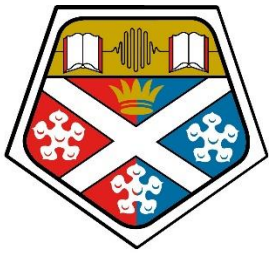

University of Strathclyde Glasgow

Strathclyde Institute of Pharmaceutical and Biomedical Sciences

Faculty of Science

Investigating the Anti-Inflammatory Effects of Non-Ionic Surfactant Vesicles

Jonathan McGahon

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree

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Contents

Abstract

Inflammation can be an unwanted consequence or cause of debilitating diseases of infectious and non-infectious aetiologies. Sepsis can be a result of an uncontrolled immune response to bacterial infections (including MRSA, *E. coli* and *P. Aeruginosa*) or viral infections (including SARS-CoV-2 and Influenza). Immune dysfunction can also cause autoimmune diseases including rheumatoid arthritis, ulcerative colitis and Crohn's disease. Current anti-inflammatory medications have a number of deficiencies including lack of specificity and undesirable side effects. Herein the potential of Non-ionic surfactant vesicles (NISV) as an anti-inflammatory drug and their mode of action is investigated. NISV were found to have a wide array of anti-inflammatory effects on macrophages *in vitro* including down-regulation of IL-6, IL-12 and multiple chemokines regardless of whether cells were stimulated with LPS, Poly(I:C) or Pam3csk4. The individual components of NISV, monopalmityol glycerol (MPG), dicetyl phosphate (DCP) and cholesterol) did not replicate the immunomodulatory effects found in macrophages, proving the formulation of NISV is essential for the anti-inflammatory effects. Liposomes were shown to augment LPS and Poly(I:C) stimulation of macrophages, inducing upregulation of pro-inflammatory cytokines including IL-6 and TNF-α, demonstrating that the anti-inflammatory effects of NISV are not a common feature of all vesicular formulations. Transcriptomic analyses showed consistent anti-inflammatory effects, and indicated down-regulation of NF-κB as an important aspect of the anti-inflammatory effects mediated by NISV. Metabolomic analysis show NISV disrupt the Warburg effect by reducing production of itaconate and succinate, indicating anti-inflammatory downstream effects. The mechanism through which NISV down-regulate NF-κB is unknown. However, the NISV's primary component, MPG, demonstrates structural similarity to the sphingolipid, sphingosine-1-phosphate (S1P). As S1P is an immune mediator that acts through NF-κB, future work should explore the hypothesis that MPG disrupts S1P signalling.

1. Introduction

1.1 Inflammation

The term ‘Inflammation’ is derived from the Latin, ‘Inflammare’ meaning to set on fire and was used by Celsus in 1AD who described four aspects of this phenomenon: *rubor et tumor cum calore et dolore* (redness and swelling with heat and pain). The original texts were more recently translated and published in 1876 (Celsus, 1876).This phenotypic description is now recognised to be a result of a number of different molecular mechanisms involving different cells and mediators. Some inflammation could be considered to be an inevitable accompaniment of a useful immune response, although inflammation can also contribute to the pathology of diseases. The immune response to infectious agents is a complex process involving the interactions between many cell types and mediators that culminates in elimination of infection and initiation of the healing response, although there are exceptions in organisms that specialise in host evasion, such as *Toxoplasma*. Certain immune mediators are known to play beneficial roles in these processes, but can play a detrimental role if their production is too high or prolonged. Notably, in extreme cases dysregulation of the immune response can result in life-threatening or life-changing conditions including sepsis, autoimmune diseases or anaphylaxis. Consequently, measurement of inflammatory mediators and interpretation of their roles in infectious diseases needs to be performed in the context of the immune response as a whole.

1.1.1 The context of Inflammation in the immune response

Surface level injuries, such as scrapes or impact injuries, elicit an inflammatory response easily identified by the onset of heat, redness, swelling and pain (Celsus, 1876). This is the result of the activation of immune components including the complement pathway and immune cells such as macrophages and neutrophils, which produce compounds inducing the phenotypic response as described previously, this response acts to induce the healing process and detect if any infectious agent has breached the skin (Koh & DiPietro, 2011). If an infectious agent is detected, the innate immune system is responsible for the detection and initiation of elimination of the foreign agent. This response begins with the complement cascade, this system holds many immune functions and can be activate through multiple triggers. The three pathways of complement activation are the classical, alternate and lectin pathway and these three pathways involve primarily the same set of proteins C1 through C9. The classical pathway is activated through the C1 complex binding to an immunoglobulin

that has formed a complex with an antigen or directly to a pathogen (Janeway et al, 2001). This binding leads to a conformational change in C1 that activate serine proteases which then cleaves C4 into C4a and C4b, C1 then combines with C4b (C14b) to cleave C2, in to C2a and C2b. C2a and C14b then combine to form a C3 convertase (C14b2a), which then splits C3 into C3a and C3b (Janeway et al., 2001). C3b then combines with C14b2a to form a C5 convertase (C14b2a3b) that breaks down C5 into C5a and C5b. C5b then forms the membrane attack complex (MAC) by complexing with C6, C7, C8 and C9. The MAC forms on the surface of the pathogen creating pores in the cell membrane, this causes leakage and cell lysis when enough MACs have formed (Janeway et al., 2001). The alternative pathway of complement is triggered by the recognition of endotoxins; primarily lipopolysaccharide (LPS), the first step of this pathway is the spontaneous hydrolysis of C3 into C3b. C3b then forms a complex with Bb forming the C3 convertase of the alternative pathway, which breaks down C3 into C3a and C3b (Janeway et al., 2001). C3b the forms a complex with itself and Bb to create a C5 convertase, it is at this point the alternative pathway reconciles with the classical to culminate in the MAC. The lectin pathway is activated by the binding of mannose binding lectin (MBL) to mannose and glucose on the cell wall of organisms that present mannose and glucose. This recognition induces the production of a serine protease which forma a complex with MBL and cleaves C2 and C4 in to C2a and C2b, and C4a and C4b (Janeway et al., 2001). A C3 convertase is then formed of C2a and C4b and the pathway reconciles with the classical pathway. The different components of the complement pathway possess functions outside of the cascade including opsonic, chemotactic and activatory functions. C3a, C4a and C5a all function as activators of immune cells including basophils and mast cells. C5a is also a chemoattractant of neutrophils, recruiting them to the site of infection. C3b acts as an opsonising agent signalling and facilitating phagocytosis by immune cells (Janeway et al., 2001).

The phagocytic cells, such as macrophages and dendritic cells, and their recognition of pathogen-associated molecular patterns (PAMPs), upon recognition by pattern-recognition receptors (PRRs) inflammatory pathways are activated. Through various signalling cascades, primarily MyD88 signalling, pro-inflammatory effectors, such as IL-1β, IL-6 and TNF-α, are produced to combat the infection and/or recruit other immune cells, like neutrophils (Kumar et al, 2011). An example of this process is the reaction to the PAMP; LPS is a major component of the Gram negative bacteria's cell membrane and when the bacteria dies and disintegrates LPS is released into the

cellular environment and when detected by the PRR, toll-like receptor 4 (TLR4), a signalling cascade begins, as shown in Figure 1.2. This cascade includes the two pathways that facilitate all toll-like receptor signalling the MyD88 and TRIF pathways, which culminate in the activation of NF-κB, AP-1 and IRF transcription factors, these factors induce production of pro-inflammatory effectors, which recruit and alter the expression patterns of immune cells they interact with (Kawai & Akira, 2007). During the release of immune effectors, the phagocytic cells engage in phagocytosis engulfing the source of the antigen and, in most cases, digestion of the infectious agent (Figure 1.1). After digesting a foreign agent its components are utilised for antigen presentation through the major histocompatibility complex (MHC) class II, the antigens are processed and epitopes are created and coupled with MHC class II and shuttled to the cell surface where they are presented to $CD4^+$ T-cells for processing (Jones et al, 2006). During this process a number of stimulatory actions take place inducing changes in the APC as well as the interacting T-cell. Present on the surface of T-cells is CD40L, this molecule interacts with CD40 on the surface of the APC inducing a more inflammatory state, increasing expression of TNF receptors and upregulating NO and ROS production (Chatzigeorgiou et al, 2009). CD80 and CD86 are also important in the activation of the inflammatory response in T-cells and interacting APCs. These molecules have overlapping effects, CD80 and CD86 interact with cells expressing CD28 where it causes a co-stimulatory reaction, inducing changes in both the CD80/86 expressing cell and the CD28 presenting cell. For example, in an interaction between a $CD8^+$ T-cell and a DC expressing these co-stimulatory molecules, the T-cell is preferentially differentiated into a cytotoxic T-cell and the DC increases production of pro-inflammatory mediators (Mir, 2015; Orabona et al., 2004). CD80 and CD86 can also induce an inhibitory response upon interacting with CTLA-4; this interaction induces a reduction in stimulatory molecules produced by T-cells and supresses further activation of T-cells (Zheng et al., 2004). The interactions between APCs and T-cells are essential components of the adaptive immune response. The adaptive immune system works in two ways, depending on if the system has been previously exposed to an infection or not. If an infection is novel to the body the innate immune response is primarily relied upon to minimise spread and damage caused by infection. This is done by the expression of an array of pro-inflammatory cytokines, creating an inhospitable environment for the infection, expression of cytokines recruiting more immune cells and bioactive molecules, such as macrophages, DC's and neutrophils and recruitment of the complement proteins. Following phagocytosis the

cells of the innate immune system, primarily macrophages and DC's, break down the infection and express its components via MHC class II for presentation to cells of the adaptive immune response.

Utilising the MHC class II system, after digestion of the foreign agent, antigen-presenting cells (APCs) migrate to lymph nodes be present to CD4+ T-cells. When presented with a foreign antigen, naïve CD4+ T-cells undergo maturation into T helper (T_h) cells, which then divide rapidly and produce cytokines to recruit and differentiate other immune cells. T_h cells fall into multiple classifications, each of which produced different cytokines to fulfil a different purpose. T_h1 cells are induced by IL-12 and primarily produce IFNγ. This cytokine is important in macrophage activation, inducing the pro-inflammatory M1 form (Zhu & Paul, 2008). Th2 cells are induced by the presence of IL-4 and IL-2, they produce cytokines, such as IL-4 and IL-13 aid in the differentiation and antibody production by B cells (Zhu & Paul, 2008). Th9 cells also play an important role in fighting parasitic infection and are known for their production of IL-9. This T helper cell phenotype is induced by the presence of TGF-β and IL-4, in combination they can cause maturation of naïve T-cells into T_h9 cells and TGF-β alone can cause T_h2 cells to differentiate into T_h9 cells (Kaplan et al, 2015). T_h17 are known for their production of IL-17, IL-21 IL-22 and GM-CSF. These cells are induced in the presence of IL-6, IL-21, IL-23 and TGF-β. T_h17 cells are responsible for the recruitment of neutrophils and play a role in pathogen clearance from mucosal surfaces (Ivanov et al., 2006; Zambrano-Zaragoza et al, 2014). Other than these subtypes of T_h cells naive T cells can differentiate into memory T-cells upon stimulation by an antigen, although the exact mechanisms through which this is done is still debated (Restifo & Gattinoni, 2013). Memory T-cells serve a purpose identical to that of memory B cells, but with different functions, augmenting the immune response when the remembered antigen is subsequently detected. Cytotoxic $CD8^+$ T-cells are a subtype of T cell responsible for destruction of infected cells. They are made upon a naïve $CD8^+$ T-cell interacting with an APC, where they undergo maturation into cytotoxic $CD8^+$ T-cells (Hivroz et al, 2012). These cells main function is performed through the body's self-recognition system, MHC class I. MHC class I is expressed on all cells in the body, excluding red blood cells, and acts as a recognition point for the immune system, specifically cytotoxic T-cells (Hansen & Bouvier, 2009). It works by presenting native peptides which have been broken down within the cytoplasm of a given cell, the peptide is presented on the MHC class I and, if recognised by a cytotoxic T-cell, the cell is signalled as safe and is not destroyed (Wieczorek et al., 2017). This form

of presentation can also be utilised in a form similar to MHC class II, wherein if a virus infects a cell intracellularly, for example, the infections proteins are naturally broken down and presented as peptides on MHC class I. These peptides are not recognised as self by cytotoxic T-cells and the cell presenting is signalled for destruction (Hansen & Bouvier, 2009).

Upon maturation, B-cells form into either memory B-cells or plasma cells. B-cell activation functions through expression of the antigen on MHC class II, which is detected and the presented peptide is endocytosed and processed by the B-cell (Blum et al, 2013). The B-cell undergoes maturation in response to the CD40L produced by the T-cells, which causes an increase in proliferation, immunoglobulin (Ig) class switching and somatic hypermutation (Crotty, 2015). At this point, the B-cells differentiate into either memory B-cells or plasma cells, the initial batch of plasma cells produce low-affinity antibodies to assist in the immediate infection, but the others undergo more Ig class switching and somatic hypermutation to produce highly specific high affinity antibodies. The memory B-cells also undergo Ig class switching and somatic hypermutation forming the specific antibodies for an infection after which they are primed to quickly react and produce these antibodies upon subsequent infection (Nutt et al, 2015). This process is highly specific and effective at clearing an infection but, when the infection is novel, takes several days to perfect

The innate immune response is immediate but non-specific and causes noticeable side-effects in the host such as, fever and swelling. These systems are both highly important and effective but they are not without flaw, which can lead to some debilitating and even fatal disease.

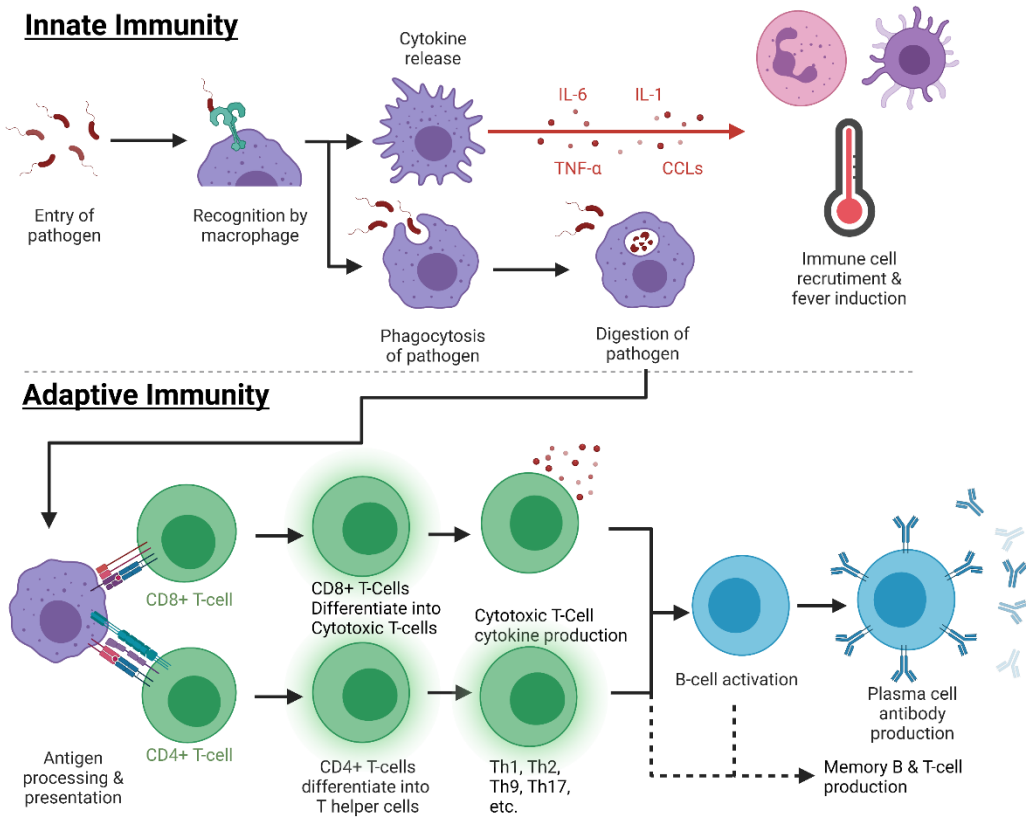


Figure 1.1 Processes of Immunity. *A diagrammatic representation of innate and adaptive immunity responding to an unknown bacterial pathogen. Upon recognition, macrophages polarise to produce pro-inflammatory cytokines, recruiting cells, including neutrophils & DC's. After phagocytosing a pathogen, it is broken down and its peptides are presented on MHC class II. These peptides are then presented to T-cells. CD4+ T-cells differentiate into various different forms depending on the inflammatory environment, these cells 'help' by activating and recruiting other immune cells depending on the infection. CD8+ T-cells differentiate into cytotoxic T-cells, which destroy cells with foreign peptide presented on MHC class I. B-cells are activated upon antigen presentation by T-cells or APC's (not shown), where they produce antibodies targeted to the peptide presented. Helper T-cells, cytotoxic T-cells and B-cells can all form memory cells in preparation for future infections.*

1.1.1 Inflammatory disorders of the Immune System

Some situations can cause the immune system to malfunction both during the innate and adaptive immune responses, causing debilitating disease and even death. These diseases come in many forms and can cause sudden onset illness, such as sepsis or debilitating lifelong conditions, in the cases of autoimmune diseases. Treatment of immune malfunction is inherently difficult as it involves bypassing or disabling the host's immune response, weakening their immune system and possibly leading to further complications.

1.1.1.1 Sepsis

Sepsis is a life threatening condition caused by an overreaction of the immune system to a toxin produced by an infection, such as *Staphylococcus aureus*, present in the bloodstream, called septicaemia (Otto, 2014). This illness often begins as another form of infection, such as a urinary tract or respiratory infection, which is not properly cleared by the immune response and migrates into the bloodstream and continues propagation (Vincent, 2016). At this point, the infection is likely out of control of the immune system and requires medical intervention. Despite this, the immune system will continue combating the infection to the detriment of the host. Sepsis is characterised by an increase in immune cells and their unrestricted release of pro-inflammatory cytokines. This immune response leads to common side-effects of these cytokines, such as fever and chills, but without treatment will lead to organ dysfunction and soon organ failure throughout the body through the action of the cytokine being overproduced. Sepsis has a very high mortality rate and no specific treatment due to its large number of potential causes and profiles. Its mortality rate depends on how early sepsis is diagnosed and treatment begins; when found early the mortality rate is 30%, 50% when the disease advances to severe sepsis and when septic shock occurs mortality is 80% (Jawad et al, 2012). The treatment of sepsis has been thrust to the forefront of modern medicine in this past year by SARS-CoV-2. COVID-19 infections are largely non-lethal but when complications occur the body becomes septic and an immune overreaction takes place causing severe illness that leads to death without treatment (Olwal et al, 2021). Treatment of sepsis is generally untargeted and relies on infection control through the use of antibiotics, antivirals or antifungals, depending on the infectious agent, or surgical removal of the infected area and the support of organ function (National Health Service, 2019b). In sepsis caused by COVID-19, the

corticosteroid Dexamethasone has been used in combination with anti-virals, primarily remdesivir, to reduce inflammation and combat the virus in parallel (Scavone et al, 2020).

1.1.1.2 Autoimmune Diseases

As stated previously, immune disorders are not only seen as sudden illness but can cause debilitating lifelong conditions, called autoimmune diseases. These disease are cause by misrecognition of native peptides as foreign; these peptides generally share structural similarity with a previously detected antigen, and when detected again they cause a flare up in the immune system inducing pro-inflammatory cytokine production. Rheumatoid arthritis (RA) is one of the most common of these diseases, affecting as much as 1% of the worldwide population (Smolen & Aletaha, 2017). It is caused by the disproportionate targeting of the synovium by the immune system and typically causes swelling, pain and stiffness (National Health Service, 2019a). There is no cure for this lifelong condition and treatments range from the use of disease-modifying anti-rheumatic drugs (DMARDs) for the easing of symptoms and to slow progression to the use of corticosteroids during serious flare-ups of the disease (National Health Service, 2019c). Illness within the gastrointestinal tract caused by immune dysfunction is also a common form of autoimmune disease. The incorrect targeting of the gastrointestinal tract by the immune system causes illnesses such as ulcerative colitis and Crohn's disease. This targeting is caused by multiple factors, genetics, gut bacteria composition and diet but its source varies between individuals. Ulcerative colitis and Crohn's disease can both cause diarrhoea, which is extremely common in any inflammation of the gut, rectal bleeding and weight loss (National Health Service, 2020). Its treatments are similar to those of RA and allergies, a controlled diet is prescribed in combination with immunosuppressants, such as corticosteroids, but although these may alleviate symptoms there is no cure. Specific disabling of key cytokines in these conditions has been achieved using monoclonal antibodies. In RA, Crohn's and ulcerative colitis mAbs which bind and disable TNF-α have been used as successful treatments (Du et al, 2017; Lúdvíksson et al, 1999) . These autoimmune diseases rarely cause death in their sufferers but do require lifelong maintenance and care by the sufferer in order to maintain a normal life; these diseases also increase the chance of cancers, especially ulcerative colitis and Crohn's disease (National Health Service, 2020).

1.1.2 Repeat Offenders of the Immune System

There are certain cytokines that are found overexpressed in all of the previously mentioned diseases and the control of these cytokines is a sought-after prize in medicine. Every cytokine storm is different but cytokines commonly found overexpressed in this situation include IL-1 and its superfamily, IL-6, IL-10, IL-12 and its family and TNF-α. IL-1, IL-6 and TNF-α are the most prolific of these cytokines in causing harm to the host due to their natures as fever inducers; they also function as activators and inducers of differentiation in various immune cells and promote their recruitment to the site of the reaction (Tisoncik et al, 2012). Important members of the IL-1 superfamily are IL-1α, IL-1β and IL-18; these three cytokines are important for induction of fever, as said previously, and they promote migration of inflammatory cells (Sims & Smith, 2010). IL-6 is induced by IL-1 and TNF-α and is an important modulator of the acute phase response, it also plays a role in B and T-cell activation (Garbers et al, 2018). IL-12 is also an important modulator of T-cell maturation, promoting the adaptive immune response. IL-10 is an anti-inflammatory cytokine that, despite its pro-inflammatory nature, is overexpressed in the diseases mentioned previously. It is responsible for various immunosuppressive actions, such as downregulating expression of cytokines in T-cells and macrophages, down-regulating the presentation of antigens bound to MHC class II and IL-10 also drives macrophage polarisation towards the anti-inflammatory M2 form, as well as promoting B cell proliferation and antibody production (Akdis et al., 2016; Viola et al, 2019). This anti-inflammatory effect works as negative feedback in cellular pro-inflammation, but does not induce a great enough effect to control the out of control immune response in pro-inflammatory diseases.

1.1.3 The Macrophage

One of the most important cells in immunity is the macrophage; it is one of the primary producers of many of the cytokines mentioned previously and through their cytokine production play a significant role in immune-related disease. Macrophages play many roles within the body, their main role lies within the immune response as phagocytic antigen-presenting cells, meaning they are part of the first line of cell mediated defence against infection and essential to the adaptive immune response in processing and presenting antigens to T-cells (Shapouri-Moghaddam et al, 2018). Macrophage polarisation describes the plasticity in macrophages which allows them to have multiple functional forms in immunity. The typical activated macrophage form is that of the M1 macrophage, a phenotype that is characterised by the expression of pro-inflammatory cytokines and recruitment and activation of other immune cells. This is the

form taken upon initiation of an immune response and the most problematic form in immune diseases (Orecchioni et al, 2019). Circulating macrophages polarize to this form when exposed to microbial products, such as lipopolysaccharide (LPS), peptidoglycan, or stimulating cytokines, such as interferon gamma (IFN-γ) or TNF-α (Figure 1.2). The other forms of macrophage are encompassed under the M2 descriptor but come in four varieties: M2a, M2b, M2c and M2d or tumour-associated macrophages (TAMs), as shown on Figure 1.3 (Orecchioni et al., 2019). The M2 form is an anti-inflammatory form of macrophage; the four types are induced by different mechanisms and hold different functions. M2a macrophages are induced by IL-4 and IL-13. When this form is induced, they begin the resolution of the pro-inflammatory response by sequestering IL-1 via overexpressing the mannose receptor. They also express the anti-inflammatory effectors IL-10 and TGF-β inducing tissue recovery and wound healing (Viola et al, 2019). M2b macrophages are induced by IL-1 receptor (IL-1R) agonists. These macrophages produce both pro and anti-inflammatory cytokines, such as IL-1β, TNF-α and IL-10, regulating both the pro and anti-inflammatory actions in the body. M2c is a strict anti-inflammatory form, induced by glucocorticoids and IL-10, these macrophages have similar expression profiles to M2a macrophages, producing IL-10 and TGF-β (Viola et al., 2019). TLR ligands, A2 adenosine receptor agonists or IL-6 induce the M2d form in macrophages. These macrophages are expressed in the tumour microenvironment and ensure an anti-inflammatory profile, expressing IL-10 and TGF-β, and encourage tumour growth and metastasis by expressing vascular endothelial growth factor (VEGF) (Zhou et al, 2020). Macrophage expression patterns are extremely important in many diseases, with M1 and M2d acting to propagate immune dysfunction and cancers, respectively, and M2a, M2b, and M2c acting as regulators of the immune response. The ability to control the cytokines expressed and the polarisation of these cells would prove a very powerful tool in the fight against immune dysfunction.

