 2019; Saz-Leal et al., 2018). Particularly, one study has shown that BMDMs trained with β-glucan produce significantly higher levels of TNFα following LPS restimulation compared to non-trained cells (Saz-Leal et al., 2018). Since BMDMs have been established in literature as a model used to study trained immunity, they were used to help verify our use of J774A.1 cells as an *in vitro* model to study trained immunity. While similar trends were observed between the BMDMs and J774A.1 cells, one marked difference was the production of TNFα by trained BMDMs following restimulation with MDP (Fig. 15A). Compared to the other agents—Dukoral, LPS and Influenza virus, restimulation with MDP elicited a robust production of TNFα (Fig. 15A). Perhaps this could be attributed to a difference in NOD2 expression between BMDMs and J774A.1 cells.

Our results also indicate, GLA and delivery system trained cells produced significantly higher levels of IL-10 following restimulation, compared to NT controls (Fig. 15B). According to literature BMDMs produce significantly higher levels of IL-10 following LPS stimulation, compared to non-treated controls (Chamberlain et al., 2009). This might explain why BMDMs trained with GLA, an LPS analog, produce significantly more of IL-10 compared to NT controls.

It should be noted, GLA and delivery system trained BMDMs secrete significantly more TNFα following LPS restimulation, while J774A.1 cells did not (Fig 14A, Fig. 15A).

Conceivably, this could be due differences in phenotype between BMDMs and J774A.1 cells. According to our M-CSF differentiation protocol, BMDMs should have the phenotype of a mature macrophage (Madaan et al., 2014). Whereas J774A.1 cells have an intermediate monocyte-macrophage phenotype (Chamberlain et al., 2009). However, both BMDMs and J774A.1 cells have similar expression levels of cell surface markers for differentiation (CD14, FcR), integrins (CD11b, CD11c, CD18) and activation (TLR4, CD40) (Chamberlain et al., 2009).

On the whole, our data indicates that J774A.1 cells can be used as a murine *in vitro* model to study trained immunity. The J774A.1 model was used to screen for trained immunity stimulants—GLA, LPS, MDP, CL, and delivery system. Finally, the J774A.1 model was used to develop a delivery system which acts through NOD2-TLR4. Furthermore, BMDMs validate our J774A.1 cell trained immunity findings; however, we merit caution in regard to directly comparing cytokine production, phenotypes, and activation states.

4.3 THP-1 cells as a human *in vitro* model for trained immunity

THP-1 cells trained with GLA, MDP and delivery system only showed a trained immunity cytokine profile—significantly higher TNFα production compared to NT cells—when restimulated with LPS (Fig. 16A). However, THP-1 cells are a human monocyte cell line, meaning they are in an undifferentiated state (Chamberlain et al., 2009). Therefore, THP-1 monocytes are much less reactive to immune stimuli (Chamberlain et al., 2009). Nonetheless, THP-1 cells express moderately high levels of TLR4, explaining the rise in TNFα following LPS stimulation (Daigneault et al., 2010). Another theory is that LPS potentially differentiated the THP-1 monocytes to macrophages, enhancing their ability to produce TNFα. THP-1 cells do

express NOD2, therefore are capable of responding to MDP (X. Chen et al., 2018). However, studies show that MDP stimulated THP-1 cells have poor accumulation of NF-κβ in the nucleus, thus poor TNFα production (Takashiba et al., 1999).