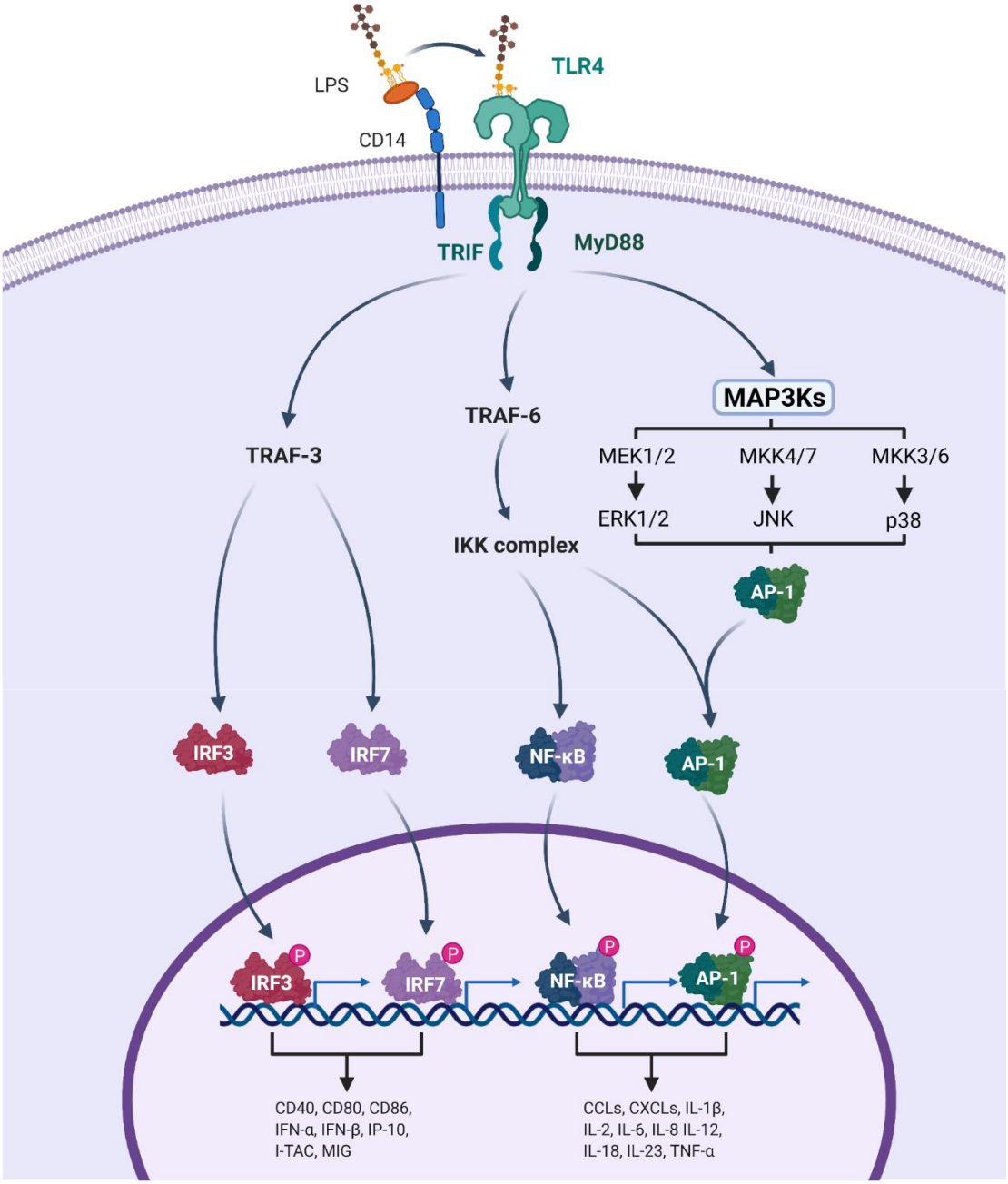


Figure 1.2 Signalling Cascade in Response to LPS Recognition. *Upon recognition of LPS the signalling cascade shown results in the up-regulation of many cytokines. The cascade is initiated by the binding and shuttling of LPS by CD14 to TLR4. This then activates the MyD88 and TRIF complexes. MyD88 begins a signalling cascade that through IKK and Map3K signalling culminates in the production of transcription factors NF-κB and AP-1, which activate a series of pro-inflamatory cytokines creating a hostile environment and recruiting immune cells. TRIF signalling is tranduced by TRAF-3 and culminates in the production of the transcription factors IRF3 and IRF7, which induce pro-inflammatory surface proteins essential to the adaptive immune response and cytokines affecting immune cell activation and maturation.*

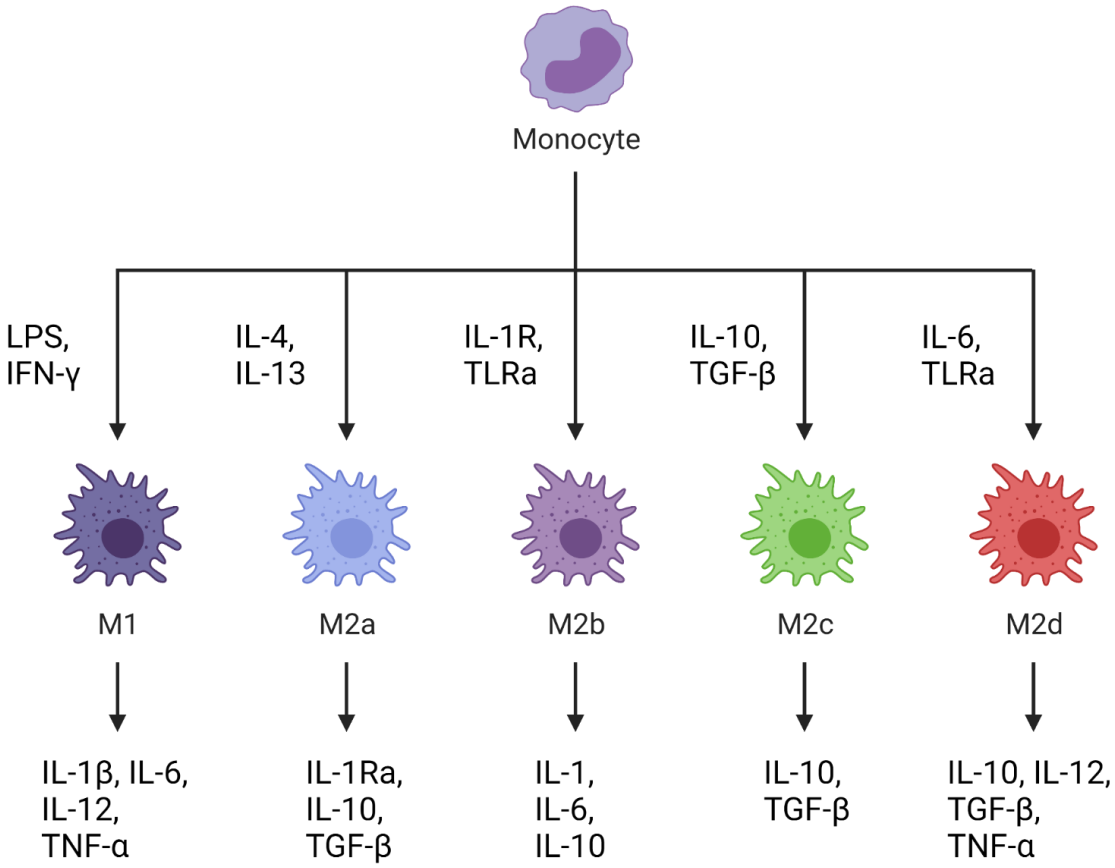


Figure 1.3 Differences in Macrophage Polarisation. *Diagrammatic representation of macrophage polarisation, showing the effectors that induce different macrophage types and the cytokines produced by said macrophage.*

1.1.3.1 Dendritic cells

Dendritic Cells (DCs) are important APCs and share many similarities with macrophages. They are primary responders to areas of injury and infection where they phagocytose and lyse foreign agents. They also share many response pathways with macrophages and can exert similar effects when then same stimulation is exerted. DCs differ from macrophage in their primary function, DCs form the primary link between the innate and adaptive immune system. While macrophage are APCs and present antigens to the adaptive immune system, DCs primary function is this process. When at a site of infection DCs will phagocytose and lyse an agent, process its antigens and immediately migrate to a lymph node to present the antigen to T Cells and B cells activating them (Smith et al, 2004). Macrophages and dendritic cells hold many overlapping functions; they also share pathway similarity when stimulated via TLRs, they would likely react to a novel anti-inflammatory drug in a similar fashion. The following project utilised *in vitro* macrophage cultures to mimic the conditions used by Roberts et al in the patent 'NON-IONIC SURFACTANT VESICLES AS A THERAPUTIC AGENT' (Roberts et al., 1997). Whether the effects found in this project would translate into dendritic cells would require further testing, but similar phenotypes would be expected.

1.1.4 Treating Immune Dysfunction

The drugs used to treat the diseases described previously have overlapping functions due to the similarity between immune responses and their potency at disabling it. However, as these drugs are immunosuppressive they are often used in combination with anti-infectives. Thus, sepsis is first treated with an attempt to reduce the infection, using antibiotic, antifungals or antivirals, depending on the infectious agent. Vancomycin is a commonly used antibiotic due to it having a broad-spectrum of activity and its ability to clear the common hospital acquired pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) (Rubinstein & Keynan, 2014; Van Hal et al, 2011). Despite its common use, vancomycin does have side-effects; these include severe local pain and thrombophlebitis to the severe, but uncommon, side-effect of nephrotoxicity (Rubinstein & Keynan, 2014). Used in the combating of COVID-19, Remdesivir is an antiviral that inhibits viral RNA polymerase halting viral RNA replication and slowing viral particle production. Its side-effects include nausea, infrequent side effects of fever and rare side effects including; chills, vomiting, high blood pressure and allergic reactions (Scavone et al., 2020). Drugs available for reducing inflammation itself are limited, but include glucocorticoids, Disease Modifying anti Rheumatic Drugs

(DMARDs), Non-steroidal anti-inflammatories (NSAID) and 'Biologicals' including soluble cytokine receptors and neutralising antibodies.

1.1.4.1 Glucocorticoids

A commonly used anti-inflammatory used for treatment of many inflammatory diseases including COVID-19 sepsis is dexamethasone. This is a glucocorticoid also commonly used to treat RA, Crohn's disease and ulcerative colitis (Barnes, 2006). Glucocorticoids are a class of steroid hormone based on their natural homolog cortisol. Cortisol and glucocorticoids work through the glucocorticoid receptor activating a signalling event that culminates in the upregulation of anti-inflammatory effectors and down-regulation of pro-inflammatory effectors specifically through the recruitment of histone deacetylase, which closes NF-κB's binding site on DNA, as demonstrated on Figure 1.4 (Barnes, 2006; Mittelstadt & Ashwell, 2001). Dexamethasone is one of the most potent glucocorticoids, with a potency up to 80 times that of cortisol and has a half-life up to 54 hours compared with cortisol's 8 hours (Ahmet, 2019). While dexamethasone's anti-inflammatory potency is obvious, using such a potent immunosuppressant can cause a significant number of side-effects. Common side-effects include: an increased risk of infection, headaches, amnesia, irritability among many others, some rarer side-effects include: Cushing's syndrome, osteoporosis, mania, psychosis and cardiomyopathy (Barnes, 2006). Treating sepsis with glucocorticoids requires a fine touch, treating with too high a dose fully disables the immune system allowing infection to run rampant, if the antibiotics/antivirals/antifungals are ineffective, using too low a dose results in minimal reaction from the patient and does not halt septic shock's damaging effects. The COVID-19 pandemic identified a gap in treatment of novel illnesses that cause sepsis. Consequently, finding new anti-inflammatories to expand treatment options should be a priority in order to prevent unnecessary deaths in subsequent pandemics and upon the oncoming antibiotic-resistance crisis.

1.1.4.2 Disease-modifying anti-rheumatic drugs (DMARDs)

Disease-modifying anti-rheumatic drugs (DMARDs) are anti-inflammatory drugs used to treat arthritis. These drugs are slow acting over a period of weeks and months and so unlike glucocorticoids are not used for immediate relief. DMARDs encompass many drugs with no unified mode of action. Most commonly prescribed include the purine metabolism inhibitor methotrexate, the IL-1 and TNF-α inhibitor sulfasalazine and the anti-malarial hydroxychloroquine. Methotrexate was originally an anti-cancer drug used in chemotherapy, but for treatment of arthritis it is utilised in much lower

concentrations where it slows disease progression (Wessels et al, 2008). It is thought to do this by causing accumulation of adenosine, leading to the inhibition of T cell activation, causing downstream anti-inflammatory effects. This drug holds a range of side-effects, causing liver damage, leukopenia, both of which can lead to liver failure and a susceptibility to infection (American Society of Health-System Pharmacists, 2020). Sulfasalazine is used for its IL-1 and TNF-α inhibitory effects, however the mode of action of these effects are poorly understood. Its primary use is in treatment of IBDs, including Crohn's disease and ulcerative colitis. It too has severe side effects, causing urinary tract and intestinal obstruction and severe liver and kidney issues including organ failure. Also sulfasalazine is a sulfa drug and can exacerbate sulfa allergies (American Society of Health-System Pharmacists, 2021). Hydroxychloroquine is an anti-malarial with anti TLR effects and anti-MHC class II effects in APCs. In APCs the lysosomal pH is increased, causing more efficient breakdown of antigens but leaving less peptides for antigen presentation (Meyerowitz et al., 2020; Takeda et al, 2003). This in combination with the down-regulation of TLRs has an anti-inflammatory effect. However, hydroxychloroquine can cause minor side-effects including, vomiting, headaches and weakness, but also severe side-effects such as, retinopathy and cardiac arrhythmia (Owens, 2020; Yusuf et al, 2017). Biologics are relatively new to the DMARD family, their function is primarily anti-TNF and this function is preformed via two methods. Both involve direct interaction with TNF-α, one involves production of monoclonal TNF antibodies, which find and attach to TNF directing it for destruction, the other involves circulation of unbound TNF receptors that bind and deactivate TNF molecules. Biologics are effective but cause some side-effects, notably a susceptibility to infection and kidney damage (Li et al, 2017).

1.1.4.3 Nonsteroidal anti-inflammatory drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a drug class used for reduction of pain, fever and inflammation. Their mechanism of action is through the inhibition of the cyclooxygenase enzymes. These enzymes are essential in the production of prostaglandins and their reduction causes anti-inflammatory effects (Day & Graham, 2004). Due to the essential nature of prostaglandins in many bodily functions the side effects caused by NSAIDs are wide-ranging, including the inhibition of platelet aggregation, causing issues in blood clotting, increased chance of thrombosis, which can cause heart attack or stroke and gastrointestinal problems such as nausea, diarrhoea and gastrointestinal bleeding (Traversa et al., 1995; Trelle et al., 2011). Despite the wide-ranging side-effects NSAIDs are commonly used as severe side-effects are rare

in those without pre-existing conditions. NSAIDs anti-inflammatory effects, like DMARDs, are slow acting, taking weeks to have an effect (“Non-steroidal anti-inflammatory drugs,” 2021).

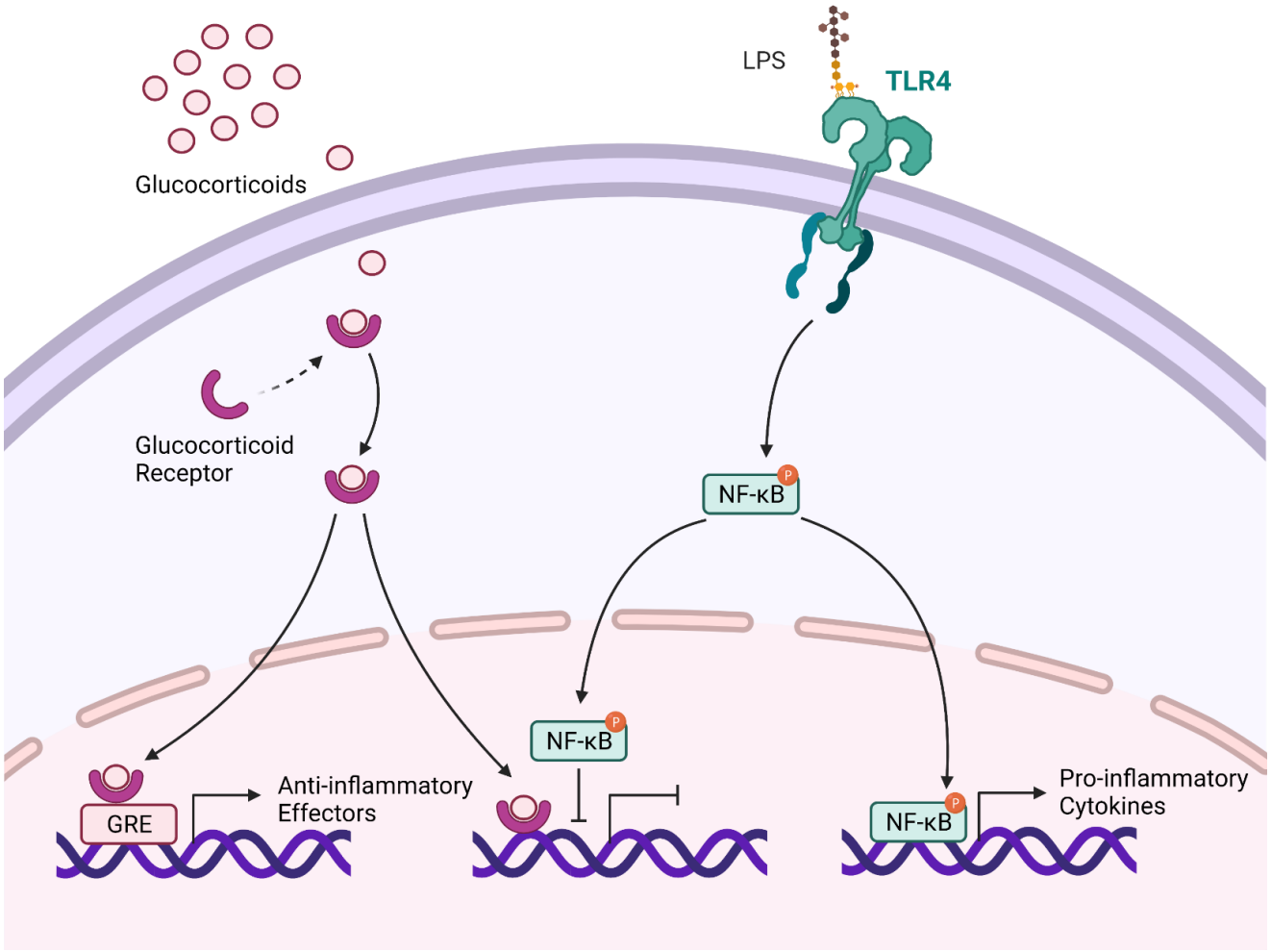


Figure 1.4 The Effects of Corticosteroids on NF-κB Signalling. *A representation of the effects of glucocorticoids on NF-κB signalling induced by TLR4 excitation. Glucocorticoids bind the glucocorticoid receptor. Following this, they bind to GRE and induce transcription of anti-inflammatory effectors. This complex an also block the binding sites of important pro-inflammatory transcription sites, such as NF-κB.*

1.1.5 The need for better Anti-Inflammatory Drugs

As mention above, current treatments all have clinical disadvantages and some are not financially viable for wide-spread use. New better treatments are therefore required. Ideally, these should have minimal side effects and be cost effective. One potential line of research is the use of non-ionic surfactant vesicles (NISV) which were shown to have promise as anti-inflammatory drugs in a number of in vitro and in vivo models (Roberts et al., 1997).

1.2 Non-Ionic Surfactant Vesicles

Non-ionic surfactant vesicles (NISV) are a synthetic vesicle-based delivery system used to enhance the bioavailability of drugs and extend the half-life of said drugs *in vivo*. NISV are similar to liposomes in many ways in both function and structure but instead of being comprised mainly of phospholipids, NISV's primary component is a non-ionic surfactant. NISV original use was for skin treatments, enhancing tanning products and moisturisers (Handjani-Vila et al, 1979). Since this, NISV have been used in a variety of ways solidifying themselves as a medical delivery platform for vaccines, genetic material and a plethora of drugs (Ge et al, 2019; Verma et al, 2010). They owe this range in ability to their vesicular structure, which, as shown in Figure 1.5, mimics the cell membrane allowing the entrapment of a variety of compounds with a large range of characteristics. The primary component of the vesicles is a surfactant, an amphiphilic molecule containing a hydrophilic head and a hydrophobic tail. This structure allows the formation of vesicles with broad entrapment capabilities. When a vesicle with a phospholipid or surfactant bilayer is formed it holds two sites of exclusion where certain drugs can be entrapped; the aqueous compartment formed in the centre of the vesicle and the hydrophobic compartment formed between the tail groups of the surfactants (Bulbake et al, 2017; Ge et al., 2019). These two compartments allow for the entrapment of drugs with great variations in polarity. The bilayer itself forms an area in which membrane proteins can be embedded, allowing a large array of molecules to add new functionalities to the vesicle. These include receptors, ligands and antibodies, which can be used for targeting, transporters, which can be characterised after being embedded in a vesicle, and surface molecules such as polyethylene glycol, which allows the vesicle to evade uptake and circulate longer *in vivo*.(Verma et al., 2010; Williamson et al., 2020).

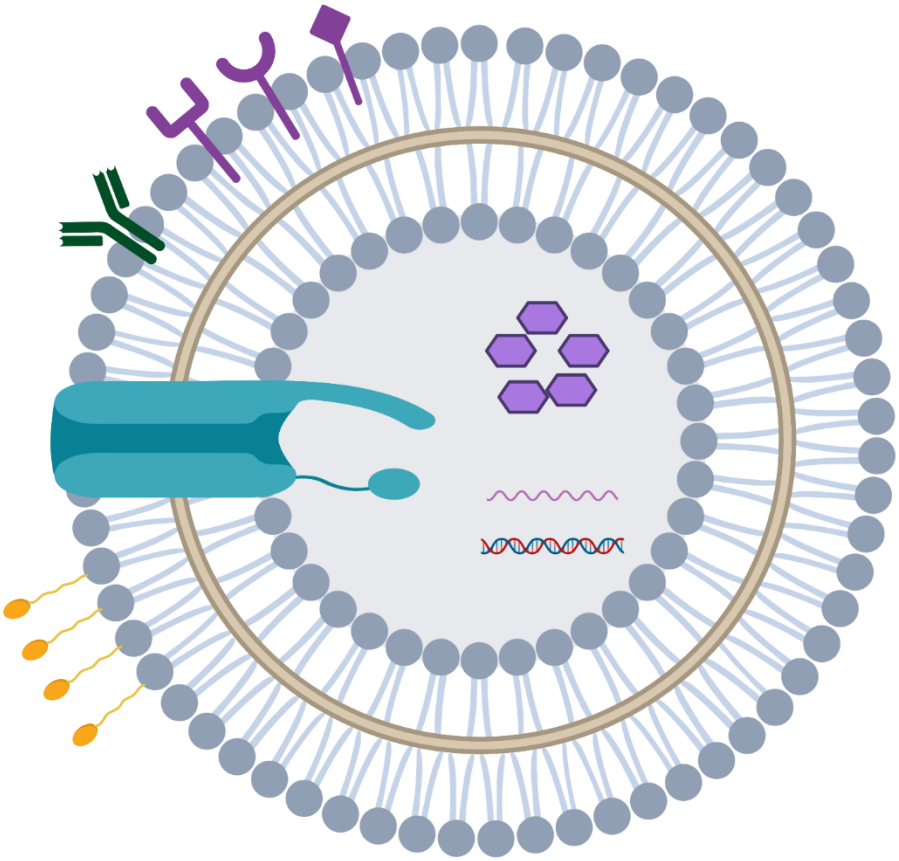


Figure 1.5 Example of a Unilamellar Vesicle. *A diagrammatic representation of a vesicle and the different forms of cargo that can be entrapped. The light brown section identified the hydrophobic compartment, and the light blue compartment identifies the hydrophilic aqueous core. The yellow molecules symbolise poly(ethylene glycol) (PEG) attached to the heads of the surfactants. The blue transmembrane protein symbolises an ion channel. The green antibody show that antibodies can be embedding in the surface. The purple membrane proteins symbolise the receptors and ligands that can be embedded in a vesicles surface. Drugs are symbolised by the purple & turquoise hexagons within the aqueous core and bilayer of the vesicle. The single stranded and double stranded molecules show vesicles capability to encapsulate nucleic acids.*

The most popular avenue of research, currently, for vesicle-based treatments is their use in drug delivery. NISV have been shown to increase stability and efficacy of many drugs, allowing treatments previously infeasible, including the delivery of beclomethasone dipropionate in treatment of pulmonary disease, in which NISV increase mucous permeation amplifying therapeutic effects (Pawar & Vavia, 2016). NISV have been used to increase efficacy of cancer drugs, which is the most common use of liposomes in medicine. (Paolino et al., 2008). Non-ionic surfactants alone have been identified as increasers of drug uptake through their down inhibition of multidrug efflux pump P-glycoprotein, which may be essential to their enhancement of drug efficacy (Kumar & Rajeshwarrao, 2011). NISV have also shown potential in overcoming the blood-brain barrier (BBB) and Woods *et al* showed significantly increased delivery of an anti-VEEV monoclonal antibody across the BBB by entrapment in NISV (Woods et al., 2020).

NISV have been shown to be powerful adjuvants in numerous studies utilising both model antigens and vaccine candidates in disease models. When used to encapsulate ovalbumin, NISV produced an increase in IL-2 production in BALB/c mice. In the same set of experiments NISV induced an increase in IL-12 and decrease in IL-10 in splenocytes, further showing NISV adjuvant candidacy (Brewer et al., 1996). In contrast, a patent filed, by the same laboratory that demonstrated NISV pro-inflammatory capabilities against entrapped antigen, indicated NISV could induce anti-inflammatory effects, reducing cytokines closely related to many of the inflammatory diseases mention previously (Roberts et al., 1997). Roberts et al discovered that NISV could reduce weight loss in *Toxoplasma*-infected mice. Following this discovery, they found that NISV reduced IL-1, IL-6 and TNF-α in LPS-stimulated human peripheral blood leucocytes and reduced IL-6 in mice for a sustained period of time (Roberts et al., 1997). Protection against weight loss by NISV has been shown since in experiments by D'Elia et al, in which three drugs, levofloxacin, ciprofloxacin and doxycycline, were encapsulated in NISV and used to treat *Burkhoderia pseudomallei* (D'Elia et al., 2019). In their experiment, it was found that NISV significantly reduced antibiotic induced weight loss and aided in the recovery period. In this study, they suspected that this may be due to a protective effect of the gut microbiome, due to its many links to weight modulation, but this avenue proved fruitless (Aoun et al., 2020). The formulations used in the patent and in this efficacy study are born of the same formulation, with identical compounds (MPG:Cholesterol:DCP at 5:4:1) used other than the addi-

tion of sodium deoxycholate in the efficacy study, which is used to increase oral stability (Conacher et al, 2001; D'Elia et al., 2019; Roberts et al., 1997). The protection against weight loss found in these two experiments is likely due to the same factor, namely NISV anti-inflammatory capabilities. These properties of NISV are poorly studied but the immunogenic properties of their counterpart liposomes have been extensively studied.

Liposomes have been shown to interact with the immune system but are not known as anti-inflammatory. In fact, they are known to stimulate the immune response, both with and without the presence of additional surface proteins (Zahednezhad et al, 2019). Circulating liposomes interact with the complement proteins, activating their cascade and increasing the reaction to foreign antigens. Their pro-inflammatory effects can be enhanced though the addition of poly(ethylene glycol) (PEG) which enhances interaction with complement proteins. The size and charge of liposomes can alter their immune effects but the outcomes remain pro-inflammatory. Liposomes have also been shown to stimulate a pro-inflammatory cytokine response when used as a delivery platform for immunosuppressive siRNA, increasing IL-6 and G-CSF in a dose-dependent manner in mice (Tabernero et al., 2013). The make-up of liposomes has proven important in controlling this cytokine reaction, Yanamoto et al have shown that hydrogenated egg phosphatidylcholine (HEPC) based liposomes can induce an inflammatory response comparable to that of LPS, inducing production of many macrophage related cytokines including: IL-6, IL-10, IL-1β and TNF-α. However, this reaction can be reduced by reduction of vesicle size from ~800nm to ~50nm showing the great importance size and composition of a vesicle has on its immune profile (Yamamoto et al., 2002). Despite these inflammatory effects, liposomes of many forms are used safely in the medical field today.

1.3 Summary of Study

In the following thesis, work was undertaken to identify and characterise the anti-inflammatory abilities of the NISV used by Roberts et al (Roberts et al., 1997). The formulation was produced using monopalmityol-glycerol (MPG), cholesterol and dicetyl phosphate (DCP) at a ratio of 5:4:1, respectively, manufactured using the melt method. This method produces large, negatively charged vesicles with a large disparity in sizes. Liposomes were used as a point of comparison for the NISV formulation. The formulation chosen was a modification of the liposomes used in the anti-cancer drug DaunoXome (Bulbake et al., 2017). The Perrie laboratory at the University of

Strathclyde are experts in liposomes formulation and their formulation of 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol at a ratio of 4:1 was manufactured using the nanoassemblr to make small, anionic and uniform vesicles, the phenotype generally sought after in liposomes (Khadke et al, 2018). An alternative surfactant was also utilised to make a novel NISV formulation, 1-O-hexadecyl-sn-glycerol (HG) was used due to its similarity with the MPG used in Roberts et al's formulation. Simply put, HG is identical to MPG in every way other than the linker between the head and tail groups. Here HG links the groups via and ether bond as opposed to the ester bond present in MPG's structure. These different formulations and manufacturing methods were put under scrutiny to find physiochemical properties required for immunomodulation. Representative formulations with immunomodulatory properties were further investigated to find the breadth of their activity and to investigate the mechanism through which activity is exerted.

1.4 Aims & Hypothesis

The aims of this study are to:

i. Confirm previous findings showing NISV has anti-inflammatory capabilities and investigate the breadth of these abilities to suppress cytokine production in bone marrow-derived macrophage (BMDM) stimulated with LPS. This will be done by treating LPS-stimulated BMDM with various forms of NISV, and other vesicle types, and comparing the levels of cytokine production to each other and relative controls. An IL-6 ELISA will be used for comparison of different formulations and stimulants and those of interest will be analysed using the Biolegend LEGENDplex bead array to further investigate effects on a broader range of cytokines.

ii. Investigate the effects of vesicular formulations on gene transcription, in particular on transcription of genes related to the immune system. Using transcriptomic analysis an in-depth look into the pathways and mechanisms most affected by NISV treatment will be possible, this will allow further insight into potential mechanisms of immune modulation by vesicle formulations.

iii. Investigate the effects of NISV on the metabolic profile of BMDM in unstimulated and LPS-stimulated settings. Using metabolomics analysis, the impact of NISV and liposomes on cellular metabolism will be analysed. Primarily metabolic function affecting the immune response, such as the Warburg effect, will be engaged in further depth to investigate possible mechanisms of vesicle-based immune reactions.

2. Materials & Methods

This section details the materials and methods used to produce the results discussed in the following chapters.

2.1 Vesicle Manufacture

Manufacture of vesicles can be performed in different ways. In this experiment methods used were the 'Melt' method and using the Benchtop Nanoassemblr® (Precision Nanosystems).

The melt method was carried out by melting the NISV components, DL-α-palmitin (MPG), cholesterol and dihexdecyl phosphate (DCP) at a ratio of 5:4:1, at 135°C then adding 5ml of PBS and immediately applying to a vortex for 2 minutes (Roberts et al., 1997).

The Nanoassemblr® is used by dissolving the vesicle components, DSPC and cholesterol at a ratio of 4:1, in 5ml of methanol at room temperature and following the Nanoassemblr user manual. A staggered herringbone micromixer chip was used, with a flow rate of 12ml/min and flow ratio of 3:1 (aqueous: solvent).

Vesicle size, poly-dispersity and zeta-potential were measured using the Zetasizer nano-ZS (Malvern).

2.1.1 Vesicle Centrifugation

Vesicles made using the Nanoassemblr were centrifuged through Vivaspin filtered falcon tubes for 30 minutes at 3000G.

2.2 Macrophage Culture

Bone marrow cells were harvested from the tibias and femurs of 8-12 week old BALB/c mice and incubated at 37°C for 3 days in 10 ml Complete DMEM (73% DMEM, 15% L-cell conditioned media, 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin) per bone wash out in petri dishes. On the third day 10ml more Complete DMEM media was added and the cells were incubated for 5 days at 37°C. For the final feed, the media was removed from the plates and replaced with another 20ml Complete DMEM they were then incubated for another 3 days at 37°C after which they were harvested. The media was removed from the plates and replaced with 10ml cold RPMI and the plates were scraped with a cell scraper to release the cells into the media. Cell suspensions were centrifuged at 1400rpm for 5 minutes and the pellets re-suspended in 5ml complete RPMI and a cell count was carried out.

2.2.1 Re-plating Cells

Viable cells were counted on a haemocytometer using trypan blue exclusion and cells were diluted as appropriate.

Cells were re-plated in 96-well plates (depending on experiment) with $1x10^5$ cells/well in complete RPMI and incubated at 37°C overnight to allow adherence to the plate.

2.3 Macrophage Stimulation

Macrophages were stimulated by adding 50µl of vesicles at different concentrations ranging from 3mM to 0.012mM in wells with or without LPS at 3µg/ml. These were then incubated overnight at 37°C.

2.3.1 AlamarBlue Toxicity Assay

100µl of supernatant was removed and stored at -4°C. 10µl of alamarBlue was then added to the remaining cells, which were incubated for 6 hours at 37°C in the dark. The absorbance was read at two wavelengths; 570nm and 600nm and the data analysed.

2.3.2 IL-6 ELISA

Plates were coated with of 1µg/ml IL-Capture antibody suspended in pH9 PBS (BD Biosciences) at 100µl/well and refrigerated at 4°C overnight. Plates were then washed with wash buffer (0.5ml Tween 20 and 999.5ml PBS) 3-5 times and the wells were filled with 200µl blocking buffer (10% FCS in PBS) and incubated at 37°C for 1 hour. Plates were washed and 30µl of samples were loaded at a 1/2 or 1/5 dilution as was 30µl of standard with top concentration 20ng/ml with halving concentrations 10 times and a blocking buffer blank as the bottom standard (all dilutions were made with blocking buffer). The plates were then incubated at 37°C for 2 hours. 3-5 washes was performed after incubation and plates were coated with 50µl of 0.5µg/ml purified anti-mouse IL-6 biotin (BD Biosciences) and incubated at 37°C for 1 hour. Following incubation another wash was performed and then plates were coated with 50µl of a 1/2000 dilution of AKP streptavidin (BD Biosciences) and incubated at 37°C for 45 minutes. The final wash step was then carried out and to the plates was added 50µl of 1mg/ml pNPP dissolved in glycine buffer; the plates were then covered with tinfoil and incubated at room temperature for 20 minutes to allow colour development. Following sufficient colour development the plates were then read using a Spectramax450 at 405nm and the results recorded.

2.3.3 LEGENDplex™ Cytometric Bead Array

Macrophages stimulated with LPS at 3µg/ml, poly(I:C) at 10µg/ml and pam3csk4 320ng/ml and treated with NISV and liposomes were taken forward for analysis using the LEGENDplex™ Mouse Macrophage/Microglia bead array panel. Manufacturer methods were followed with samples being diluted 1/2 with 'Assay Buffer' to ensure the results remained in scale with the standard curves.

2.4 RNA and Metabolite Extraction

RNA extraction was performed as per the Qiagen RNAeasy mini kit's instructions, with one deviation. Due to a weaker response to NISV & liposomes upon scaling from $1x10^5$ cells/well in 96-well plates to $1x10^6$ cells/well in 24-well plates, the initial step of RNA extraction was carried out in 10 identically treated wells of a 96-well plate and pooled to make one sample. RNA was extracted 6 hours after the treatment of cells. After RNA was extracted 1µl was taken and the quality and quantity of RNA was tested using the Bioanalyser 2100 (Agilent) following the manufacturer's instructions. The remaining RNA was frozen using the Nalgene Mr Frosty freezing container at -80°C. For transcriptomic analysis, RNA was diluted to 1µg/20µl in RNase-free water and sent to Eurofins for their INVIEW Transcriptome Explore service. The library preparation was performed by Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany) using proprietary methods. Subsequent sequencing was also performed by Eurofins Genomics Europe Sequencing GmbH using Illumina HiSeq4000 instruments in 50 bp single end read mode.

Metabolite extraction was performed as per the instructions given by Glasgow Polyomics, for metabolite extraction the same process of pooling 10 wells of a 96-well plate was followed in order to keep consistency. Metabolites were extracted by removing supernatant from cells, kept for further testing, and adding 40µl of chloroform:methanol:water (20:60:20) and rocking the platers on a rocker for 1 hour at 4°C. Extraction mix was then transferred into Eppendorf tubes and vortexed for 5 minutes. Extractions were then centrifuged at 13000 rpm for 3 minutes at 4°C and the supernatant was removed to be stored at -80°C. Blank and pooled samples were also created as a control and for fragmentation data, respectively. The mass spectrometry analysis was carried out at Glasgow Polyomics. Hydrophilic interaction liquid chromatography was carried out on a Dionex UltiMate 3000 RSLC system using a ZIC-pHILIC column (150 mm × 4.6 mm, 5µm column). The column was maintained at 25°C and

samples were eluted with a linear gradient (20mM ammonium carbonate in water, A and acetonitrile, B) over 26 minutes at a flow rate of 0.3 ml/min as follows:

Time / minutes %A %B	Time / minutes %A %B	Time / minutes %A %B
0	20	80
15	80	20
15	95	5
17	95	5
17	20	80
26	20	80

The injection volume was 10µl and samples were maintained at 5°C prior to injection. For the MS analysis, a Thermo Orbitrap QExactive was operated in polarity switching mode and the MS settings were as follows:

- Resolution 70,000
- AGC 1e6
- m/z range 70–1050
- Sheath gas 40
- Auxiliary gas 5
- Sweep gas 1
- Probe temperature 150°C
- Capillary temperature 320°C

For positive mode ionisation: source voltage +3.8 kV, S-Lens RF Level 30.00, SLens Voltage 25.00 (V), Skimmer Voltage 15.00 (V), Inject Flatopole Offset 8.00 (V), Bent Flatapole DC 6.00 (V). For negative mode ionisation: source voltage-3.8kV. The calibration mass range was extended to cover small metabolites by inclusion of low-mass calibrants with the standard Thermo calmix masses (below m/z 138), butylamine (C4H11N1) for positive ion electrospray ionisation (PIESI) mode (m/z74.096426) and COF3 for negative ion electospray ionisation (NIESI) mode (m/z84.9906726).

3. The Immunomodulatory Effects of NISV

3.1 Abstract

Previous studies demonstrated that NISV composed of mono-palmitoyl glycerol (MPG), dicetyl-phosphate (DCP) and cholesterol have anti-inflammatory effects. The following studies were undertaken to better understand the properties of the vesicles responsible for these effects and to obtain further information as to the immunological extent of these effects. The individual components, MPG, DCP and cholesterol did not have anti-inflammatory effects alone, as determined by their inability to reduce IL-6 production in macrophages stimulated with LPS. NISV formulated with 1-O-hexadecyl-sn-glycerol (HG) rather than MPG had similar anti-inflammatory effects demonstrating that these effects were not dependent on the ester bond between the surfactant head and tail portion. However, liposomes consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol augmented IL-6 production in response to LPS, indicating that not all vesicular formulations are anti-inflammatory. NISV composed of MPG, DCP and cholesterol were found to also exert anti-inflammatory effects on polyinosinic:polycytidylic acid (poly(I:C)) and pam3csk4 stimulated macrophages indicating their ability to modulate myD88 dependent and myD88 independent immune events. Through use of a cytometric bead array, NISV comprising MPG, DCP and cholesterol were shown to downregulate macrophage production of a number of cytokines when stimulated with LPS (CCL22, G-CSF, IL-6, IL-12p40 & IL-12p70), poly(I:C) (IL-6 & IL-12p40) or pam3CSK4 (IL-10 & IL-12p40). The effect of liposomes consisting of DSPC and cholesterol on macrophage production of these cytokines was more PAMP dependent. While some cytokines were augmented and others reduced following LPS or Pam3CSK4 stimulation, all cytokines examined were augmented by liposomes following poly(I:C) stimulation. This work demonstrates broad anti-inflammatory properties of NISV composed of MPG, DCP and cholesterol. These properties are dependent on the components being formulated into vesicles, but are not a common property of all vesicle formulations as they are not replicated in liposomes consisting of DSPC and cholesterol.

3.2 Introduction

Previous data by Roberts et al suggested that NISV hold anti-inflammatory properties (Roberts et al., 1997). The purpose of this study is to determine the validity of these findings and to expand on them. Using the bone marrow derived macrophage model and various PAMPs we hope to gain insight into the mechanism of action of NISV.

The use of macrophages allowed a look into the effect of vesicles on interleukin 6 (IL-6) which is often associated with adverse inflammatory events.

3.2.1 Immune Pathway Probing

Interleukin 6 is a pyrogenic cytokine with important functions in fever regulation and as a mediator of the acute phase response. It is primarily produced by macrophages when stimulated by pathogen associated molecular patterns (PAMPs) (Heinrich, Castell, & Andus, 1990). PAMPs interact with pattern recognition receptors (PRRs) found on the macrophages surface and induce a response. To simulate this reaction *in vitro* lipopolysaccharide (LPS) was used which is a toll-like receptor 4 (TLR4) agonist. When LPS binds to TLR4, a pro-inflammatory cascade is initiated activating the TRIF- and MyD88- dependent pathways which result in up-regulation of many pro-inflammatory cytokines, including IL-1β, IL-12, TNF-α and IL-6. IL-6 has an important role in many inflammatory diseases. It is produced ubiquitously in cytokine storm reactions including sepsis and COVID-19 complications, as well as being responsible for pro-inflammatory states in many auto-immune diseases such as; rheumatoid arthritis, multiple sclerosis and atherosclerosis (Garbers et al., 2018). In the following studies, IL-6 is used as a marker of inflammation to determine if NISV, liposomes or components thereof, have anti-inflammatory effects on the innate immune system.

PAMPs that have an alternate mechanism of activation were also included to expand upon the findings of Roberts et al. Shown on Figure 3.2.1, LPS stimulates TLR4 and activates signalling pathways TRIF and MyD88 resulting in activation of transcription factors NF-κB and interferon regulatory factor 3 (IRF3) leading to a pro-inflammatory response. LPS is a major component of Gram-negative bacteria outer membrane, and when present in clinical samples suggests infection (Zanoni & Granucci, 2013). Investigation of other PAMPs associated with other broad classes of pathogens allows a comparative and more robust look at NISV immunomodulation. For this reason, TLR1/2 agonist pam3csk4 and TLR3 agonist poly(I:C) were used as stimulants in the following experiments. Pam3csk4 is a synthetic agonist of TLR1/2 which, when stimulated activates the MyD88 signalling cascade triggering the activation of NF-κB and production of pro-inflammatory mediators. TLR1/2 interact to respond to the major component of the microbial cell wall peptidoglycan and lipopeptides, which are commonly produced by infectious Gram-positive bacteria such as *Pseudomonas aeruginosa* and *Bacillus subtilis*(Coutte et al., 2017; Farhat et al., 2008). Poly(I:C) is a synthetic PAMP that stimulates TLR3, which would normally bind double stranded

RNA molecules produced during viral infection and thus activate the anti-viral immune response. This activation utilises the TRIF pathway inducing IRF3 to up-regulate pro-inflammatory cytokines (Deguine & Barton, 2014; Liu et al, 2017). Using these three PAMPS not only gives a wider look at NISV anti-inflammatory capabilities, but it allows us to confirm if NISV have a direct effect on the pathways activated by the three PAMPs associated with diverse pathogens and pathologies.

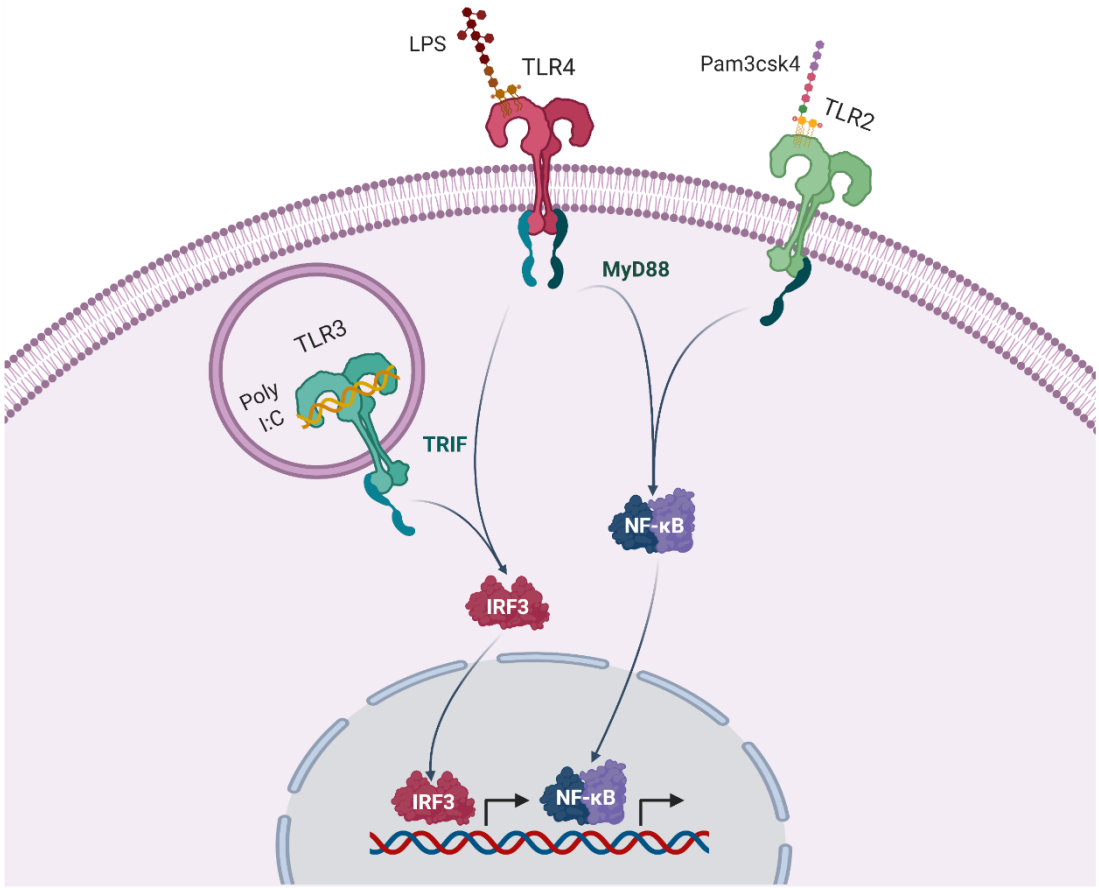


Figure 3.2.1 TLR & PAMPs Interactions. *The diagram shows interactions between TLRs 2, 3 & 4 with PAMPs Pam3csk4, Poly(I:C) and LPS, respectively, and the activation pathways they initiate. Created with BioRender.com*

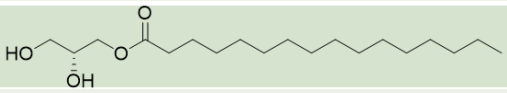
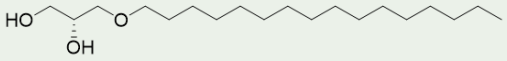
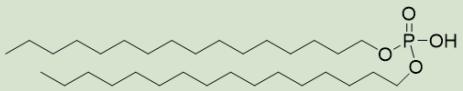
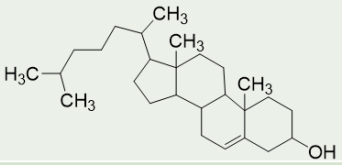
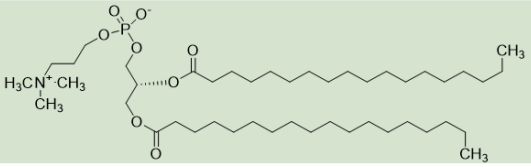
3.2.2 The Effects of Formulation

The following studies were performed to determine which particular characteristic of NISV plays a role in modulation of the inflammatory response. Vesicle formulations can be made with many different components and through many different methods in order to produce differences in size, surface charge, uniformity and, according to the following data, immunomodulation (Sakthivel et al, 2015). For the purpose of investigating surfactant properties, a novel formulation was made using 1-O-hexadecyl-sn-glycerol (HG) as the non-ionic surfactant. HG is identical to MPG in all ways but one, where MPG contains an ester bond HG has an ether bond, as shown on Table 3.2.1. The presence of an ether bond on HG makes the vesicles non-biodegradable, increasing their stability and, by extension, their toxicity (Zhi et al., 2018). Thus, the hypothesis is explored that vesicle stability may play a part in the anti-inflammatory effects of NISV, with anti-inflammatory effects being limited when a more stable vesicle is used.

Liposomes are the most widely used and studied form of vesicle in the literature and their interactions with the immune system are well documented. Comparing liposome treatment with NISV treatment of macrophages stimulated with LPS could provide further insight into the characteristics of NISV that are important for immunomodulation.

With the introduction of liposomes, we introduce a new method of formulation. In this study, liposomes are made using a microfluidics method utilising the nanoassemblr platform. The nanoassemblr can make vesicles of varying sizes and uniformity depending on the parameters of formulation, and in following published methods we made vesicles with a small very uniform phenotype (Obeid et al, 2017).

Table 3.2.1 Chemical structures of all vesicle components used in this study.

Vesicle Component	Chemical Structure
DL-α-Palmitin (MPG)	
sn-1-O-Hexadecyl-glycerol (HG)	
Dicetyl Phosphate (DCP)	
Cholesterol	
1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)	

3.2.3 Investigating Breadth of NISV Function

In search of an even broader look into the immunomodulatory effects of vesicles, we utilised the LEGENDplex™ bead array. This assay allows the rapid measurement of 13 different cytokines related to macrophage polarisation and immune response. The assay follows the same basic principle as sandwich immunoassays, utilising specific capture beads and biotinylated detection antibody and the relevant cytokine to form the bead-cytokine-detection antibody sandwich. Streptavidin-phycoerythrin is added conferring the fluorescent signal, rather than an enzymatic signal. Each bead associated to a cytokine is differentiated by the internal fluorescence and size of the bead which can be segregated and quantified by measuring the phycoerythrin signal on a flow cytometer. The panel used in this experiment is the macrophage/microglial panel which allows identification of the following array of cytokines shown on Table 3.2.2. Measurement of these cytokines gives insight into vesicles effects on macrophage polarisation as well as many aspects of the immune response. Eight of the 13 measured cytokines are identifiers of M1 macrophage activation, those eight are the chemokine CXCL1, interleukins 1β, 6, 12p40, 12p70, 18 and 23 and fever inducing TNF-α. With NISV expected to show anti-inflammatory effects it is expected that these eight cytokines will be down-regulated showing a less inflammatory state than that of an M1 macrophage. On the other hand, NISV could induce cells to show a phenotype more similar to the traditionally anti-inflammatory M2 macrophage showing expression of their related cytokines, CCL17, CCL22 (Two chemoattractants of regulatory T cells), G-CSF, IL-6, IL-10 and TGF-β1 (Orecchioni et al., 2019). If the anti-inflammatory effects of NISV as shown by Roberts et al are confirmed it would be expected that down-regulation of IL-1β, IL-6 and TNF-α would result.