Each of the markers analyzed has a unique function on its respective cell; CD14 is a TLR4 co-receptor, CD16 is a fragment crystallizable gamma receptor 3 (FCγRIII), and CD11b is an integrin. Human monocytes express CD14 and have low/absent expression of CD16 and CD11b (Marimuthu et al., 2018). However, THP-1 are not "normal" human monocytes, they are a unique immortalized cell line characterized by low CD14 and low CD16 expression (Bosshart & Heinzelmann, 2016). Conversely, human macrophages have high expression of CD14, CD16 and CD11b (Bosshart & Heinzelmann, 2016). Therefore, these surface markers were used to track the PMA induced differentiation of THP-1 cells from monocytes to macrophages. PMA is a mitogen activator that induces the differentiation of THP-1 cells to macrophages in a dose and time dependant manner (Starr et al., 2018). THP-1 cell expression of CD11b, CD14 and CD16 increased by 77.7%, 87.8% and 21%, respectively, over 72h PMA differentiation (Fig. 17, Table 6). Therefore, 72 hours was the optimal PMA differentiation time length tested.

In comparison to naive THP-1 cells, PMA differentiated THP-1 cells showed a strong trained immunity response. Following Dukoral, MDP and influenza restimulation, delivery system trained PMA differentiated THP-1 cells produced significantly higher levels of TNFα compared to NT cells (Fig. 18A). PMA differentiated THP-1 cells have a macrophage phenotype, and therefore express higher levels of PRRs and pro-inflammatory signaling cascades (Daigneault et al., 2010). That is, PMA differentiated THP-1 cells are prepared to respond to the imposed training stimuli. Interestingly, a trained immunity cytokine profile was absent following LPS restimulation (Fig. 18A). LPS is a potent pro-inflammatory activator of human macrophages

(Agbanoma et al., 2012). Restimulation with LPS increased the production of TNFα by all groups of PMA differentiated THP-1 cells, including the NT control. Perhaps the level of TNFα produced by the cells was saturated at the LPS dose used for restimulation. On the whole, the data suggests PMA differentiated THP-1 cells are a better *in vitro* model to study trained immunity in comparison to their monocyte precursors.

Our data indicates, CL+GLA and CL+GLA+MDP (DS) trained PMA differentiated THP-1 cells produce significantly more TNFα compared to NT cells when challenged with Dukoral, MDP and influenza virus (Fig. 19A). These results suggest delivery system training in PMA differentiated THP-1 cells could be attributed to the GLA and CL components of the system. GLA is a synthetic derivative of LPS, which signals though TLR4 (Coler et al., 2011). As previously mentioned, cationic liposomes may trigger pro-inflammatory responses through TLR2 (Pizzuto et al., 2017). Both TLR2 and TLR4 signal through MyD88 adapter protein promoting NFκB mediated induction of proinflammatory cytokine production (Underhill, 2007). Simultaneous stimulation of TLR2 and TLR4 in THP-1 cells have shown to produce a synergistic effect with respect to immune stimulation (S. Yang et al., 2001). Therefore, training PMA differentiated THP-1 cells with GLA, and CL might together enhance MyD88 signaling, preparing the cells to react more robustly to non-specific restimulation.

A noteworthy difference between J774A.1 cells and THP-1 cells is MDP vs. GLA mediated training. As previously mentioned, delivery system training in J774A.1 cells was attributed to MDP+CL components. Whereas, delivery system training was attributed to GLA+CL in PMA differentiated THP-1 cells. Perhaps this is due to differences in NOD2 and TLR4 expression patterns between the two macrophages.