Table 3.2.2 Cytokines Covered in Cytometric Bead Array.

Target Cytokine	Macrophage Polarisation
CCL17	M2
CCL22	M2
CXCL1	M1
G-CSF	M2
IL-1β	M1
IL-6	M1 & M2
IL-10	M2
IL-12p40	M1
IL-12p70	M1
IL-18	M1
IL-23	M1
TGF-β1	M2
TNF-α	M1

3.2.4 Aims & Hypothesis

The aims of this chapter are to:

i. Confirm the ability of NISV, comprised of MPG, DCP and cholesterol to down-regulate LPS-induced IL-6 production by murine macrophages and to better understand which aspects of the NISV formulation contribute to this ability. To achieve this the ability of the individual components that the NISV are composed of will be tested for their relative ability to inhibit LPS induced IL-6 production. Furthermore, the main component of these vesicles MPG, will be substituted with 1-O-hexadecyl-sn-glycerol (HG), which has an identical structure to MPG with the exception of the substitution of an ester bond by an ether bond at the junction of the head and tail groups.

ii. Determine if the anti-inflammatory effects of NISV are specific to LPS stimulation or if they are evident following stimulation with other TLR ligands that signal exclusively through the TLR2 receptor (pam3csk4) or through the TLR3 receptor (poly(I:C)). As these ligands signal through myd88 dependent and independent pathways respectively, this should also provide insight into breadth and potential mechanisms of the effects of NISV.

iii. Determine the extent of the effects that NISV have on the ability of macrophages to produce a wide range of cytokines in the absence and in the presence of TLR-stimulation using a cytometric bead array and to compare this with the effect of a widely used formulation of liposomes. This should provide insight into the apparently contradictory properties of NISV in having both immunological adjuvant properties as well as anti-inflammatory properties.

3.3 Are NISV Anti-Inflammatory Effects Real?

The size, polydispersity index (PDI) and charge of all vesicles were measured and recorded prior to all individual experiments. This showed the effect of formulation and component changes on the vesicle characteristics.

Table 3.3.1 shows us that all characteristics are affected by formulation method. Size and PDI was larger in vesicles made via the melt method, and small when using microfluidics. Formulation methods used show effects on vesicle charge. When comparing MPG-based vesicles, charge increases when using the microfluidics methods mirroring the net positive charges of other vesicles made by these methods. Due to these changes in characteristics, we continued to study these vesicles, testing their toxicity and immunomodulatory abilities.

To measure the anti-inflammatory effects of NISV and ensure minimal toxicity an IL-6 ELISA was performed in tandem with an alamarBlue cell viability assay. This assay measures the reduction of resazurin by the cells and is a proxy for general cell metabolism. The results of the alamarBlue assay on Figure 3.3.1.A shows NISV have no negative effects on cell viability. NISV treatment of BMDM increased the percentage reduction of alamarBlue by 152% when compared to the control cells at 3mM. However, as the concentration of NISV was reduced so too was the reduction of alamarBlue to levels close to that of the control cells. LPS stimulation did not change alamarBlue reduction when compared to the control cells.

Figure 3.3.1.B shows the results of the IL-6 ELISA performed with the supernatants. Minimal IL-6 was produced by unstimulated cells with or without NISV treatment. The LPS stimulated cells produced >15ng/ml IL-6 from BMDM. Treatment of LPS stimulated cells with NISV significantly reduced IL-6 production and followed a dose-dependent response with reducing treatment starting at 3mM.

Table 3.3.1 Table of Vesicle Sizes. *Prior to experimentation, vesicle size, PDI and zetapotential were measured using the Malvern zetasizer nano zs.*

Vesicle Type	Formulation Method	Size (nm)	Polydispersity Index	Charge (mV)	Figure Reference
NISV (MPG)	Melt	1406 (+/-21.8)	0.371	-29.5	3.3.1, 3.3.2, 3.3.3, 3.6.1, 3.6.2
NISV (HG)	Melt	1338 (+/-99)	0.469	-23.5	3.4.3
Liposome	Microfluidics	143 (+/-2.6)	0.293	6.1	3.4.5, 3.6.1, 3.6.3
NISV (MPG)	Microfluidics	87.54 (+/-1.3)	0.203	4.9	3.5.2

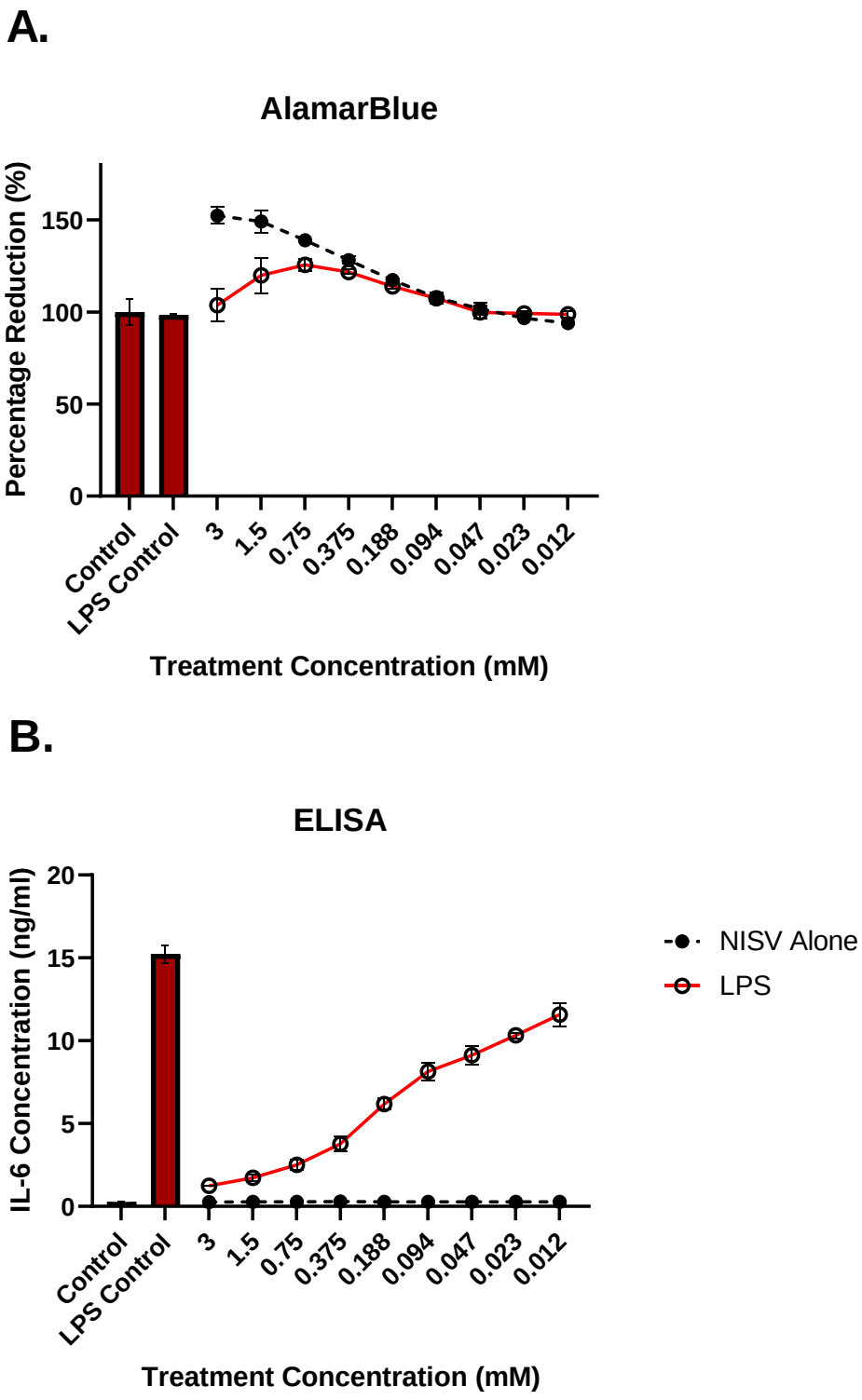


Figure 3.3.1: NISV reduce IL-6 production in LPS stimulated BMDM. 100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.

3.3.1 Characterising Immunomodulation of NISV

To determine if the immunomodulatory effects of vesicles is dependent on their composition, the individual components of NISV as well as other formulations were tested to examine their ability to alter IL-6 production. Testing of the singular components of NISV was expected to elucidate whether any of the three components were responsible for the immunomodulatory effect. This would further the ability to fine tune and control this effect. Other forms of vesicles would help answer a similar question, allowing us to determine if changing components altered or nullified the anti-inflammatory effect.

Testing of the primary components of the NISV formulation was performed first. Each component was prepared as an NISV using the melt method to more closely mimic the NISV preparation. Figure 3.3.1 demonstrates that high concentrations of MPG (0.375-1.5mM) resulted in a reduction of cell viability. Concentrations lower than this were non-toxic to cells. IL-6 production was not observed in control cells, but was produced by cells stimulated with LPS. The concentrations of MPG and DCP used in testing was equivalent to the amount present in NISV (i.e. 3mM NISV has 1.5mM MPG). MPG had no significant effect on IL-6 production at non-toxic concentrations. However, IL-6 production was reduced where cells were incubated with toxic concentrations of MPG.

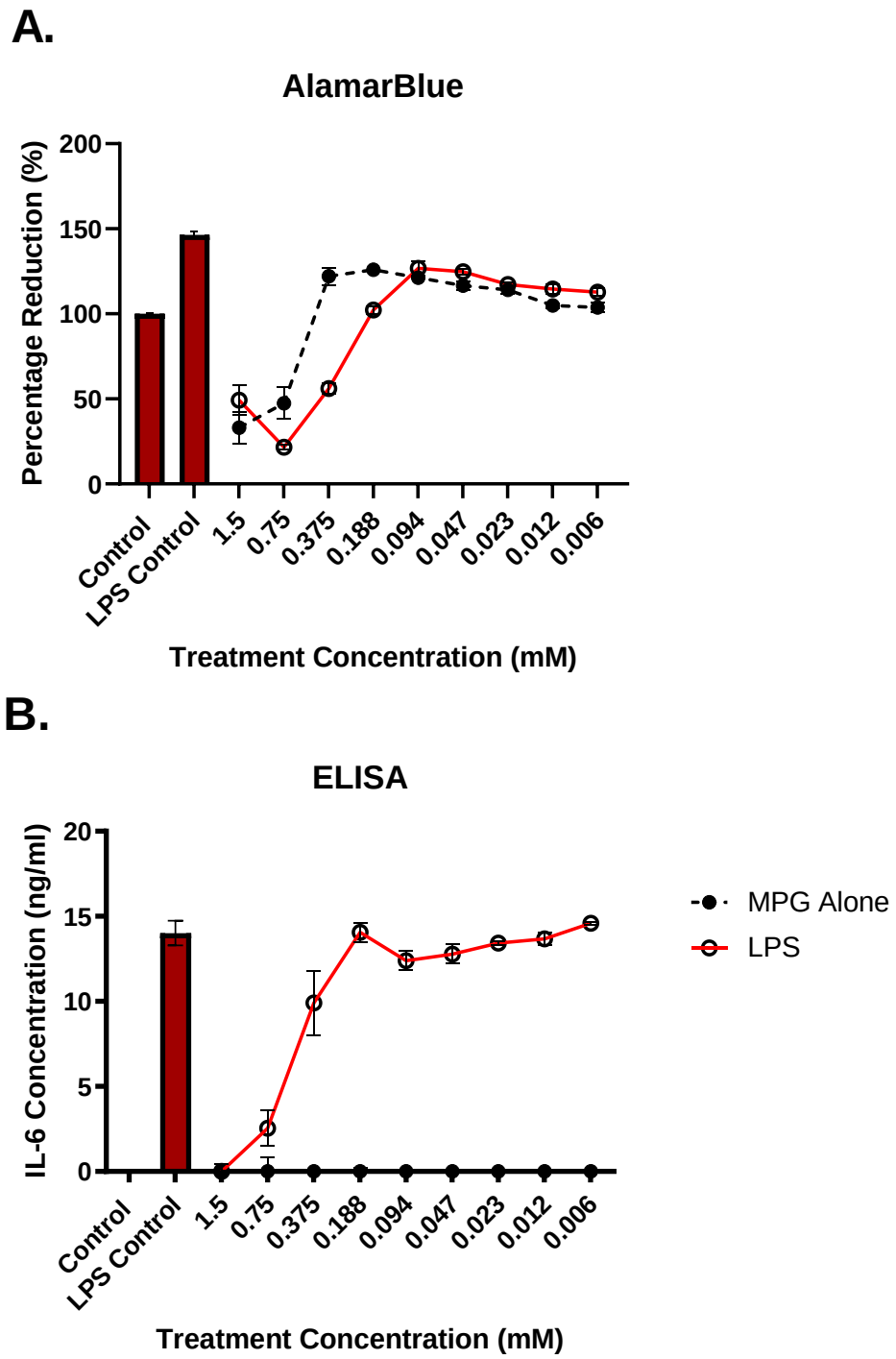


Figure 3.3.1 MPG treatment proved toxic to BMDM. 100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with MPG at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.

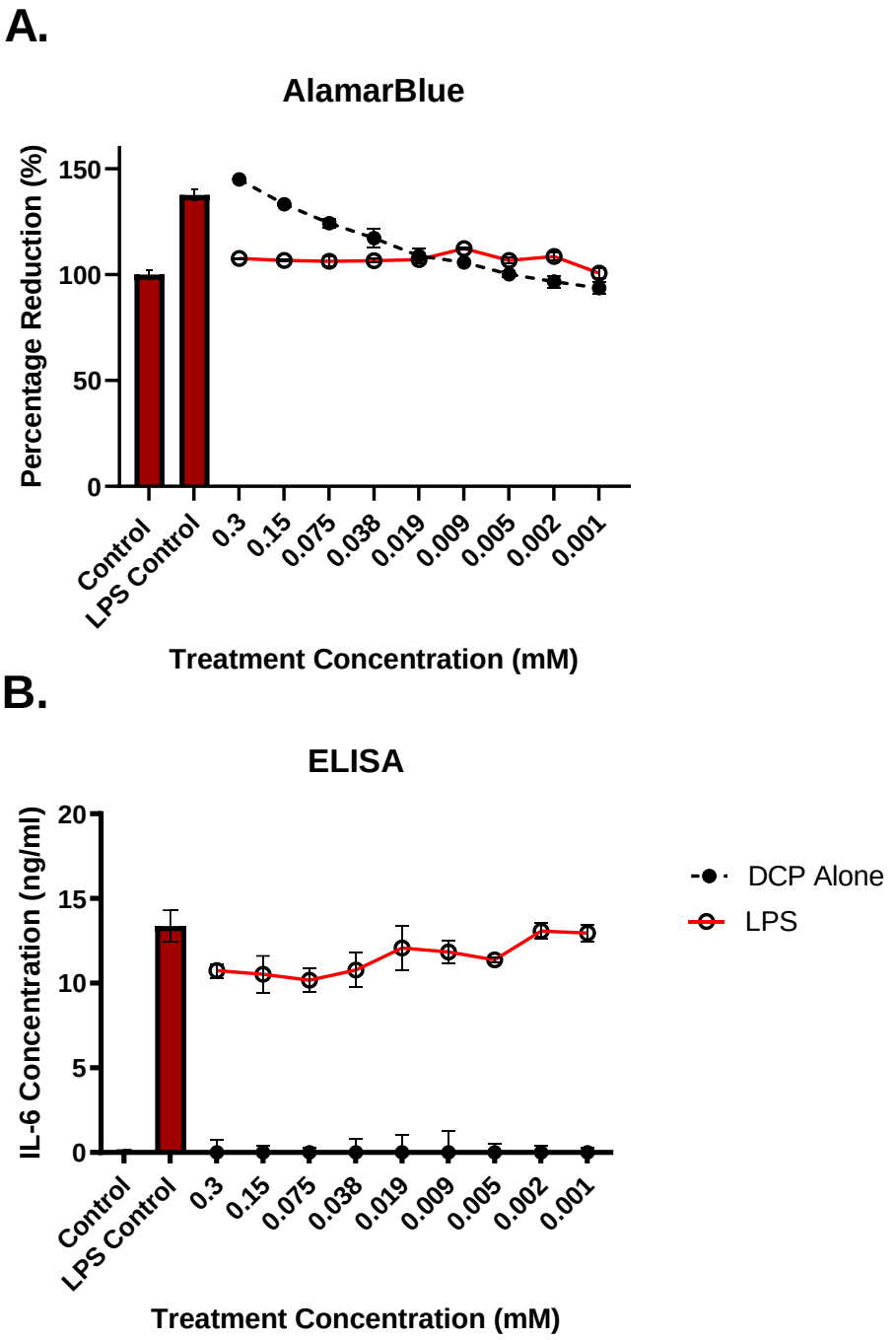


Figure 3.3.2 DCP shows minimal effects on BMDM. 100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with DCP at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.

Cell viability was not profoundly affected by DCP in the presence or absence of LPS as shown on figure 3.3.2. DCP did not notably affect IL-6 levels in LPS-stimulated macrophages.

3.3.2 Inflammatory Effects of Other Vesicles.

Cell viability was affected at high concentrations of HG-based NISV at concentrations of 1.5 - 3mM. IL-6 levels were minimal in unstimulated cells whether treated with NISV or not (Figure 3.3.3.B). In LPS-stimulated macrophages HG-based NISV reduced the IL-6 response significantly in a dose dependent manner. This data shows that the HG-based NISV were more toxic as hypothesised, but still mediate anti-inflammatory effects at non-toxic concentrations, indicating that the presence of an ester bond is important for target cell viability, but not immunomodulation.

Liposomes showed no toxicity in BMDM at any concentration examined (Figure 3.3.4.A). Liposomes induced a pro-inflammatory response when administered at high concentrations to unstimulated control cells where they induced IL-6 production. In LPS-stimulated cells, liposomes at 3mM and 1.5mM augmented IL-6 production induced by LPS in a dose dependent manner. This result further emphasised how important the components of a vesicle are to the immunomodulatory function.

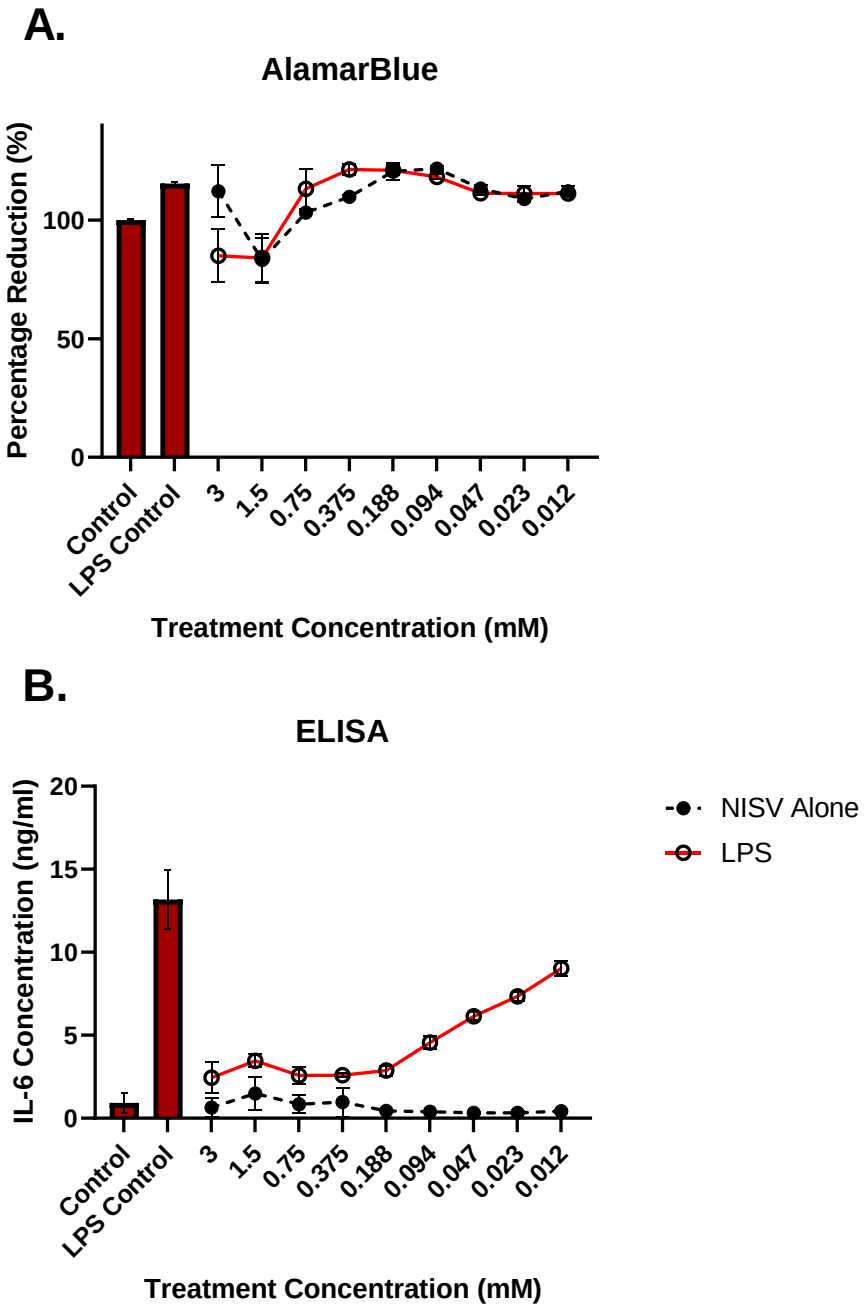


Figure 3.3.3 HG-based NISV reduce IL-6 production in LPS stimulated BMDM. *100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with HG-based NISV at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.*

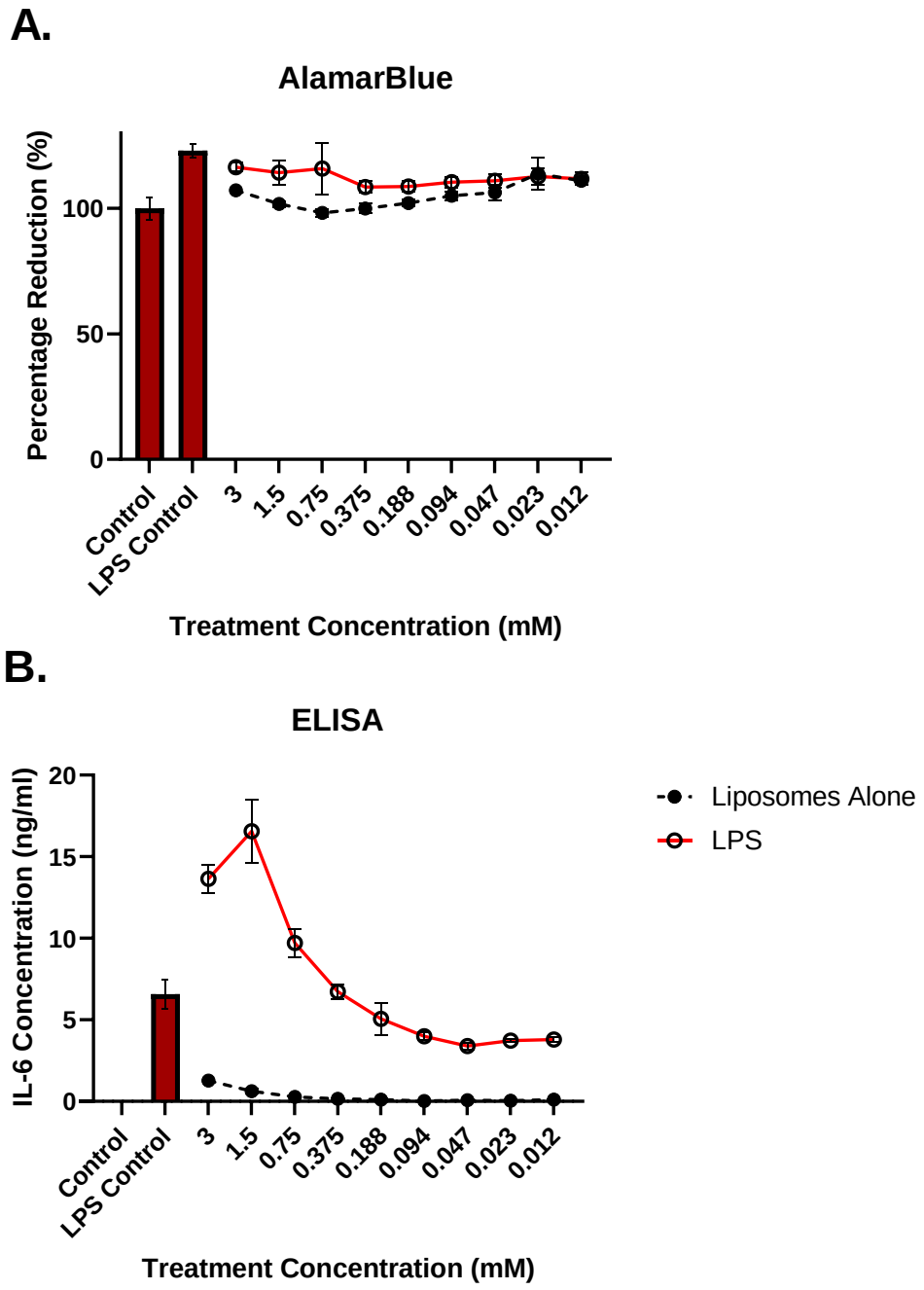


Figure 3.3.4 Liposomes are pro-inflammatory. *100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with Liposomes at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.*

3.4 Effects of Formulation

Through the testing of other vesicle types, a different formulation method was introduced as a variable. In order to know if the cause of variance between NISV and liposomes was due to the change of component or a change in the formulation method NISV, consisting of MPG, DCP and cholesterol, were made using the Nanoassemblr to the same specifications as the liposomes.

Nanoassembled NISV had a toxic effect at 3mM in unstimulated cells and at both 3mM and 1.5mM in LPS-stimulated cells (figure 3.4.1). The NISV inhibited IL-6 production in LPS-stimulated BMDM in a dose dependent manner at non-toxic concentrations. This result shows that the anti-inflammatory effect is replicable using the nanoasemblr to formulate NISV. However, the toxicity of NISV is increased using the nanoassemblr, relative to NISV made using the melt method.

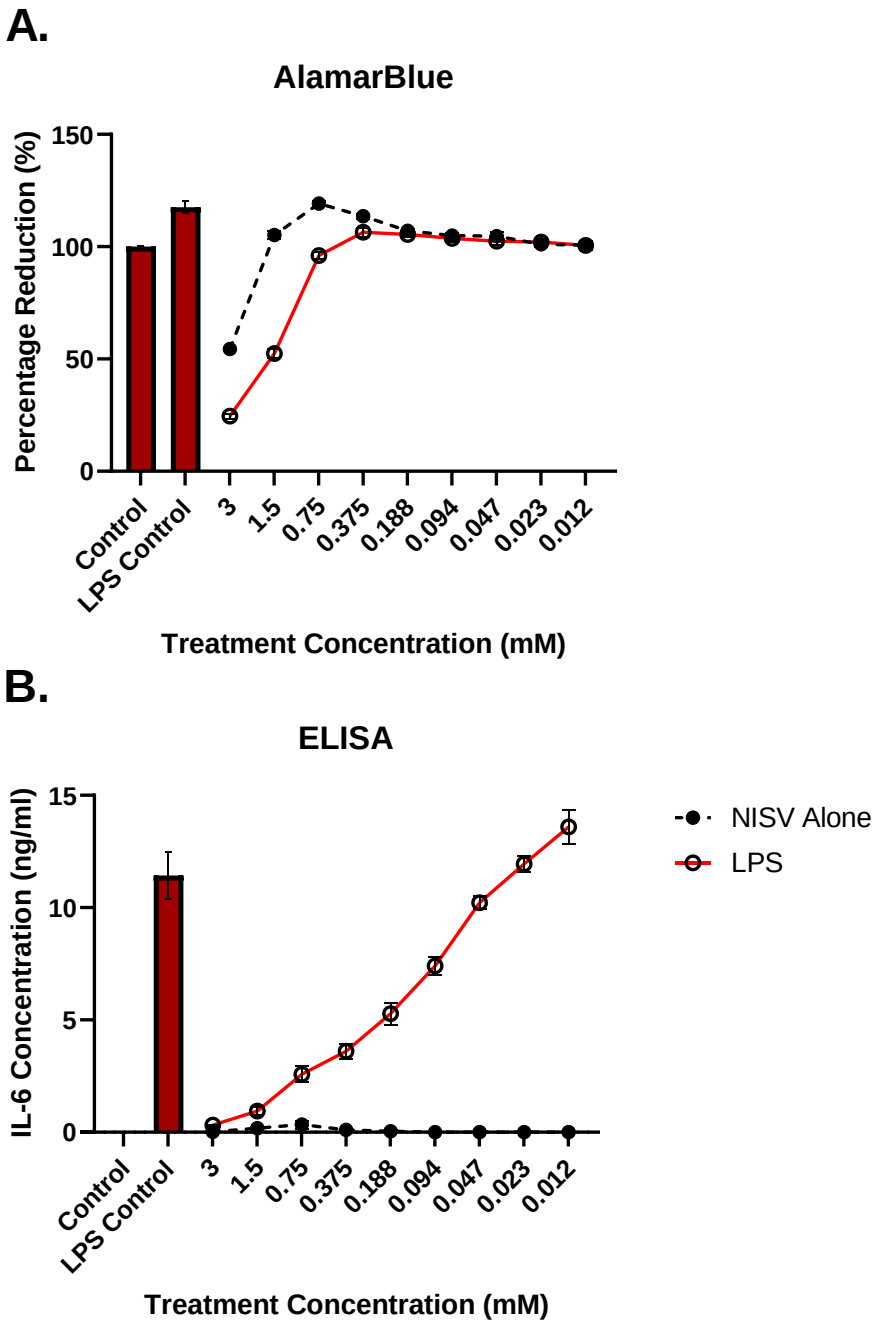


Figure 3.4.1 NISV made by microfluidics are toxic to BMDM. 100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.

3.5 NISV Effects on Various PAMPs Stimulation Profiles

Following the confirmation of anti-inflammatory effects of NISV on macrophages stimulated with LPS, the breadth of NISV effects were investigated. Poly(I:C), an agonist of TLR3, and Pam3csk4, an agonist of TLR2, were used to stimulate BMDM in the presence or absence of NISV.

Poly(I:C) showed an excitatory effect on BMDM increasing alamarblue reduction to 132% compared with the control cells (Figure 3.5.1.A). Levels of interleukin 6 were again shown to be minimal in non-stimulated cells (Figure 3.5.1.B). NISV reduced IL-6 production in poly(I:C)-stimulated BMDM in a dose-dependent manner.

Stimulation with pam3csk4 increased alamarBlue reduction (Figure 3.5.2.A). IL-6 was reduced in pam3csk4-stimulated BMDM treated with NISV in a dose dependent manner (Figure 3.5.2.B). This data further proves the anti-inflammatory effect of NISV. It shows the effect is not exclusive to LPS stimulation and similar effects are seen with poly(I:C) and pam3csk4.

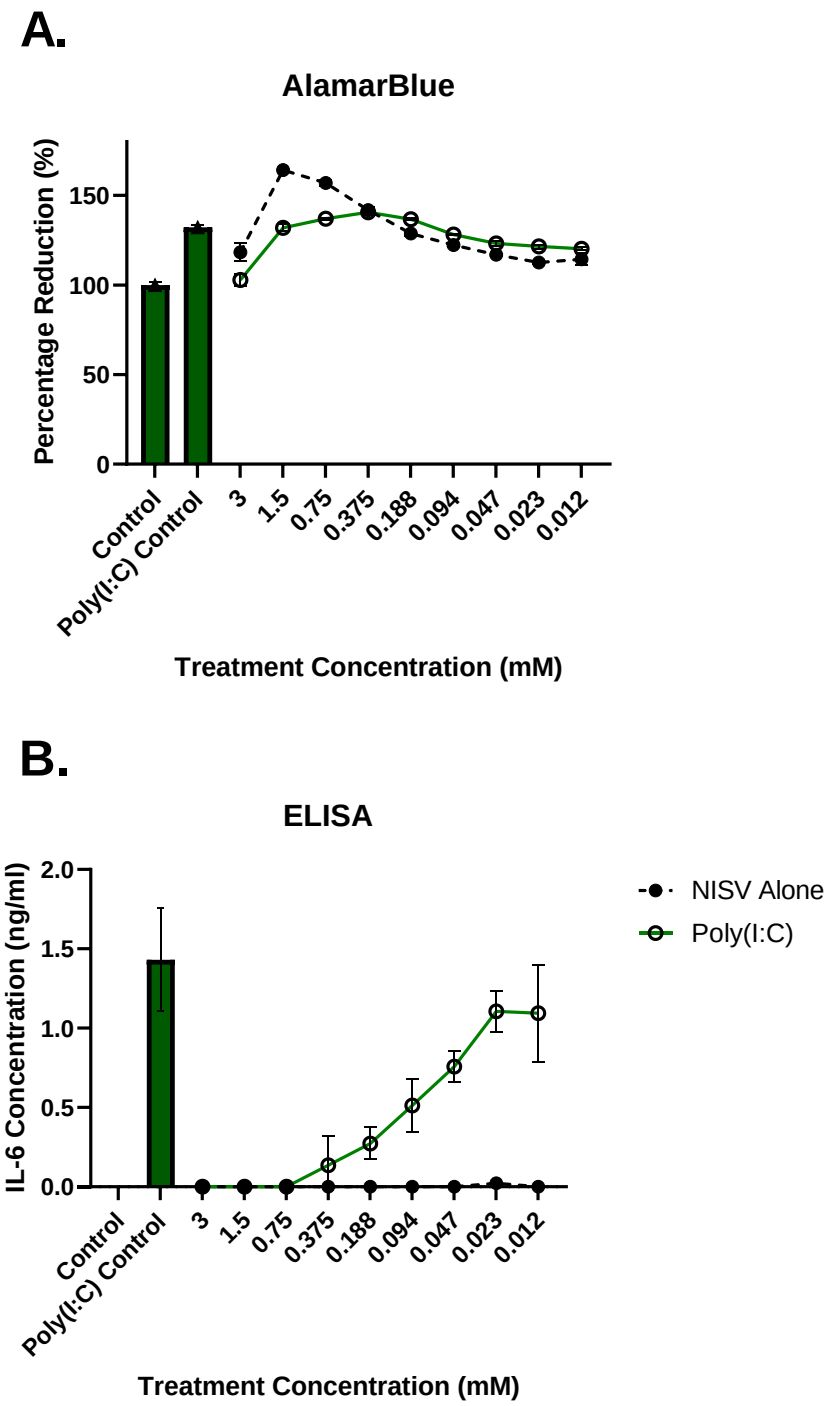


Figure 3.5.1 NISV reduce IL-6 production in poly(I:C) stimulated BMDM. 100,000 BMDM were plated per well in triplicate and stimulated with poly(I:C) at 10μg/ml or media, in controls, followed by treatment with NISV at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.

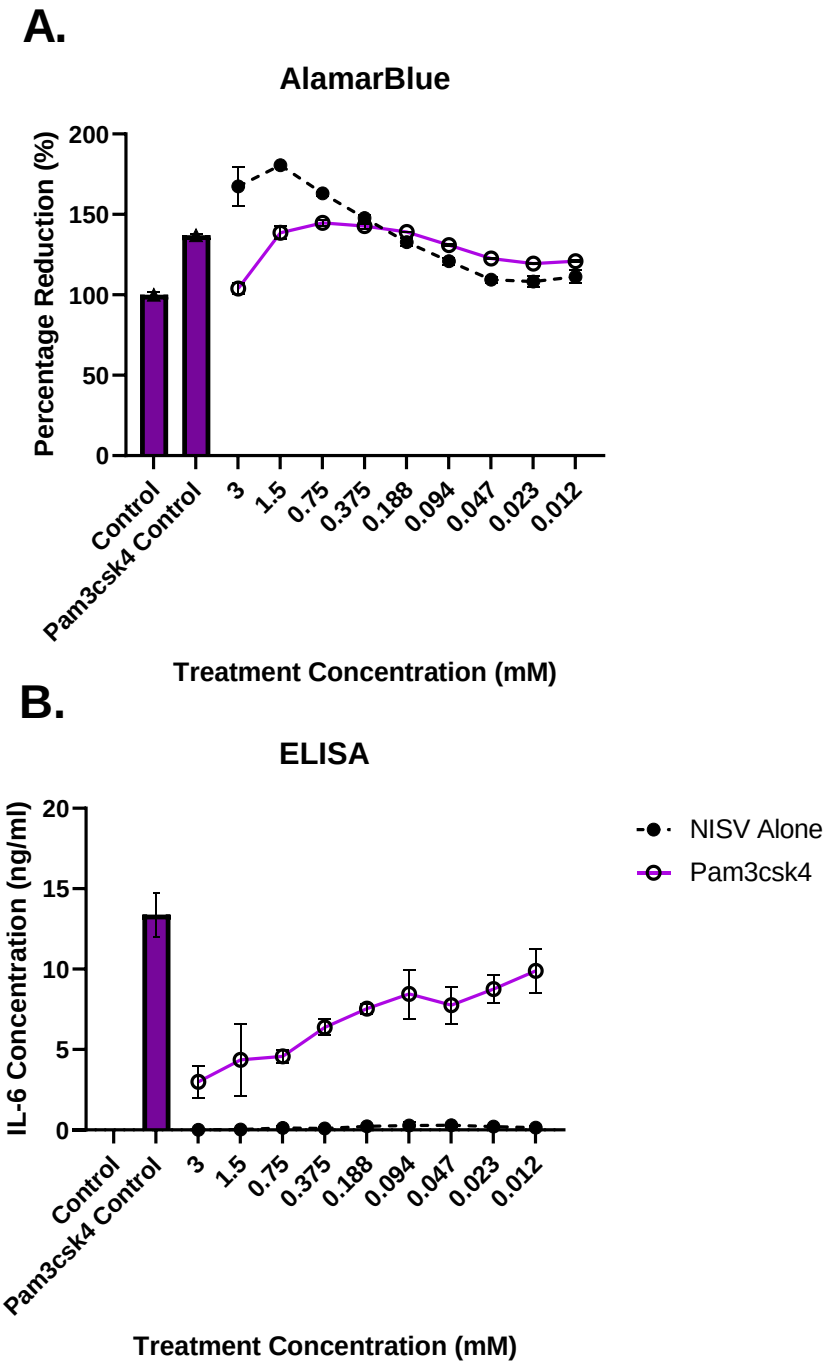


Figure 3.5.2 NISV reduce IL-6 production in pam3csk4 stimulared BMDM. *100,000 BMDM were plated per well in triplicate and stimulated with pam3csk4 at 320ng/ml or media, in controls, followed by treatment with NISV at descending concentrations with controls untreated. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants after 24 hours.*

3.6 Bead Array Analysis of BMDM

The panel used in this study was the LEGENDplex™ Mouse Macrophage/Microglial panel, which is optimised for the detection of cytokines related to M1 and M2 macrophage expression profiles. Using this tool allowed a wider examination of the immunomodulatory profile of both NISV and liposomes in the presence of three stimulants administered independently; LPS, pam3csk4 and poly(I:C).

3.6.1 BMDM stimulated with Vesicles or PAMPS

Stimulation of BMDM with vesicles alone or PAMPSs induced significant changes from basal levels of cytokine production, as shown on Figure 3.6.1. BMDM stimulated with NISV showed a pro-inflammatory phenotype with significant upregulation of all cytokines other than CCL17, IL-12p40, IL-18 and IL-23, though not to the degree shown by the three PAMPs. Similarly, liposomes stimulated BMDM showed upregulation of pro-inflammatory cytokines and uniquely showed significant up-regulation of CCL17. The only cytokines which were not significantly up-regulated in BMDM stimulated with liposomes were CXCL1, IL-18 and IL-23. The three PAMPs all induced significant stimulation of BMDM with LPS and pam3csk4 significantly upregulating all cytokines other than CCL17 and TGF-β1, respectively. Poly(I:C) induced a less inflammatory profile than the other PAMPs significantly upregulating only 7 out of 13 cytokines and significantly downregulating CCL17 and TGF-β1.

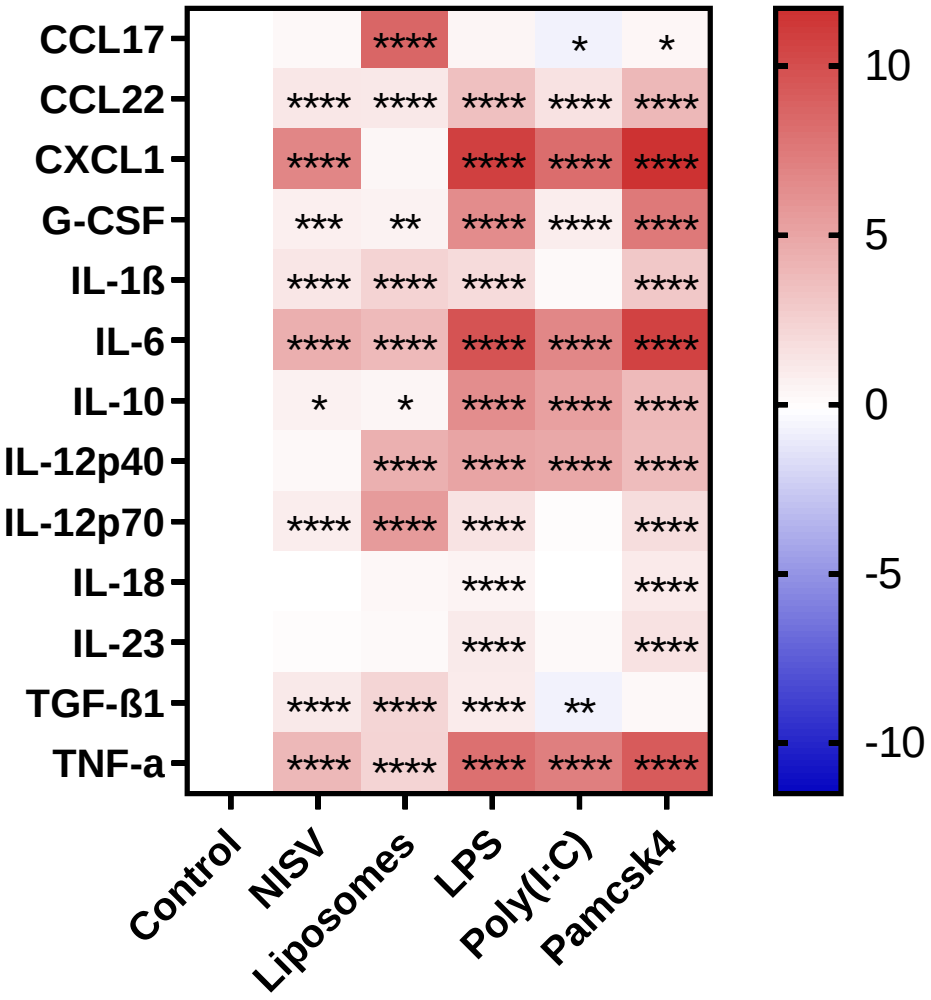


Figure 3.6.1 NISV and liposomes, both, are metabolically stimulating. *100,000 BMDM were plated per well in triplicate. NISV, liposomes, LPS, poly(I:C) and pam3csk4 were then added to the cells. Supernatants were taken from the cells after 24 hours and measured using cytometric bead array analysis. Heat map shows the Log2(fold change) of vesicle treatments and PAMP treatments compared to an unstimulated control. Statistical analysis was performed using Prism 8 where a two-way ANOVA with Tukey's multiple comparisons test was carried out*

3.6.2 Bead Array Analysis of BMDM treated simultaneously with NISV and PAMP

The effects of NISV treatment on stimulation by LPS, pam3csk4 and poly(I:C) is shown in Figure 3.6.2. NISV had a mostly anti-inflammatory effect on the effects of LPS, reducing production of cytokines, CCL22, G-CSF, IL-6, IL12p40 and IL-12p70. However, NISV did significantly increase IL-1β production in BMDM stimulated with LPS. NISV significantly up-regulated IL-1β and TGF-β1 in poly(I:C), but significantly down-regulated IL-12p40 and IL-6 in poly(I:C)-stimulated BMDM. NISV had a more limited effect on pam3csk4-stimulated BMDM, up-regulating IL-1β, IL-23 and TNF-α, but significantly down-regulating IL-12p40 and IL-10. These data demonstrate that the immunomodulatory abilities of NISV on BMDM are robust, but exhibit distinct differences depending on the PAMPS used in macrophages stimulation.

3.6.3 Bead Array Analysis of BMDM treated simultaneously with Liposomes and PAMP

Liposome effects on LPS-stimulated BMDM were predominantly pro-inflammatory showing significant increases in the cytokines G-CSF, IL-1β, IL-10, IL-23 and TNF-α (Figure 3.6.3). Liposomes did significantly reduce IL-12p40 and TGF-β1 in LPS-stimulated BMDM. When treated with liposomes, poly(I:C)-stimulated cells demonstrated an increase in all cytokines measured, with statistically significant increases in CCL22, CXCL1, IL-6, IL-12p40, TGF-β1 and TNF-α. Liposomes significantly decreased IL-6, IL-18 and TNF-α and significantly increased IL-1β and IL-10 production in pam3csk4-stimulated cells.

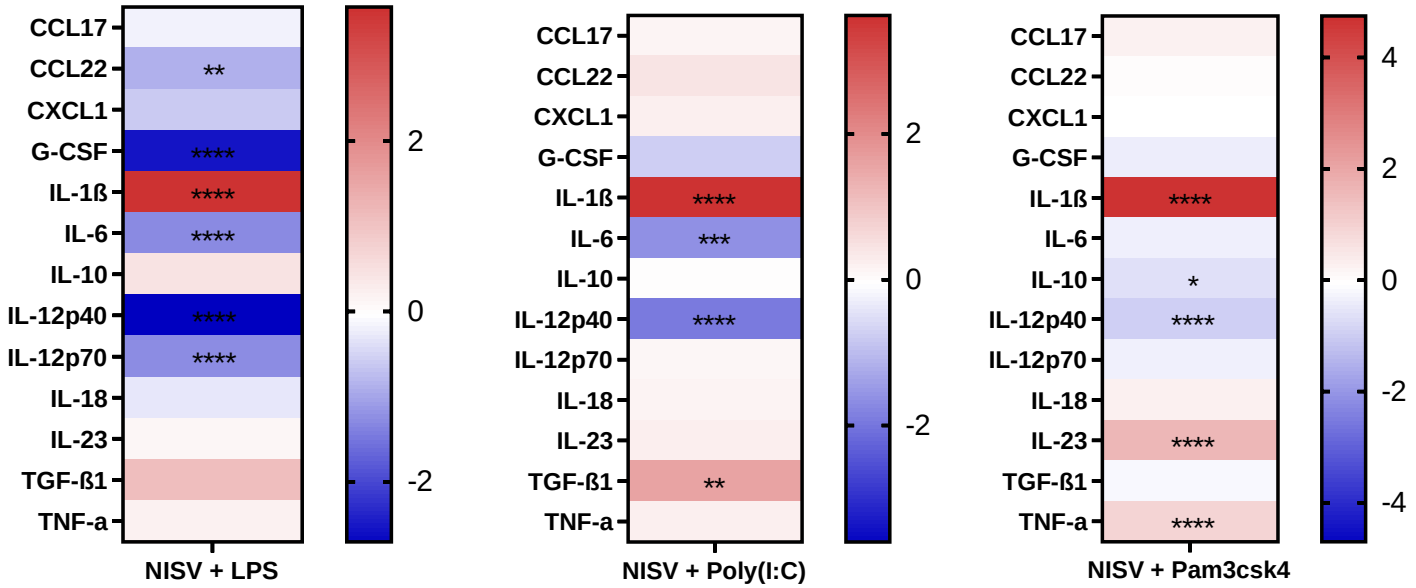


Figure 3.6.2 NISV are not exclusively anti-inflammatory. *100,000 BMDM were plated per well in triplicate and were stimulated using 3µg/ml LPS, 10µg/ml poly(I:C) and 320ng/ml pam3csk4, these were then treated with 1.5mM NISV. After 24 hours supernatants were taken and analysed using the cytometric bead array. Heat map shows the Log2(fold change) of NISV compared to their respective PAMP control. Statistical analysis was carried out using Prism 8 where a two-way ANOVA and Tukey's multiple comparisons test was performed.*

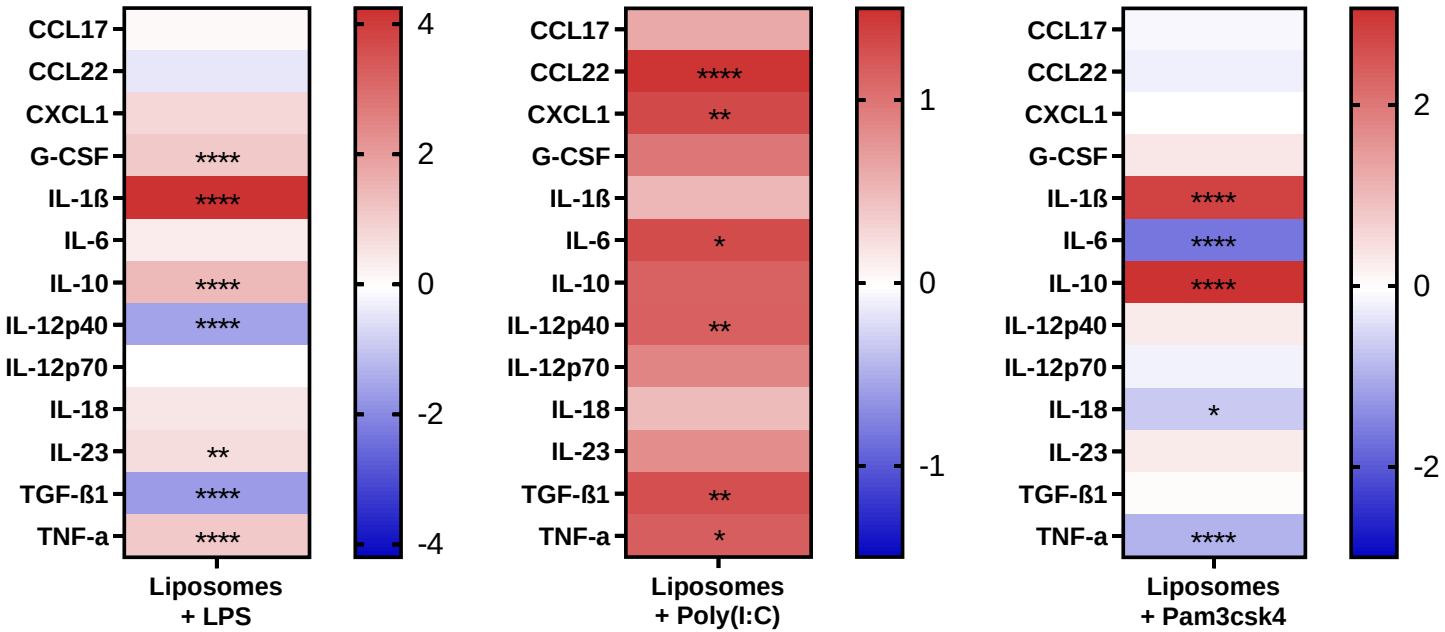


Figure 3.6.3 Macrophage immune response is drastically altered by Liposomes. *100,000 BMDM were plated per well in triplicate and were stimulated using 3µg/ml LPS, 10µg/ml poly(I:C) and 320ng/ml pam3csk4, these were then treated with 1.5mM Liposomes. After 24 hours supernatants were taken and analysed using the cytometric bead array. Heat map shows the Log2(fold change) of Liposomes compared to their respective PAMP control. Statistical analysis was carried out using Prism 8 where a two-way ANOVA and Tukey's multiple comparisons test was performed.*

3.7 Discussion

The results found in this series of experiments are important in the context of all vesicle based treatments and the development of future vesicle drug formulations. They may also shed light on unknown side effects of vesicle-based drugs. Being able to choose appropriate vesicles or tailor vesicle properties could be an incredible boon for the future.