PBMCs displayed a trained immunity cytokine profile *in vitro*; delivery system trained PBMCs produced significantly more TNFα compared to the NT cells, when restimulated with Dukoral (Fig. 20A). PBMCs show similar results to PMA differentiated THP-1 cells with respect to delivery system induced trained immunity *in vitro*. However, delivery system trained PBMCs produced significantly higher levels of TNFα compared to NT cells even in the absence of restimulation (Fig. 20A). Our PBMC results are consistent with a study done by Kleinnijenhuis et al., 2012, where MDP and BCG trained PBMCs show an increase in TNFα production in response to restimulation with Pam3Cys (P3C), *Staphylococcus aureus*, and *C. albicans*. Likewise, in another study, PBMCs trained with β-glucan, BCG and oxLDL produce significantly higher levels of TNFα compared to the NT cells when restimulated with LPS and P3C (Bekkering et al., 2016). Finally, *C. albicans* trained PBMCs produce significantly higher levels of TNFα compared to the NT cells when restimulated with LPS, P3C, *M. tuberculosis* and *C. albicans* (Quintin et al., 2012). Evidently, PBMCs are a well-established model used to study trained immunity. Literature indicates that they can be trained and respond to vastly non-specific pathogens. Since our results are consistent with findings in literature, they were used to verify our human *in vitro* model used to study trained immunity—PMA differentiated THP-1 cell results. However, when comparing results of PMA differentiated THP-1 cells to PBMCs we must be mindful that PBMCs contain an assortment of immune cells. PBMCs consist of both innate and adaptive cells and therefore, cannot be directly compared to monoculture of PMA differentiated THP-1 innate immune cells. A variety of cells within the PBMC mosaic contribute to the production of both TNFα and IL-10. Consequently, cytokine quantities produced by PBMCs and PMA differentiated THP-1 cells are difficult to compare. Nonetheless, trends—such as a trained immunity status can be verified with the PBMC model.

Overall, we determined that THP-1 cells must be differentiated from monocytes to macrophages with PMA in order to be used as an *in vitro* model to study trained immunity. The PMA differentiated THP-1 model was used to screen for trained immunity stimulants—GLA, LPS, MDP, CL, and delivery system. Finally, the PMA differentiated THP-1 model was used to develop a delivery system which acts through NOD2-TLR4.

4.4 Delivery system induced trained immunity

To determine if delivery system mediated training affects macrophage surface marker expression, CD11b, CD14 and CD16 were analyzed. CD11b expression did not fluctuate when PMA differentiated THP-1 cells were trained with the delivery system (Fig. 21, Table 7). However, following delivery system training there was a 1.9% increase and 16.2% decrease in the number of CD14+ and CD16+ cells respectively, compared to NT cells (Fig. 21, Table 7). This implies the delivery system slightly modifies the phenotype of the THP-1 cells. We speculated that delivery system training might increase the expression of CD14 because the GLA component of the system signals though TLR4, a coreceptor of CD14 (Traub et al., 2006). Accordingly, the delivery system slightly increased the expression of CD14 by approximately 2%. According to literature, training can induce changes in pro-inflammatory signaling cascades, epigenetic and metabolism. For example, MyD88 expression is elevated in response to simultaneous TLR2 and TLR4 training stimuli (Bagchi et al., 2007). Additionally, an elevation in H3K4me3 and H3K4me27 has been associated with trained immunity (Quintin et al., 2012). Changes in metabolism, indicated by increased lactate production have also correlated with trained immunity (Bekkering et al., 2016). Furthermore, a study conducted by Saz-Leal et al., 2018, indicates that trained immunity decreases expression of SH-2 containing inositol 5'

polyphosphatase-1 (SHIP-1). Inhibition of SHIP-1 in trained cells leads to increased PI3K/Akt/mTOR signaling leading to epigenetic reprograming that results in elevated expression of proinflammatory cytokine production (Saz-Leal et al., 2018) . CD16 is intimately associated with SHIP-1 signaling (Hazenbos et al., 1998). Therefore, we speculate that down regulation of CD16 is associated with decreased SHIP-1 signalling, demonstrated in trained immunity. Along with modulating the expression of surface receptors such as CD14 and CD16, perhaps the delivery system induces intracellular changes, not indicated with the surface markers analyzed. It would be interesting to analyze these potential intracellular modifications in future studies.