3.7.1 NISV have Anti-Inflammatory Properties

It was determined that the components alone could not replicate the anti-inflammatory effects of NISV. MPG reduced alamarBlue metabolism when introduced at high concentrations a likely result of it being a surfactant. It did not affect IL-6 production at non-toxic concentrations. DCP did not reduce IL-6 production in LPS-stimulated BMDM. Testing cholesterol alone was not feasible due to insolubility. However, the literature would suggest that cholesterol accumulation in macrophages promotes IL-6 and TNF-α production meaning it is unlikely that cholesterol alone would produce the anti-inflammatory effects mediated by NISV (Li et al., 2005). Overall, these results established that formulation of the components of NISV was necessary to mediate the observed anti-inflammatory effects. Therefore, further studies were designed to determine if the effects were dependent on the type of surfactant used to manufacture the vesicles.

3.7.2 Variance in NISV

Studies were performed using a standard formulation of liposomes consisting of DSPC and cholesterol which determined that unlike NISV, these vesicles augmented LPS-induced IL-6 production. This indicated that the surfactant component of the vesicle is likely to be important. Therefore, vesicles were formulated that substituted the MPG component with 1-O-hexadecyl-sn-glycerol (HG) in NISV. These were then tested for their ability to inhibit IL-6 production in LPS-stimulated BMDM.

At non-toxic concentrations, HG-based NISV performed better than MPG-based NISV in terms of their ability to inhibit IL-6 production in LPS-stimulated BMDM. HG-based NISV, however, had increased toxicity relative to MPG-based NISV. This is likely due to the absence of an ester bond in this formulation, meaning the vesicle are not as readily broken down as the MPG variant. Their ability to reduce IL-6 production was also increased, again perhaps due to their increased stability. If this effect is consistent *in vivo*, HG-NISV could prove useful in reducing the necessary dosage for NISV desired effects.

The method of formulation of an NISV was not found to affect their anti-inflammatory properties as both melt-method and nanoassembled vesicles inhibited IL-6 production in LPS-stimulated BMDM. However, nanoassembled NISV tended to be smaller, more uniformed and more toxic to BMDM. MPG-based NISV, have indeed previously been shown to be toxic at concentrations above 0.8mM in vesicles between 100 and 200nm regardless of formulation method (Obeid et al., 2017). This toxicity is potentially due to the level of uptake possible by any macrophage. Small vesicles, such as the nanoassembled NISV, are easily translocated across the cell membrane via macropinocytosis and quickly localise to lysosomes allowing quick uptake, whereas large nanoparticles, as those made via the melt method, require phagocytosis meaning larger vesicles are taken up less readily than smaller vesicles (Foroozandeh & Aziz, 2018). One possibility is that toxicity caused by small NISV is due to rapid uptake of too many vesicles, leading to the sudden release of surfactants as vesicles are broken down, resulting in cell death by lysis. Larger vesicles require active uptake to enter the cell meaning despite equal concentrations larger NISV could be less toxic as the release of surfactant into the cell is slower and so non-lethal. Importantly, the above studies established non-toxic doses of NISV formulations downregulate LPS-induced IL-6 production. However, the studies do not provide obvious mechanistic insight into the extent of these regulatory effects and how these effects are mediated.

3.7.3 Breadth of Immunomodulation mediated by NISV in BMDM

To dissect these results and determine how the NISV elicit their effect, a number of immune stimulants were used that act through different toll-like receptors. These included; LPS as a stimulant of TLR4, poly(I:C) a TLR3 agonist, and pam3csk4, a TLR2 agonist. NISV were shown to mediate dose dependent reduction of IL-6 production in the presence of all three PAMPs. The ability of NISV to mediate an anti-inflammatory effect regardless of TLR stimulated and resulting signalling pathway indicates that NISV exert their effects downstream of TLR signalling and their effects are observed in both MyD88 and TRIF signalling pathways.

To expand this, the LEGENDplex™ Mouse Macrophage/Microglial bead array panel was used. This panel is designed for the identification of M1 and M2 macrophage, but is also broadly useful in determining the phenotype of macrophages. NISV were found to stimulate the production of a number of products by macrophages that is in keeping with their known adjuvant properties. These effects were considerably smaller and

more subtle than noted in macrophages stimulated with any of the PAMPs used. NISV were found to affect macrophage stimulation with all three PAMPs, altering the profile of many inflammatory cytokines. Notably, NISV had no obvious effect on macrophage known polarisation but did evidence the plasticity of the macrophages.

As liposomes were found to augment IL-6 production in resting macrophages and LPS-stimulated macrophages, they were included in the cytometric bead array analysis for comparison. Liposomes generally augmented macrophage production of mediators induced by the PAMPs. This was most clear following LPS or poly(I:C) stimulation, where the majority of cytokines were upregulated. The effects of liposomes on pam3csk4-stimulated BMDM were more varied and production of some mediators were ameliorated. These results reinforce the unique properties of NISV and emphasises that not all vesicles are anti-inflammatory in all situations.

The above studies provide greater insight into the physico-chemical components of NISV that are important for their anti-inflammatory effects, the cellular mechanisms that they affect and provide an indication of the extent of their effects. They therefore provide a data point that contributes to finding a mechanism of action, whereby NISV affect downstream events converging from myD88-dependent and independent TLR-induced signalling. However, how NISV induce these events is not clear. However, MPG the primary component of NISV, shares structural similarity with the immunoregulatory sphingolipid, called sphingosine-1-phosphate (S1P). Sphingosine-1-phosphate is a bioactive sphingolipid that regulates many pathophysiological processes including cancer, atherosclerosis and infection (Maceyka et al, 2012). S1P, its kinases and receptors are all expressed and produced within macrophages, allowing S1Ps direct effect on the innate immune system (Weigert et al, 2019). Intracellularly, S1P is a direct activator of transcription factor NF-κB through TNF receptor-associated factor 2 (TRAF2) in that sphingosine kinase 1 (SphK1) binds to the TRAF2 and allows binding of S1P which stimulates it leading to NF-κB activation (Alvarez et al., 2010). S1P is exported by cells via ATP-binding cassette transporters, where it then can exert many effects via its five receptors (Sato et al., 2007). S1P receptor 1 (S1PR1) signals through STAT3 inducing cytokine production and induces NLRP3, which is integral in the inflammosome, as well as affecting macrophage migration (Liao et al., 2018; Weichand et al., 2017). S1PR2 promotes opsonin-based uptake of pathogenic fungus and has been linked to inflammosome function (McQuiston et al, 2011; Zhao

et al., 2016). S1PR3 promotes reactive oxygen species (ROS) causing oxidative damage to bacteria. When S1PR4 is activated it upregulates production of tumour promoting cytokines IL-6 and IL-10 but its function is not fully understood (Olesch et al, 2017). S1PR5 is associated with macrophage efferocytosis and when upregulated it impairs this action (Barnawi et al, 2017). SphK1 and S1PR1 are closely related to macrophage polarisation and when upregulated and activated by S1P induce M1 macrophages furthering their connection to the pro-inflammatory response. The array of effects S1P can have on immunity indicates it and its receptors as promising drug targets. The structural similarity between S1P and MPG does not prove MPG has any effects on these processes. However, it is possible that NISV, upon being broken down into its singular components could interrupt S1P signalling. This could have the downstream effects of reducing pro-inflammatory mediators, as is shown in our data.

3.7.4 In Conclusion

This series of experiments demonstrated that the immunomodulatory properties of NISV cannot be replicated by any single component and formulation of these components into vesicles is essential, and in addition NISV properties cannot be mimicked by liposomes. NISV formed using HG do have anti-inflammatory properties indicating that substitution of the ester bond in MPG with an ether bond does not ablate their effect, but does increase their toxicity. To further our knowledge of this phenomenon and to provide additional mechanistic insight into the anti-inflammatory effects of NISV, metabolic and transcriptomic analyses of NISV and liposomes effects on BMDM will be performed in the following chapters.

4. Transcriptomic Analysis of Vesicle Treatments

4.1 Abstract

As previously shown, NISV consisting of MPG, DCP and cholesterol have anti-inflammatory abilities. To further elucidate the potential mechanism(s) of action transcriptomic analyses of treated cells were carried out allowing the investigation of NISV-mediated effects spanning the entirety of cellular function including those not directly associated with canonical immunity. Changes in genes related to sorting nexins and Golgi trafficking were observed in NISV-treated and liposome-treated cells compared with unstimulated cells indicating endosome associated uptake of NISV. NISV treatment induced upregulation of transcripts including a number of downstream immune products associated with MHC and antigen processing and immune mediators. This is consistent with their known adjuvant properties. In contrast, liposomes had downregulatory effects on MHC and antigen processing. In LPS-stimulated cells, NISV reduced the transcript levels of a number of inflammatory mediators. Specifically, NISV reduced expression of chemokines and their receptors, a number of interleukins and their receptors and members of the TNF-α superfamily and their receptors. Many of these effects are mechanistically linked to the observation that NISV significantly reduced NF-κB subunit transcription in LPS-stimulated cells. In contrast liposomes had a wide array of effects on transcripts, which included upregulation of many inflammatory mediators including members of the TNF superfamily and their receptors. Notably, and consistent with this observation, liposomes upregulated NF-κB subunit transcription in LPS stimulated cells. This work in conjunction with previous chapters, demonstrates that different vesicular preparation have unique effects on BMDMs and gives further mechanistic insight into both the adjuvant and anti-inflammatory properties of NISV.

4.2 Introduction

4.2.1 Transcriptomics

Transcriptomics gives a snapshot of total cellular function at a single point in time. With this information we can glean further information not just on the immune system, as studied in the previous chapter, but the significant effects NISV exert on the other systems of macrophage function. This new avenue of information allows further characterisation of the interactions of macrophage and NISV.

Transcriptomic studies utilise a technique called RNA-Seq, this technique utilises high throughput sequencing to identify and quantify the RNA expression of a cell or cell population at the time of extraction. This technology involves the conversion all RNA in a sample into cDNA and the alignment of the cDNA to a reference genome, allowing identification of which genes are transcribed, the read count of this cDNA allows quantification of transcription of all genes within the cell (Wang, Gerstein, & Snyder, 2009). With this technology we aimed to compare the effects of NISV and liposomes on both unstimulated and LPS-stimulated BMDM. With data comprising the entire transcriptome, the insight into the effects of these treatments on the immune system can be characterised better than ever. Transcriptomics, however, will allow great insight into NISV effects on other cellular systems we have yet to study in depth. NISV were expected to have great effects on the cell outwith LPS stimulation. It is hoped Insight will be gained of NISV uptake and fate within the cellular environment as well as more information on the effects on cellular respiration, detailed in the previous chapter.

The principles of transcriptomics relies on Next Generation Sequencing (NGS). The specific form of NGS used in this study is Illumina, which is performed following the principles shown in figure 4.2.1, providing highly accurate short DNA reads (Meyer & Kircher, 2010). These reads can then be computationally stitched together forming a full genome or other genetic material. For RNA analysis, after RNA is extracted it is converted into complimentary DNA (cDNA) and then NGS is performed following the steps outlined in figure 4.2.1. Following sequencing, the data is analysed using the 'tuxedo suite' beginning with Bowtie, which aligns the reads to a reference genome, then TopHat, which identifies exon-exon splice junctions in said alignment, and finally by the Cufflinks software's: Cufflinks, Cuffmerge and Cuffdiff. Cufflinks identifies and quantifies the transcripts, Cuffmerge then merges the reads into full-length transcripts and annotates them and Cuffdiff determines the differential expression and measure the significant differences between different samples (Trapnell et al., 2010).

Transcriptomics Studies are not without limitations, the ability to see a snapshot of RNA transcription is just that, a snapshot. It does not allow comparative results of changes over time in the cell, unless the experiment is a time-point experiment. RNA itself can be a limiting factor due to its instability and transience. RNA is quickly broken down in the cellular environment and levels of transcription are quickly altered and adapted to cellular needs, meaning sampling time is crucial and RNA levels can be

affected by slight changes (Dunckley & Parker, 2001). These limitations must be accounted for and the data carefully analysed in order to produce reliable results, as we believe we have produced. Ideally, transcriptomics should be followed with assays to prove deductions made from the data.

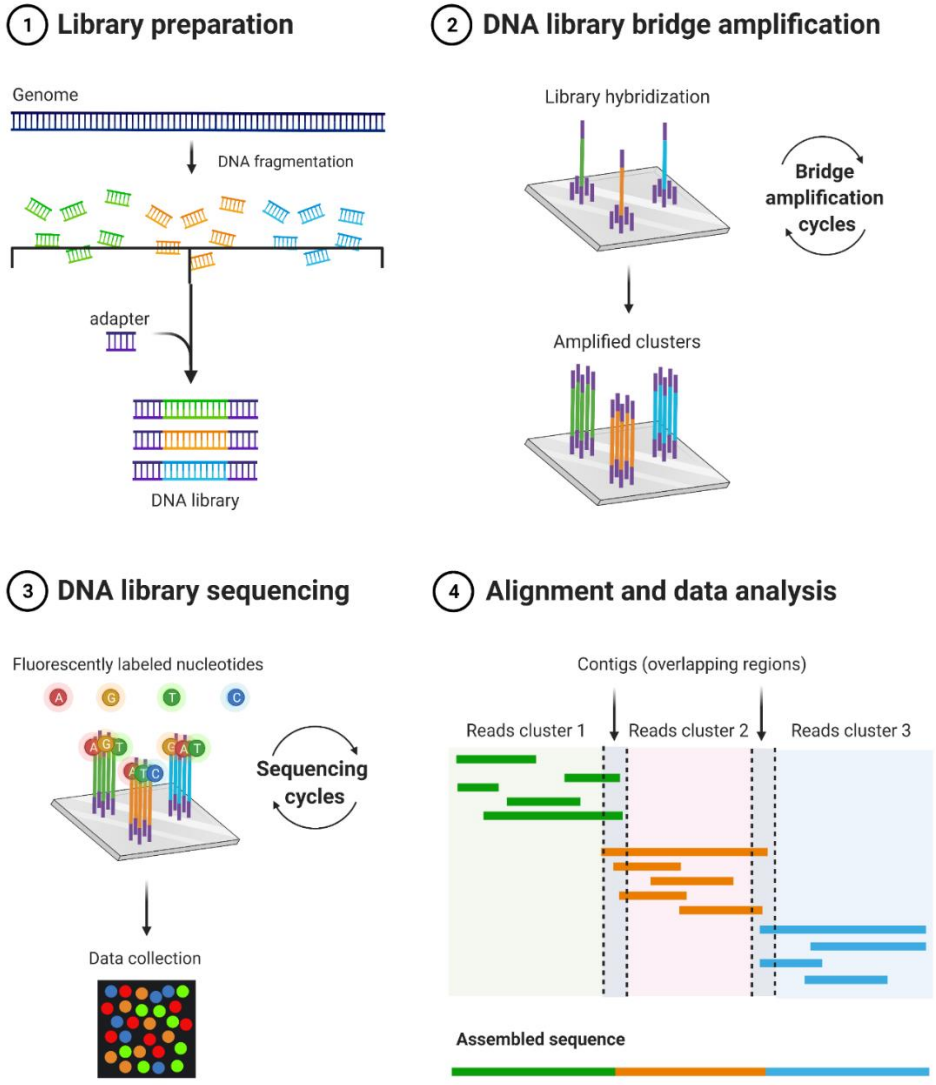


Figure 4.2.1 Principles of Illumina sequencing. *Shown is the process of Illumina sequencing. Beginning with (1) Library preparation, followed by (2) bridge amplification on the flow cell, (3) DNA sequencing and (4) the alignment and analysis. Library preparation involves the process of fixing primers to the ends of the target DNA. During bridge amplification the primers bind to those in the flow cell at both ends of the DNA and the strand is replicated, they are then denatured leaving two identical strands of DNA, this process repeats many times ensuing multiple copies of all DNA in the sample is created. In DNA library sequencing the single strand DNA are replicated again with fluorescently tagged nucleotides, whenever one binds to it releases a signal, which is read giving the nucleotide sequence of the specific strand. Alignment and data analysis is computationally performed and can utilise many different programs, they function by aligning identical DNA strands, building a longer and longer sequence until the whole sequence is characterised* (Trapnell et al., 2010).

4.2.2 Depth of Cellular Processes

Up until this point, we have shown the changes NISV cause to products of macrophage cellular function, with transcriptomics we can identify changes made in pathways and processes leading to these products. For the immune system, transcriptomics will allow in depth analysis of the genes affected by LPS stimulation and NISV effects on cytokine production.

Investigating TLR4 signalling was one of the primary analyses of the immune system performed. With this data we were able to identify the entire process from the cell signalling molecules, through MyD88 and TRIF signalling down to NISV effects on the transcription factors NFκB, AP-1 and IRF3. This analysis will bolster the assays performed to elucidate NISV effects on these signalling pathways, performed in chapter 3. Beyond this, transcriptomics show the effects on downstream cytokines affected by TLR4 signalling in extreme depth, allowing characterisation of the entire cytokine profile. With this information, future experiments will be curated in order to prove or disprove assumptions made based on this data.

Furthermore, this dataset allows an in depth look at the effects NISV have on sphingosine transcription, allowing further insight into the accuracy of the sphingosine hypothesis.

4.2.3 Aims & Hypothesis

The aims of this chapter are to investigate the effects of NISV on the transcriptome of murine resting macrophages and macrophages following LPS stimulation to gain mechanistic insight into how they mediate anti-inflammatory and adjuvant effects. In addition, these effects will be compared with the effect of liposome treatment in identical circumstances. Therefore, transcript levels determined by RNA-seq will be analysed using:

i. An untargeted analysis to identify the most important changes in transcripts induced in BMDM following NISV treatment. This should allow the elucidation of the most affected pathways by NISV and allow further analysis of pathways affected outside of the immune response.
ii. A targeted analysis of the immune system to determine the effects of NISV on pathways and products known to be influenced in macrophages (and largely identified in chapter 3) by NISV treatment. This will include TLR4 signalling, as initiated by LPS stimulation, from receptor interaction through signal transduction and finally immune effector production. Furthermore, the hypothesis established in chapter 3, pertaining to potential ability of NISV to interfere with S1P signalling.

Together, these analyses will allow a detailed description and provide mechanistic insight of how NISV affect the production of immune mediator transcription.

4.3.1 Proof of Transcriptomics Samples Validity

Prior to Transcriptomics analysis, supernatants were taken from the cell cultures, tested for inflammatory effects and parallel plates were set up for alamarBlue cell viability analysis.

The cell viability assays and IL-6 ELISA show effects of NISV are consistent with the data previously shown in section 3.3.

Following this confirmation, the supernatants were further characterised using cytometric bead array analysis, reading 13 cytokines related to macrophage polarisation. Figure 4.2.2 shows that the previous bead array data, shown on figures 3.6.2 and 3.6.3, the same trends as shown but with less statistically significant changes, most likely due to natural variation between the different batches of BMDM. In the control samples, NISV shows significant increases of CXCL1, IL-6 and TNF-α and minimal effects in the other measured cytokines. The NISV show significant reduction of G-SCF, IL-6 and IL-12p40 as well as non-significant reductions in IL-12p70 and TGF-β1 compared to the LPS stimulated cells, they also showed significant upregulation of IL-1β.

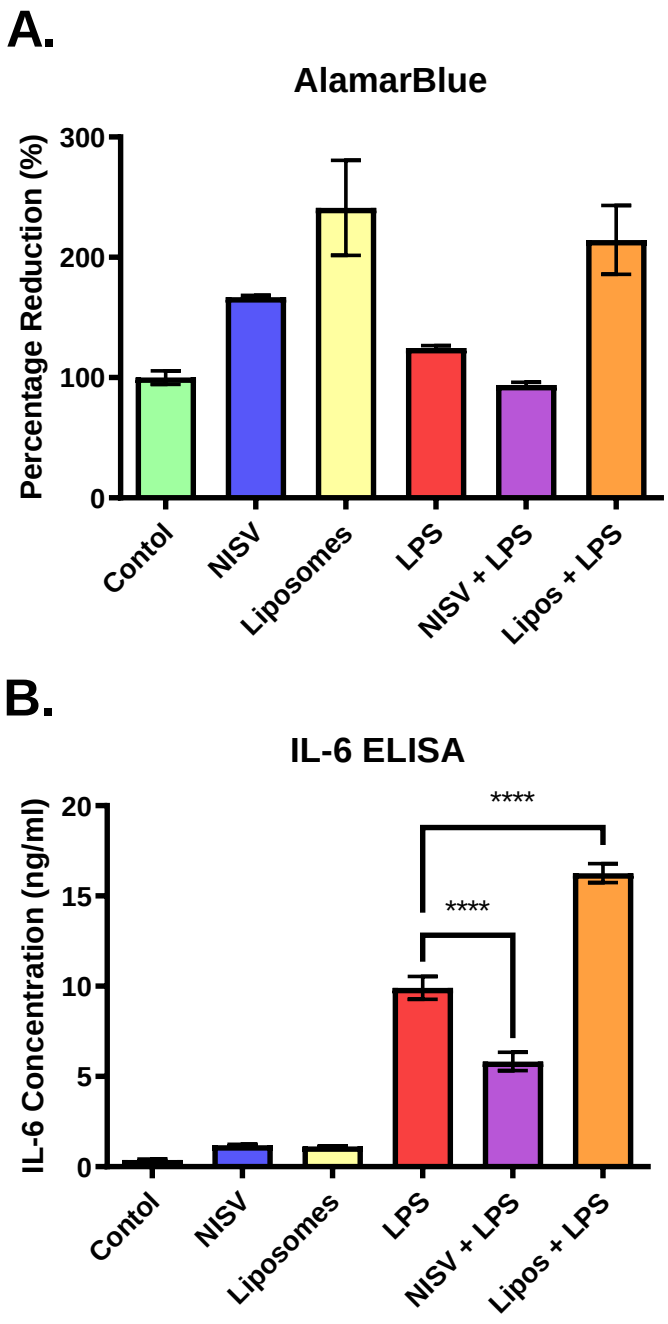


Figure 4.3.1 NISV show a Consistent Anti-Inflammatory Profile. *100,000 BMDM were plated per well in triplicate and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. A shows alamarBlue reduction by BMDM under these conditions and B shows IL-6 levels in the supernatants of transcriptomics samples after 24 hours. Statistical analysis was carried out using Prism 8 where one-way ANOVA and Tukey's multiple comparisons test was performed. * Indicates statistical significance ($p<0.05$).*

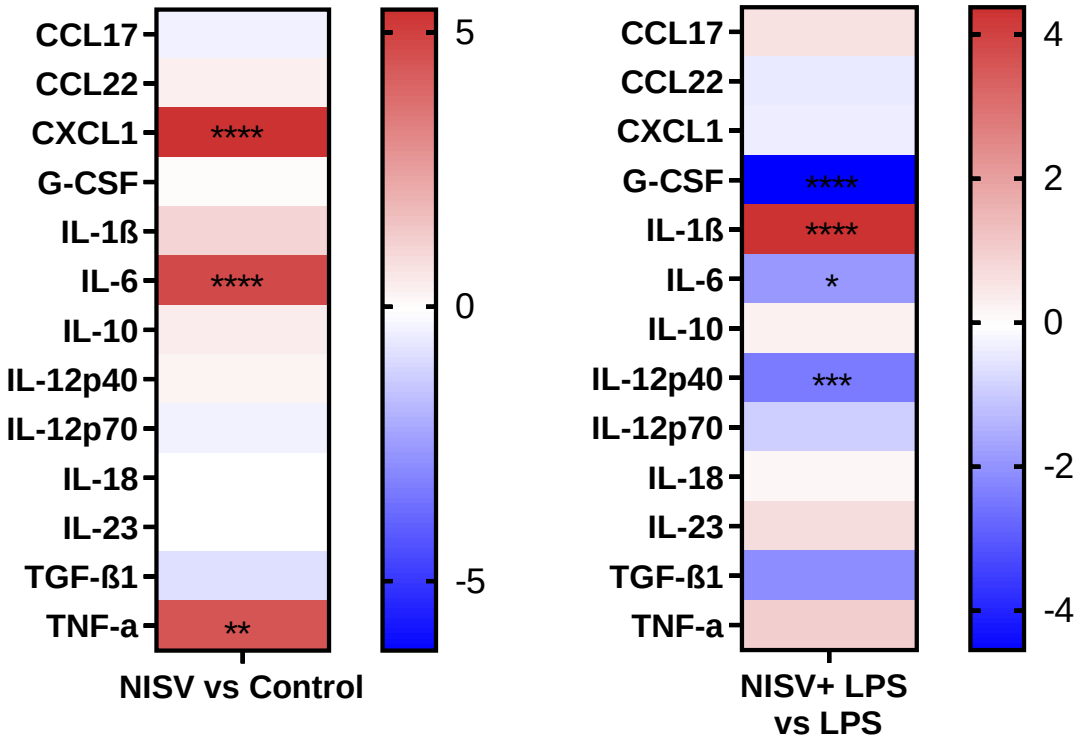


*Figure 4.3.2 NISV Repeat Anti-Inflammatory Effects in Bead Array. 100,000 BMDM were plated per well in triplicate and were stimulated using 3µg/ml LPS or left unstimulated, these were then treated with 1.5mM NISV. After 24 hours supernatants were taken and analysed using the cytometric bead array. Statistical analysis was carried out using Prism 8 where two-way ANOVA and Tukey's multiple comparisons test was performed. * Indicates statistical significance (p<0.05).*

Prior to sequencing RNA quality and yield were measured and tested using the Bio-analyzer and found to be of high quality (RNA Integrity Number > 7) and sufficient weight (1µg in 20µl), so the experiment was performed. Samples were sent to Eurofins to perform their INVIEW Transcriptome Explore service.