The next objective of our study was to evaluate signaling cascades involved in delivery system mediated training. In order to do so, we left PMA differentiated THP-1 cells non-inhibited or inhibited them with CLI-095 and/or Gefitinib, TLR4 and NOD2 antagonists, respectively. Results illustrated non-inhibited PMA differentiated THP-1 cells show a trained immunity profile (Fig. 22), consistent with previous results (Fig.18, Fig.19). However, inhibition of PMA differentiated THP-1 cells via CLI-095 and Gefitinib, resulted in termination of the training response (Fig. 22A). Therefore, we suspect the GLA and MDP components of the delivery system elicit their training effects through TLR4 and NOD2, respectively. I should be noted that gefitinib inhibits NT and delivery system trained PMA differentiated THP-1 cells' production of TNFα (Fig. 22A). This is consistent with literature, where Gefitinib has been shown to inhibit TNFα activation via MAPK (Ueno et al., 2005). Studies have shown that CLI-095 inhibits TLR4 signalling in THP-1 cells, indicated by blocking a positive feedback loop between TLR4 and NFκB (Wan et al., 2016). In particular, CLI-095, known also as resatorvid or TAK-242, binds to the intracellular domain of TLR4, potently suppressing both ligand-dependent and ligand-independent signaling through TLR4 (Kawamoto, Ii, Kitazaki, Iizawa, &

Kimura, 2008). Gefitinib is a RIP2 inhibitor, which prevents RIP2 from binding NOD2 to coordinate NFκB mediated cytokine responses (Tigno-Aranjuez, Asara, & Abbott, 2010). Additionally, Gefitinib is used clinically as an epidermal growth factor receptor (EGFR) inhibitor. EGFR is indirectly involved in TLR4 signaling by way of toll-like receptor activator molecule (TRIF) signal convergence leading to interferon (IFN) production (Chattopadhyay et al., 2015). Therefore, Gefitinib has the ability to inhibit NOD2 signaling directly and TLR4 signalling indirectly. Likewise, studies have shown NOD2 knockouts and inhibition of RIP2, impede BGC vaccine mediated trained immunity (Kleinnijenhuis et al., 2012; Mourits et al., 2018). Furthermore, studies propose that MDP provokes trained immunity through the NOD2 receptor (Rusek et al., 2018). Taken together, these studies support our hypothesis of delivery system mediated training through TLR4 and NOD2.

As expected, PMA differentiated THP-1 cells did not produce IL-2 (Fig. 23). This is easily explained by the fact that IL-2 is produced primarily by T cells and DCs (Malek et al., 2008). T cell activation requires three signals and the APC—PMA differentiated THP-1 cells—plays an important role in providing these activation signals. First, the antigen is presented via major histocompatibility complex (MHC) to the TCR/CD3 complex (signal 1) (Smith-Garvin, Koretzky, & Jordan, 2009). Next, APC's costimulatory CD28 binds to CD80/86 on the T cell (signal 2) (Smith-Garvin et al., 2009). Finally, cytokines (signal 3) modulate the T cell activation (Smith-Garvin et al., 2009). We activated the Jurkat T cells with CD3 and CD28 prior to coculture so that they would be ready to respond to antigens presented by the THP-1 cells. Our results show, co-culturing PMA differentiated THP-1 cells with CD3/CD28 activated Jurkat T cells increased the production of IL-2 (Fig. 23). The increase in IL-2 production is likely a result of the T cells receiving signals 1 (MHC-TCR/CD3), signal 2 (CD28-CD80/86) and signal 3

(cytokines from the PMA differentiated THP-1 cells) necessary for T cell activation (Ross & Cantrell, 2018). Following Dukoral restimulation, the co-culture containing delivery system trained PMA differentiated THP-1 cells produced significantly more IL-2 compared to the co-culture with untrained PMA differentiated THP-1 cells (Fig. 23). Throughout our study it has been shown that the delivery system is capable of training PMA differentiated THP-1 cells to produce significantly more TNFα compared to NT controls following restimulation with Dukoral (Fig. 17, Fig.18, Fig. 21). According to literature, TNFα promotes activation and proliferation of naïve T cells (Mehta, Gracias, & Croft, 2018). Thus, elevated TNFa (signal 3) produced by delivery system trained cells could explain the difference in Jurkat T cell IL-2 production.