Upon receiving the data from Eurofins thorough analysis was performed, initially the entire dataset was analysed using principle component analysis (PCA) to visualise differences between groups and similarities within groups (Figure 4.3.1). Each sample group, other than LPS and NISV + LPS, form tightly packed groups displaying the groups are significantly different when comparing the entire transcriptome. LPS and NISV + LPS formed a close group of six samples showing PCA could not discern great difference between the groups, this analysis ruled out use of OPLS-DA to compare differences between these two groups.

4.4 NISV and liposome effects on resting BMDM Transcriptome

To characterise the groups further the difference in gene expression between groups was compared using volcano plots. Figures 4.4.1 & 4.4.2 show the significant differences between the unstimulated cells and those treated with NISV or liposomes. NISV induced 3830 significant changes in expression compared with the unstimulated cells, whereas liposomes induced more than double that, causing 9234 significant differences in expression relative to unstimulated cells.

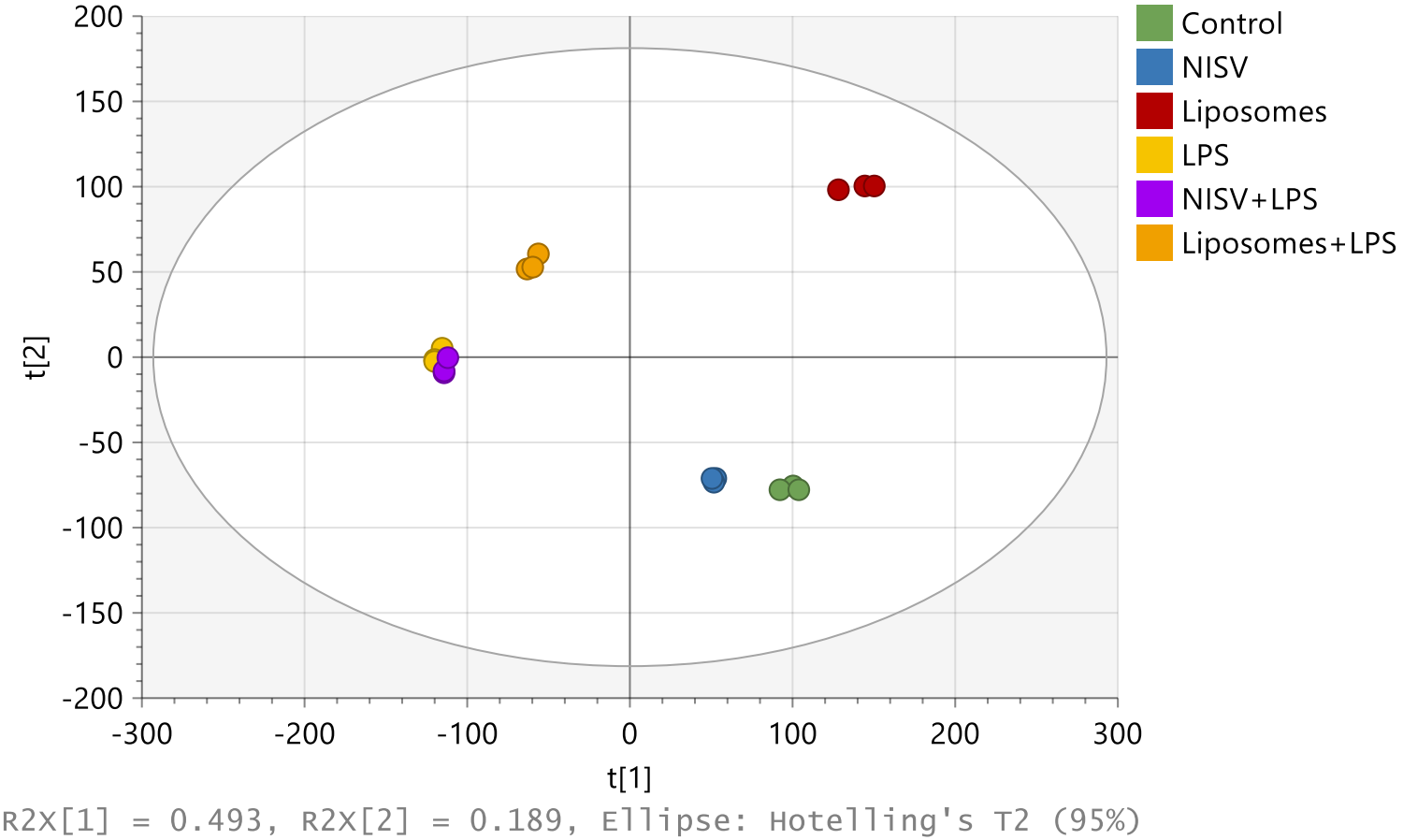


Figure 4.4.1 Visualisation of Differences found between all Groups Transcriptomes by PCA-X. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. PCA analysis was performed using SIMCA 16.*

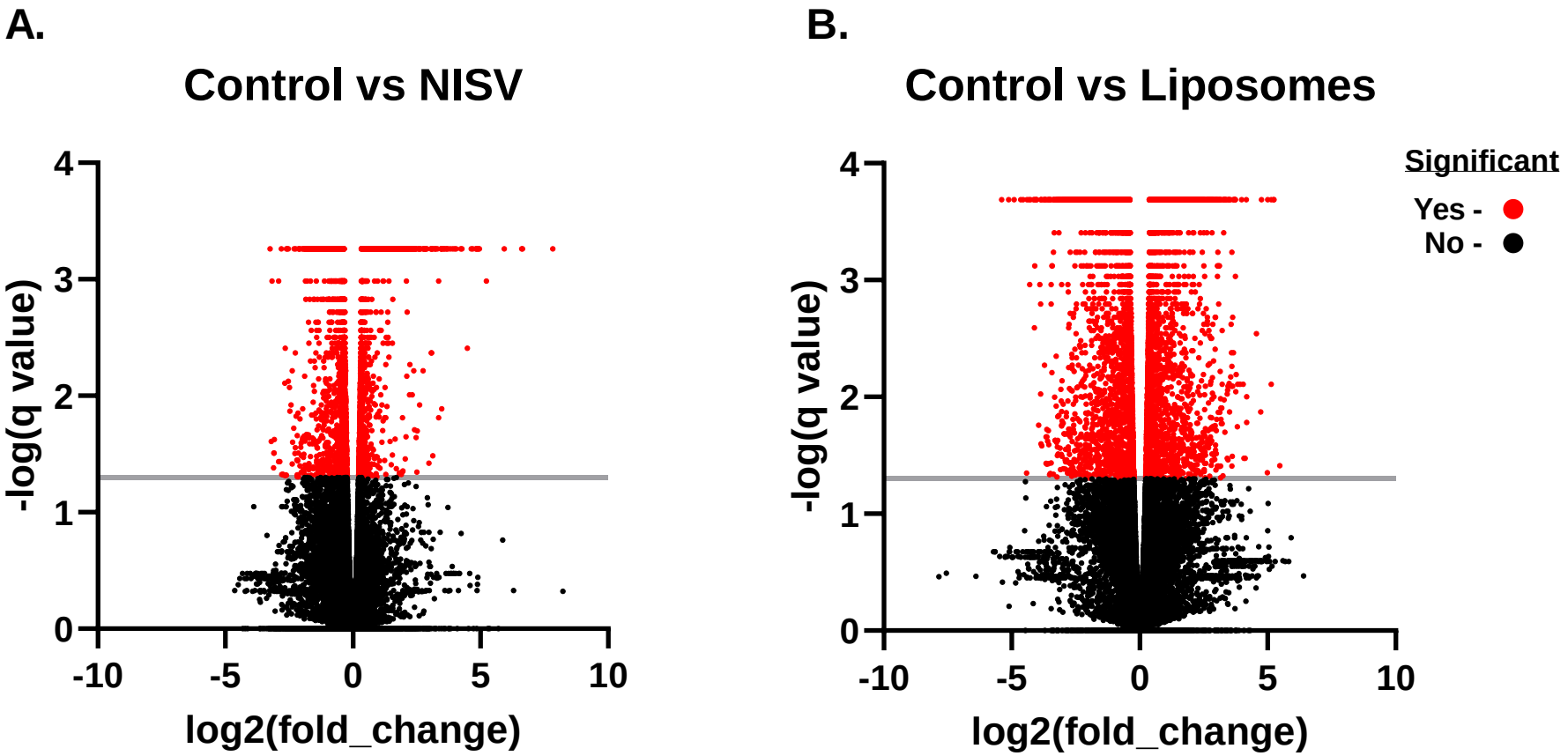


Figure 4.4.2 Volcano Plots showing the Significant Differences caused by Vesicle Treatment. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Significance determined by Cuffdiff. ($p<0.05$).*

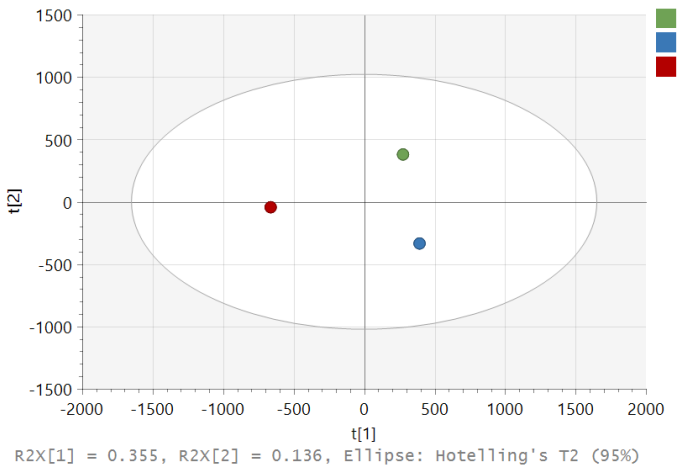
4.4.1 Effect of vesicle treatment on BMDM

To understand the differential effects of NISV and liposomes on macrophage transcription OPLS-DA was used to identify the genes that contribute most to the differences between groups. Figure 4.4.3.A shows the OPLS-DA produced comparing the control cells with both treatment controls. The top 25 most affected coding transcripts and the function with which they associate are listed by their VIP order with fold change and adjusted p value. Transcripts identified through the OPLS-DA were generally but not always found to be significantly different between at least 2 sample groups. As expected, the changes liposomal treatment show compared to both unstimulated and NISV treated cells contribute most to the VIP. We show NISV affect 5 of this selection of genes significantly, two of which affect cell structure, one the inflammatory response, one cell cycle and the final gene affects calcium transport. Liposomes treatment group compared to both the control and NISV group contribute the bulk of significant changes shown, with 11 significant difference from the control and 13 from NISV. The difference liposomes caused to levels of transcription in unstimulated cells are evident in physiological processes and cell structure, affecting 4 and 5 genes respectively, with the remaining two genes affecting inflammatory processes and lysosome activity. Changes caused by NISV treatment were poorly characterised by VIP so a Venn diagram was created comparing the significant differences NISV and liposomes make to unstimulated control cells. Further evidence that liposomes cause many changes in cellular function when compared to NISV is demonstrated in Figure 4.4.4.A. While both vesicle treatments shared 2328 significant transcript changes, NISV had 953 unique effects on cells, and liposomes had 5091.

To characterise the unique effects NISV have on transcription, we used the gene ontology software GOrilla to sort the genes into processes mostly affected in these 953 genes. One of the most highly affected groups found by GOrilla was antigen processing and presentation. The genes from this list were then taken and a heat map of Log2 fold change was produced (Figure 4.4.4.B). We show that NISV exclusively affect various components of major histocompatibility complex I & II processing and production. NISV treatment induced an upregulation of both MHC class I and class II via the upregulation of Tap transporter, proteasome cathepsin and MHC genes. Liposomes largely downregulated both MHC class I and II, inducing downregulation of MHC, cathepsin and proteasome genes but upregulating the Tap transporter genes. Further analysis of NISV effects on unstimulated cells was performed using GOrilla, where ‘defense response’ was identified as the most affected groups. Due to the large

amount of significant changes in transcripts in this group, ‘defensse response’ split into three groups, ‘Immune Effectors’, ‘Cellular Signalling’ and ‘Structure & Transport’ (Figure 4.4.5). In unstimulated BMDMs NISV induced upregulation of all genes included in the immune effector group, which included chemokines, cell surface receptors, interleukins and members of the TNF superfamily. In cellular signalling, we see some variation in effect in caspase and TLR expression but otherwise wholly positive regulation by NISV, notably including NLRP3, MyD88 and NF-κB subunits. In structure and transport, we also see an exclusive upregulation of the genes identified by GOrilla including *Gbps*, which are induced by interferons.

Due to findings by Woods *et al* (2020) indicating differential modes of uptake of NISV in different cell types, genes related to vesicular uptake were measured and compared (Woods et al., 2020). Genes related to clathrin and dynamin mediated uptake as well as sorting endosomal uptake were investigated (Figure 4.4.6). NISV had no effect on the clathrin genes but did regulate associated genes, *Ap2a2* was downregulated and *Ap2b1* upregulated. Dynamin related genes were unaffected by both NISV and liposomes. Two Golgi genes were upregulated by NISV, *Golga4* and *Golgb1*. Sorting nexins were differentially affected by both NISV and liposome treatment. NISV upregulated 4 of these genes (*Snx10, 19, 20* and *27*) and downregulated 8 (*Snx2, 5, 8, 13, 24, 29, 30* and *32*), and liposomes upregulated 5 (*Snx6, 15, 20, 21,* and *33*) and downregulated 16 genes (*Snx1, 2, 3, 5, 7, 8, 9, 13, 14, 17, 24, 25, 27, 29, 30,* and *32*).



Gene Name	Control vs NISV		Control vs Liposomes		NISV vs Liposomes		Related process
	Fold Change	q value	Fold Change	q value	Fold Change	q value	
Bbx	0.462958	0.386241	0.23258	0.702352	-0.216523	0.677878	Cell cycle
Pde1c	-0.781322	0.052293	-2.72398	0.000577	-0.882574	0.000489	Physiological processes
Hs6st3	0.0935436	0.681161	-1.57466	0.000204	-1.65679	0.000172	Physiological processes
Il1rapl1	-0.388056	0.01105	-0.363021	0.017448	0.0385129	0.823161	Inflammatory response
Zc3h7a	-0.244034	0.680388	-0.252928	0.643532	0.00509662	0.991246	RNA regulation
Asxl3	-0.403629	0.002338	-0.122927	0.450633	0.294911	0.045409	Cell cycle
Slc25a41	0.129503	0.697367	-1.48972	0.000204	-1.60924	0.000172	Physiological processes
Cntnap2	1.00835	0.000548	-0.361468	0.198572	-1.3581	0.000172	Cell Structure
Nt5dc1	-1.26615	0.114209	1.82819	0.001923	3.10761	0.00137	Physiological processes
Crocc	0.0789246	0.882085	-1.51493	0.011251	-1.58067	0.006862	Cell structure
Tbx20	0.00187183	0.989931	0.663501	0.000204	0.675017	0.000172	Cell structure
Aldoart1	0.740097	0.112226	-0.158604	0.766407	-1.08516	0.290873	Cellular Respiration
Cacna2d4	-0.436516	0.001033	-0.019794	0.908376	0.430089	0.000791	Calcium transport
Adamts13	-0.625535	0.004286	0.625575	0.001267	1.26398	0.000172	Cell structure
Actb	-0.0286995	0.879507	-1.71405	0.000204	-1.67261	0.000172	Cell structure
Slc25a13	-0.095041	0.931368	-1.06313	0.133236	-0.955039	0.147502	Calcium transport
Tmsb4x	-0.0631283	0.757902	-0.869956	0.000204	-0.793111	0.000172	Cell Structure
Epsti1	-0.500845	0.801044	-0.897348	0.585965	-0.381311	0.825206	Macrophage Polarisation
mt-Nd6	-0.14138	0.75168	0.522784	0.102395	0.677505	0.024205	Cellular Respiration
Hexb	-0.241096	0.034892	-0.783857	0.000204	-0.529379	0.000172	Hydrolytic enzyme (lysosome)
Macrod2	0.332569	0.397122	0.469934	0.141418	0.149814	0.617486	Physiological processes
Mvp	0.119778	0.975527	-2.02988	0.622546	-2.13651	0.611103	Cell Structure
Golga7b	1.54719	0.535521	0.131676	0.656704	-1.39124	0.304141	Protein export
Pou3f3	-0.285588	0.309631	-1.11373	0.000204	-0.813377	0.001652	Physiological processes

Figure 4.4.3 Vesicle Formulations Induce Significant Changes in BMDM. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. OPLS-DA and VIP analysis performed using SIMCA 16. Significance determined by Cuffdiff. ($p<0.05$) and denoted by orange colouring.*

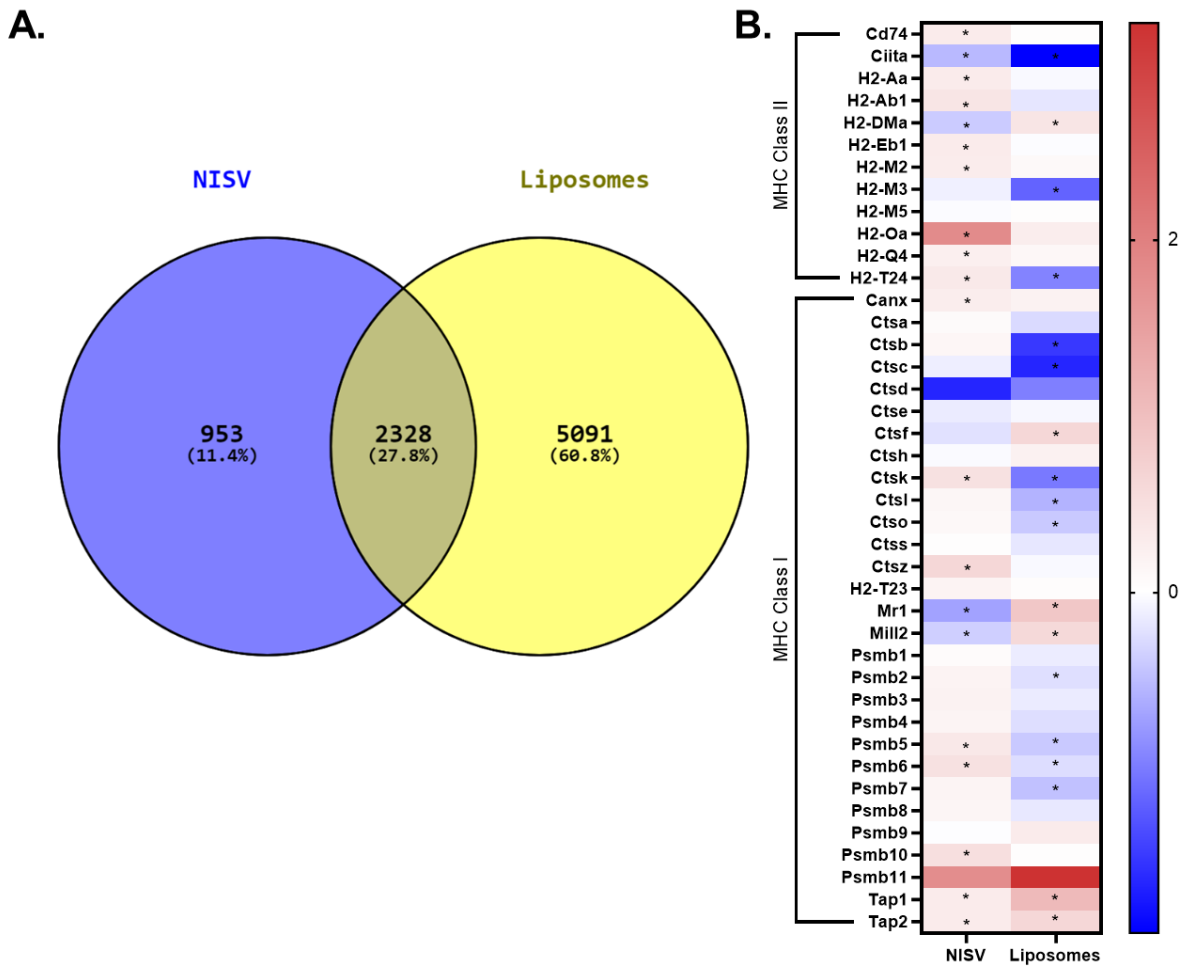


Figure 4.4.4 NISV show significant effects on MHCII Expression not seen in Liposome Treatment. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Venn diagram produced using VENNY 2.1 shows significant differences caused in unstimulated cells by NISV and liposomes in BMDM. Using Gorilla some MHC-related genes were identified as exclusively affected by NISV treatment. This was expanded upon and a heat map including genes related to MHC class I & II was produced (B). (p<0.05).*

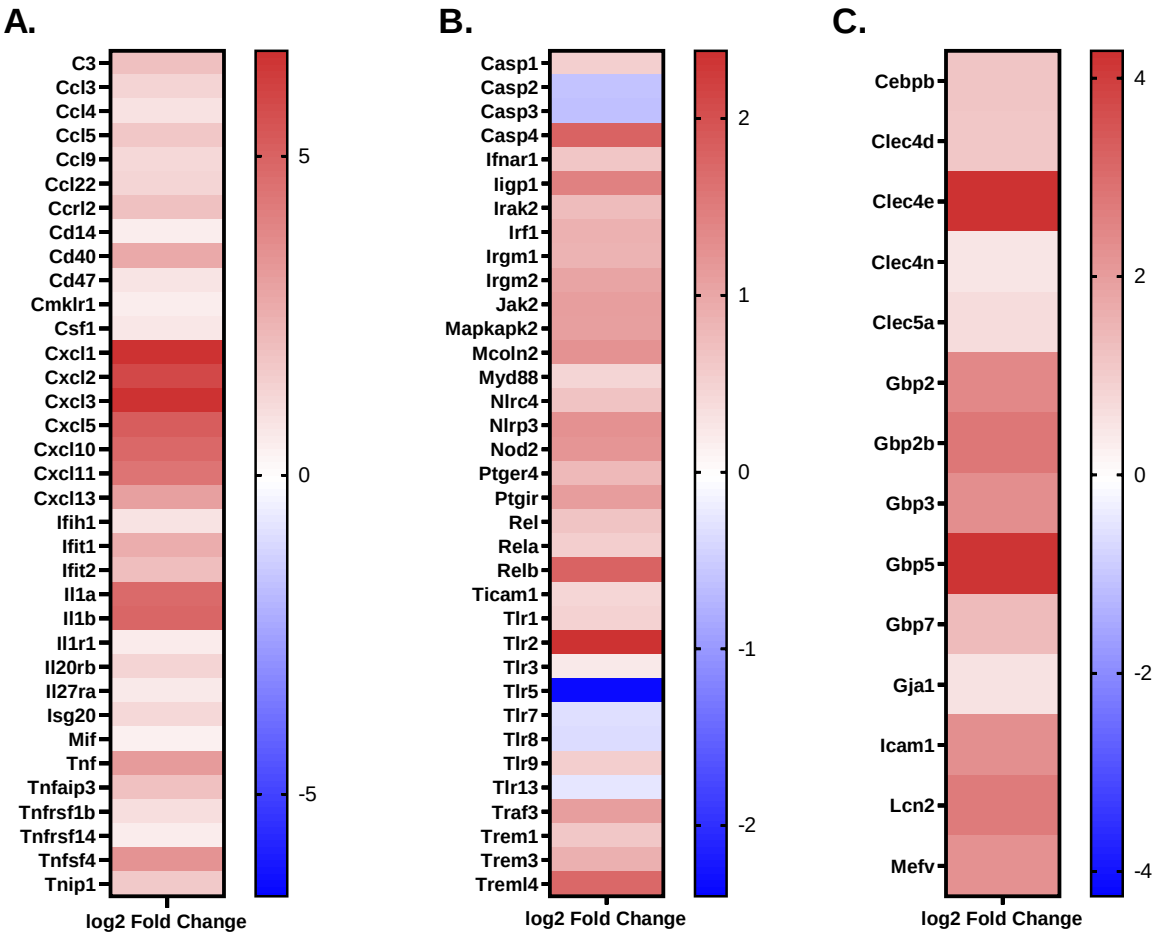


Figure 4.4.5 NISV Effects on Defence Response show Adjuvant Capabilities. Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. GO-rilla identified defence response as the most affected group by NISV in unstimulated cells, which were further separated into the group above; A shows Immune Effectors, B shows Cellular Signalling and C shows Structure and Transport. Heat maps were produced using Prism 8. ($p<0.05$).