IL-2 plays an important role orchestrating T cell effectors and memory cells. Akin to most cytokines, IL-2 works within a complex signaling network to manipulate transcription and metabolism to determine T cell fate. Ultimately, CD4+ and CD8+ T cell activation leads to a robust adaptive immune response (Ross & Cantrell, 2018). The present investigation provides evidence for a link between delivery system mediated trained immunity and the adaptive immune response. The mechanism has not been fully elucidated; however, it is valuable knowledge for researchers in the field and a point of investigation for future studies.

4.5 Conclusions

The present investigation demonstrates that J774A.1 and PMA differentiated THP-1 cells can be used as *in vitro* models to study trained immunity. It was determined that a 3-day rest period was optimal when using these *in vitro* models to study trained immunity. Following 3 days of rest, the training effects began to diminish. The two cell lines were used to screen trained

immunity stimulants. The delivery system (CL+MDP+GLA) significantly increased TNFα production following non-specific restimulation in both the murine and human *in vitro* models, indicating the ability to train the cells. A critical difference noted between cell lines, was delivery system training in J774A.1 cells was attributed to MDP+CL components, whereas delivery system training was attributed to GLA+CL in PMA differentiated THP-1 cells. Perhaps explained by differences in NOD2 and TLR4 expression patterns between the two macrophage cell lines. BMDMs and PBMCs validated our J774A.1 and THP-1 trained immunity findings, respectively. However, we air on the side of caution comparing *in vitro* cell lines to *ex vivo* BMDMs and PBMCs because we recognize variances in cytokine production, phenotypes, and activation states. On the whole, this study allowed for better characterization of a murine and human *in vitro* cell model to study trained immunity, providing a sustainable avenue for researchers to elucidate the phenomenon and develop novel therapeutics. The many advantages of *in vitro* cell models include cost efficiency, ease of use, reliability, reproducibility and less ethical concerns (Kaur & Dufour, 2012).

Since the delivery system induced training in both cell lines, it was prudent to investigate the mechanism by which the delivery system elicits its training effects. We discovered the delivery system increased the expression of CD14 and decreased the expression of CD16 on PMA differentiated THP-1 cells. Moreover, by way of cell signaling inhibitors CLI-095 and Gefitinib we were able to deduce that the delivery system was inducing training partially through TLR4 and NOD2, respectively. Finally, to evaluate the link between trained innate immunity and adaptive immunity trained THP-1 macrophages were co-cultured with CD3/CD28 activated Jurkat T cells. Results indicate, activated Jurkat T cells co-cultured with trained PMA differentiated THP-1 cells produced significantly higher levels of IL-2 upon subsequent

pathogen challenge. Suggesting, trained innate immune cells have the potential to influence the adaptive immune responses.

On the whole this research has contributed to the development of a bioassay model will allow scientists in all fields of immunology to further explore the phenomenon of trained immunity. Additionally, these *in vitro* models can act as tools to explicate the potential of trained immunity as a novel therapeutic strategy.

4.6 Future Research

In future research we would be interested in looking at more cytokines (IL-1β, IL-6 and IL-12), metabolic changes (switch from OXPHOS to aerobic glycolysis) and epigenetic changes (H3K4me, H3K9me and H3K27ac) to confirm delivery system mediated trained immunity in J774A.1 cells and THP-1 cells. Additionally, to understand differences in delivery system mediated training we would further elucidate expression patterns of NOD2 and TLR4 in J774A.1 cells compared to PMA differentiated THP-1 cells. Furthermore, we would like to further evaluate the link between trained innate immunity and adaptive immunity. Finally, it would be interesting to translate our *in vitro* results to a mouse model, and study the delivery system as a trained immunity stimulant *in vivo*. *In vivo* studies would provide insight to the complex workings of the immune system. Perhaps the delivery system could be used as an adjuvant in trained immunity-based vaccines, or used to therapy address pathologic immune imbalances that individuals face.

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