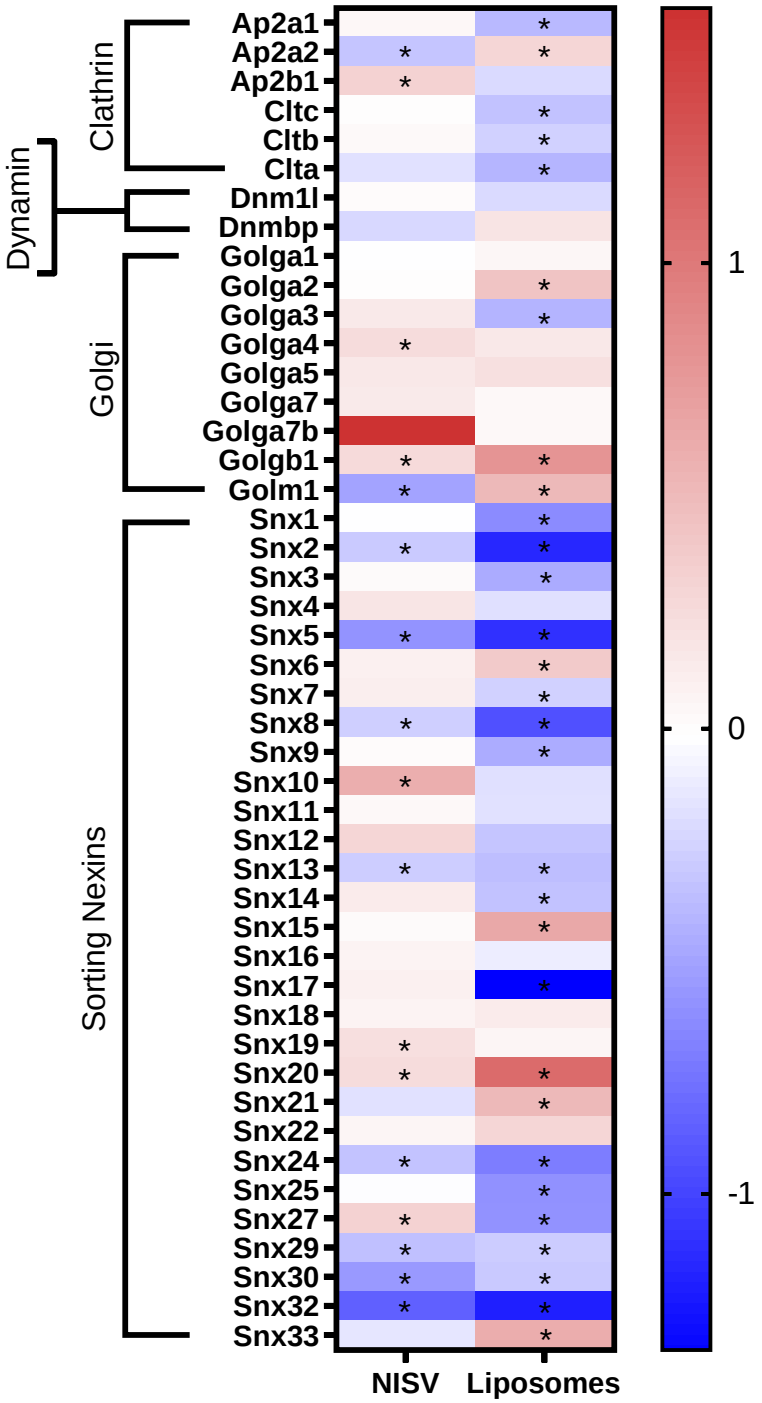


Figure 4.4.6 Vesicle Treatments Effects on Vesicular-Mediated Uptake genes. *Transcriptomic analysis was performed on RNA extracted from BMDM treated with 1.5mM vesicle formulations of NISV and liposomes and unstimulated controls. Heat map produced using Prism 8 shows genes significantly altered by NISV and liposomes that are associated to vesicular uptakes. ($p<0.05$).*

4.5 Analysis of NISV and Liposomes Immunomodulatory Effects on the Transcriptome of LPS-Stimulated BMDM

Following the analysis of the transcriptome of unstimulated cells treated with NISV and liposomes, a look into various immune processes affected by LPS-stimulation and the effects of vesicle treatment on them was performed.

Volcano plots were used to analyse the total changes cause by NISV and liposomes in LPS-stimulated cells. The similarity between the LPS and NISV + LPS found in Figure 4.4.1 is further emphasised showing only 626 significant differences (figure 4.5.1A). Liposome treatment of LPS-stimulated cells on the other hand demonstrated many more differences (8304 significant changes) in transcription compared with those seen with NISV (figure 4.5.1B).

Due to the samples of LPS and NISV + LPS not forming separate groups in the PCA the use of OPLS-DA and VIP to compare the groups was not appropriate. In order to correctly compare the differences between these groups GOrilla was again used. Using the list of genes ranked by smallest log2 fold change to largest, the genes were sorted into the most affected groups. The most affected group were identified as, ‘defense response to virus’, ‘positive regulation of cytokines’ and ‘cell surface receptor signalling pathway’, all of which are within the broad category of ‘defense response’. This data identified NISV as having a negative effect on the ‘defense response’ of BMDM (which includes LPS-induced signalling) and will be covered later in the chapter due to many of the included genes being covered in the following figures. To identify the positive effects NISV have on BMDM stimulated with LPS this data was reordered from highest log2 fold change to lowest and re-entered into GOrilla. This identified ‘catabolic processes’ and ‘cellular localisation’ as the most affected processes. ‘Cellular localisation’ shows a predominant upregulation of genes related to this process, as expected, as well as some genes which are downregulated (Figure 4.5.2). Of interest is the contradiction between some groups of genes involved in this process such as the up and downregulation of different ATP-binding cassette genes, DENN/MADD genes and low density lipoprotein related genes.

While OPLS-DA and VIP were not useful for analysis of the differences between the LPS and NISV + LPS groups, the clear separation of LPS and Liposomes + LPS ensured that OPLS-DA could be carried out and the VIP would produce meaningful results. The OPLS-DA, Figure 4.5.3.A, demonstrated the clear separation between groups and allowed us to produce the VIP, Figure 4.5.3.B, showing the most affected

genes by liposomal treatment in LPS stimulated cells. We show that most of the significantly affected genes relate to immune response or membrane and cytoskeletal function. Those related to the immune response all showed downregulation by liposomes, in contradiction to our previous findings, other than the chemokine CXCL2. Similarly, membrane and cytoskeletal function is affected negatively by liposomal treatment, reducing expression of cell structure effectors and components, actin beta, myosin-8 and collagenase 3, as well as endocytosis and exocytosis related genes which express, PDZ domain-containing protein 4 and synaptotagamin-7.

Finally, to identify the effects NISV exclusively hold over liposomes in LPS stimulated cells another Venn diagram was produced (Figure 4.5.4.A). From the Venn diagram, the genes significantly affected only by NISV were taken and analysed using GOrilla, which identified the ‘positive regulation of cytokine production’ as the most affected process by these 124 genes (Figure 4.5.4.B).

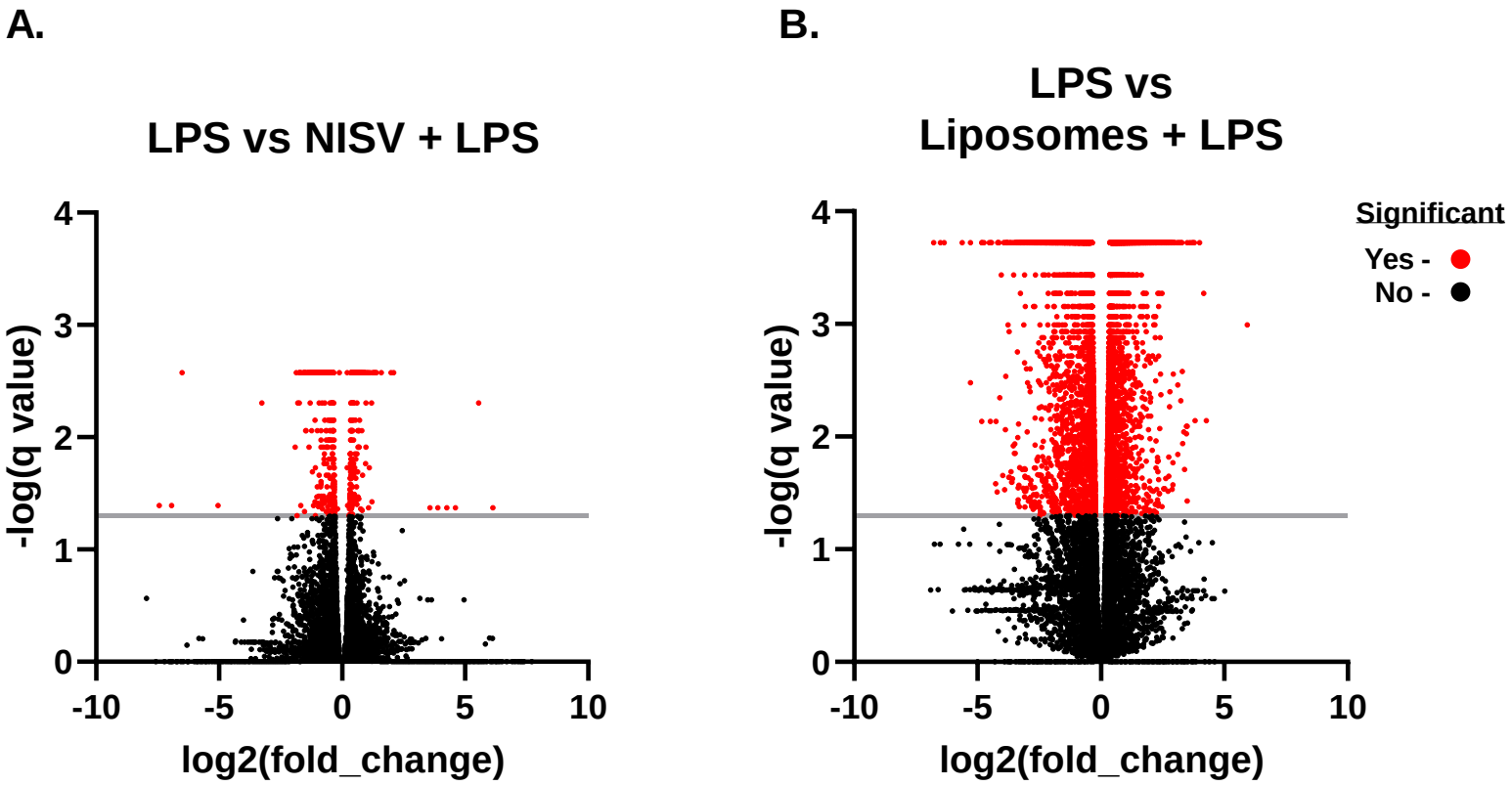


Figure 4.5.1 Volcano Plots showing the Significant Differences found by Treating LPS-Stimulated BMDM with NISV and Liposomes. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Significant difference determined by Cuffdiff. ($p<0.05$).*

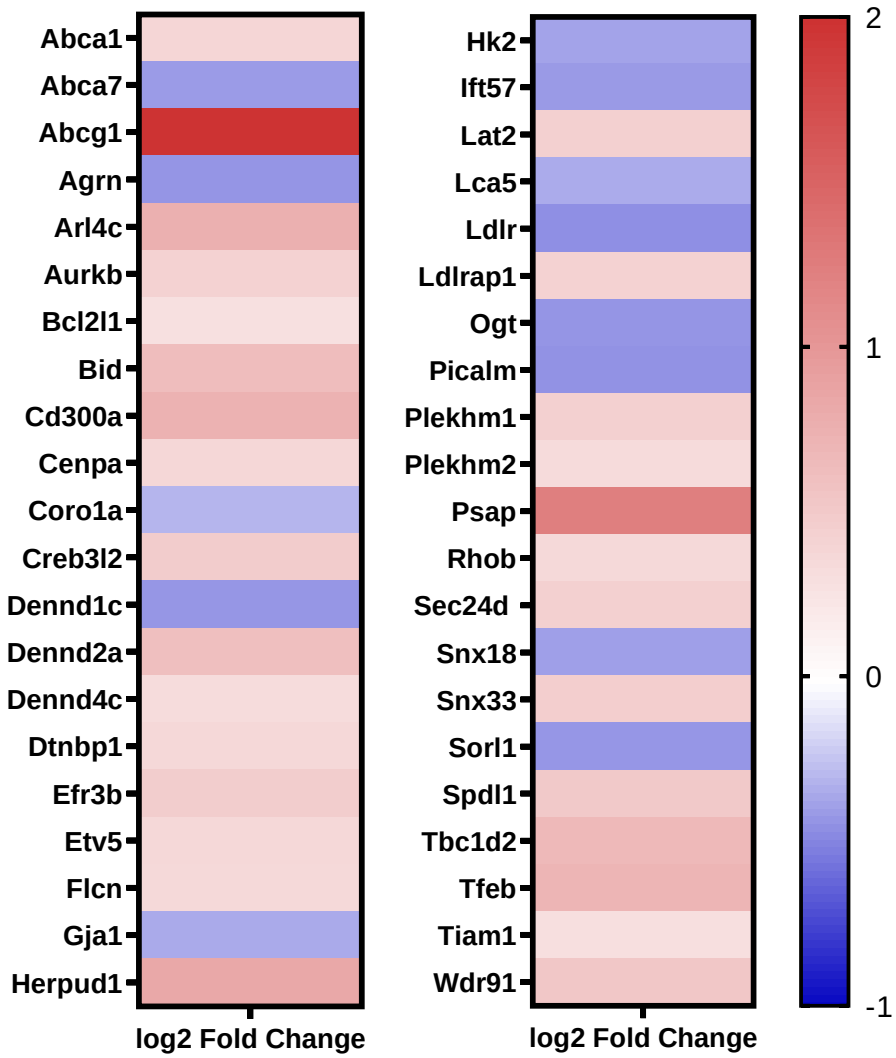
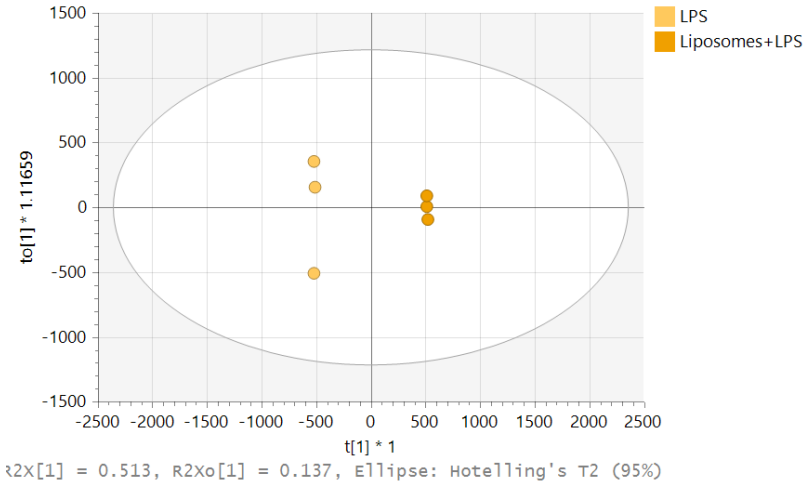


Figure 4.5.2 NISV Significantly Alter Genes related to Cellular Localisation. Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Heat map produced using Prism 8 shows genes significantly altered by NISV in a group identified by GOrilla as cellular localisation. ($p<0.05$).



Gene Name	Fold Change	q value	Encodes for:	Related process
Pde1c	-1.57788	0.063573	Phosphodiesterase 1C	Physiological processes
Bbx	-0.79092	0.133842	HMG box transcription factor BBX	Cell cycle
Cacna2d4	-1.75265	0.00019	Voltage-dependent calcium channel subunit alpha-2/delta-4	Calcium transport
Actb	-2.04053	0.00019	Actin beta	Cell structure
Cd200r3	-0.67296	0.00019	Cell surface glycoprotein CD200 receptor 3	Cell Surface interactions
Il1rapl1	-0.24424	0.086008	Interleukin-1 receptor accessory protein-like 1	Inflammatory response
Zc3h7a	-0.32594	0.48133	Zinc finger CCCH domain-containing protein 7A	RNA regulation
Cxcl10	-2.64804	0.00019	C-X-C motif chemokine 10	Inflammatory response
Clca1	-0.4344	0.521901	Calcium-activated chloride channel regulator 1	Calcium transport
Macrod2	1.59441	0.000862	ADP-ribose glycohydrolase MACROD2	Physiological processes
Golga7b	-0.98803	0.146703	Golgin subfamily A member 7B	Protein export
Syt7	-1.86586	0.00019	Synaptotagmin-7	Exocytosis
Kcnk3	-1.79742	0.00019	Potassium channel subfamily K member 3	Potassium transport
Tbx20	0.254235	0.028196	T-box transcription factor TBX20	Cell structure
Pdzrn4	-1.77109	0.00019	PDZ domain-containing protein 4	Endocytosis
Hexb	-0.23321	0.034199	Beta-hexosaminidase subunit beta	Hydrolytic enzyme (lysosome)
Myh8	-1.9724	0.00019	Myosin-8	Cell structure
Il1rn	-2.42433	0.00019	Interleukin-1 receptor antagonist protein	Inflammatory response
Mmp13	-5.29049	0.00019	Collagenase 3	Cell structure
Saa3	-0.98718	0.00019	Serum amyloid A-3 protein	Inflammatory response
Slco1a6	-4.41752	0.228697	Solute carrier organic anion transporter family member 1A6	Sodium transport
Spink13	-1.01508	0.019506	Serine protease inhibitor Kazal-type 13	Physiological processes
Ifit2	-1.80294	0.00019	Interferon-induced protein with tetratricopeptide repeats 2	Inflammatory response
Asxl3	-0.04416	0.824689	Putative Polycomb group protein ASXL3	Cell cycle
Cxcl2	1.24547	0.00019	C-X-C motif chemokine 2	Inflammatory response

Figure 4.5.3 Liposomes Primarily induce changes to Cell Surface and Structure. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. OPLS-DA and VIP analysis performed using SIMCA 16. Significance determined by Cuffdiff. ($p<0.05$) and denoted by orange colouring.*

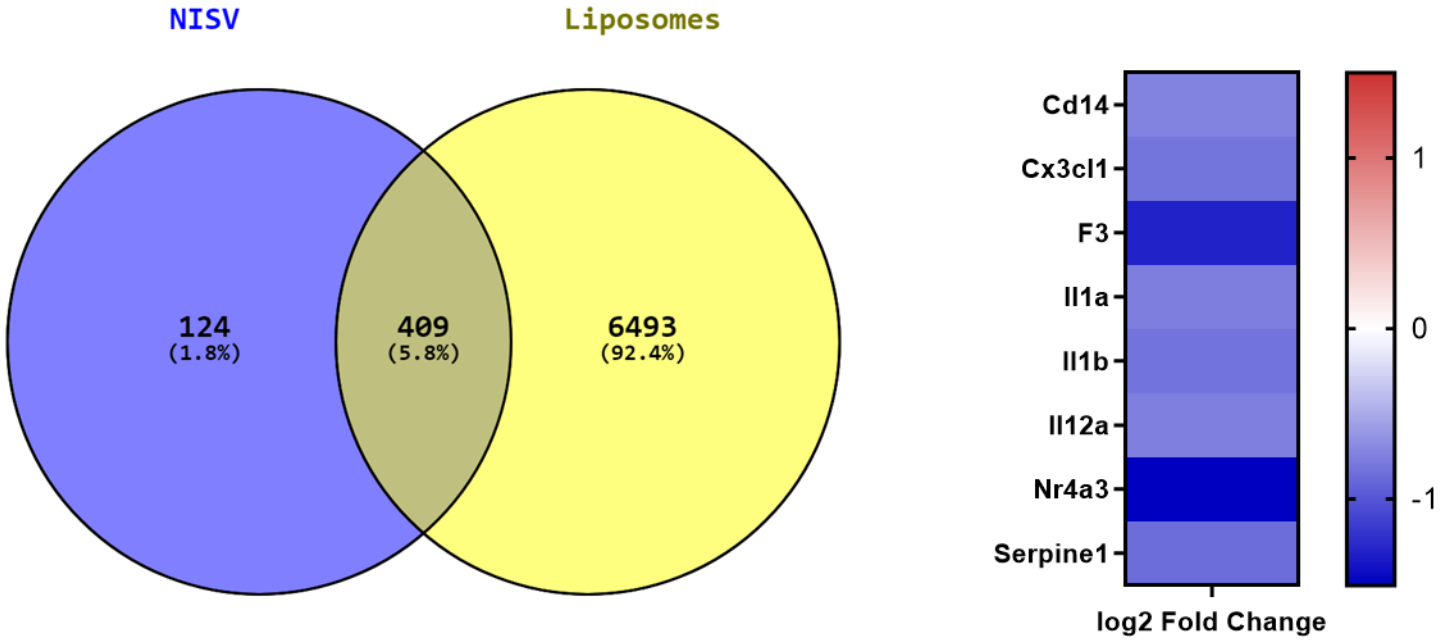


Figure 4.5.4 Unique and common genes affected by NISV and liposomes in LPS stimulated BMDMs. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Venn diagram produced using VENNY 2.1 shows significant differences cause by NISV LPS-Stimulated BMDM. Heat map produced using Prism 8 shows genes significantly altered by NISV exclusively in a group identified by GOrilla as positive cytokine production. ($p<0.05$).*

We looked at the effects NISV exert on the genes for the cytokines measured by bead array. NISV had downregulatory effects on almost all of the genes related to the bead array cytokines, with significant downregulation of all genes other than those encoding CCL17, IL-18, IL-23 and TGF-β1 (Figure 4.5.5). When compared with the results of the actual bead array, shown on Figure 3.6.2, the gene expressions do not match up perfectly, with opposing results seen in *Il1b* and *Il10* gene expression and the cytokines IL-1β and IL-10. As observed in the bead array analysis liposomes have a more variable effect on the expression of these cytokines, inducing a significant upregulation of *Cxcl1, Il23a, Tgfb1* and *Tnf* and downregulation of *Ccl22, Csf3, Il6, IL12b* and *Il18*. Again, these results do not match perfectly with those observed using the bead array. On Figure 3.6.3, we see that CCL22, CXCL1, IL-6 and IL-18 levels are not significantly affected by liposome treatment as the gene expression is on figure 4.5.1, IL-1β and Tgf-β1 also show opposing effects between these two figures.

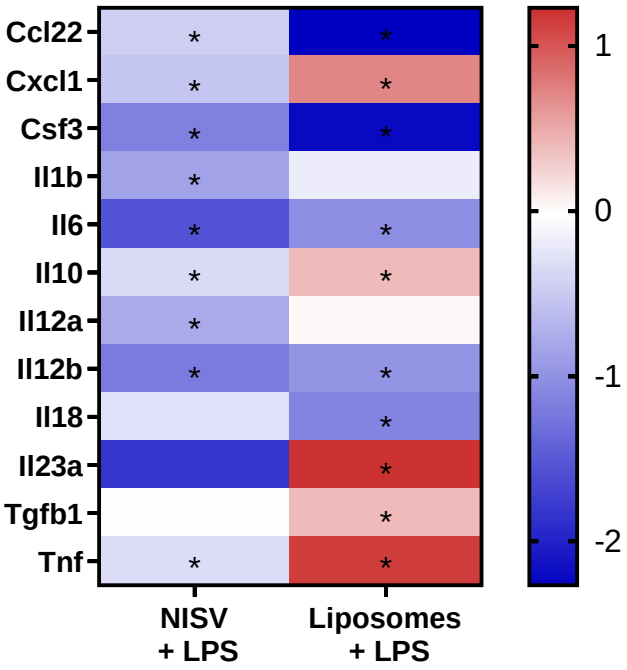


Figure 4.5.5 NISV negatively regulated genes involved in Cytometric Bead Array. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Heat maps were produced using Prism 8, demonstrating the effects of vesicle treatments on genes related to the cytometric bead array in LPS-stimulated BMDM. * indicates significant change. ($p<0.05$).*

4.5.1 NISV Downregulate LPS Binding and Signal Transduction

This analysis leads to further investigation of the systems within the cell that respond to LPS stimulation and the effects NISV have on those systems. The genes of the TLR4 receptor cascade are shown on Figure 4.5.6. heat map show NISV and liposomes effects on transcription of LPS-stimulated BMDM on the diagram. NISV were found to significantly downregulate *Cd14* and *Map3k7* (TAK1) and significantly upregulate *Ticam1* (TRIF), following LPS stimulation. In signal transduction we see no significant effects in the Map3 kinase cascade, the IKK complex or TRAF-3 signalling. Significant changes are shown in transcription factors expression, NF-κB and AP-1, we show significant downregulation of NF-κB subunits 1, 2 and *Rel*, as well as non-significant downregulation of all other NF-κB subunits. AP-1 subunits *Jun* and *Fosl2* are significantly altered by NISV treatment, with *Jun* being upregulated and *Fosl2* downregulated significantly, the expression of other subunits of AP-1 undergo similar variation in expression but not significantly. We show Liposomes effects on TLR4 signalling, on receptor interaction downregulation of TLR4, MyD88, TAB1 and TAK1 expression and upregulation of IRAK1 and TRAF6 expression is shown. Within the MyD88 signalling cascades, we see no significant effect on the IKK complex but variable changes in the MAP3 kinase cascade, we show that *Mapk3* (ERK1), *Mapk8* (JNK) and *Mapk14* (p38) are all upregulated and *Map2k1* (MEK1), *Map2k4* (MKK4) and *Mapk11* (p38β) are downregulated significantly. In the MyD88 affected transcription factors, NF-κB show predominantly significant upregulation of its subunits with the exception of *Relb*, which is significantly downregulated, and *Nfkb1*. AP-1's subunits have more variation in expression with four, *Jun, Junb, Jund* and *Fosl1*, genes being significantly upregulated and two, Fos and Fosl2, being significantly downregulated. In TRIF signalling all genes in the pathway are significantly altered by liposomes presence. TRIF expression itself is significantly upregulated but other receptor related proteins, IKKE and TANK building kinase 1, are significantly downregulated. Following the signalling, we show that TRAF-3 and IRF3 are significantly upregulated and IRF7 is significantly downregulated.

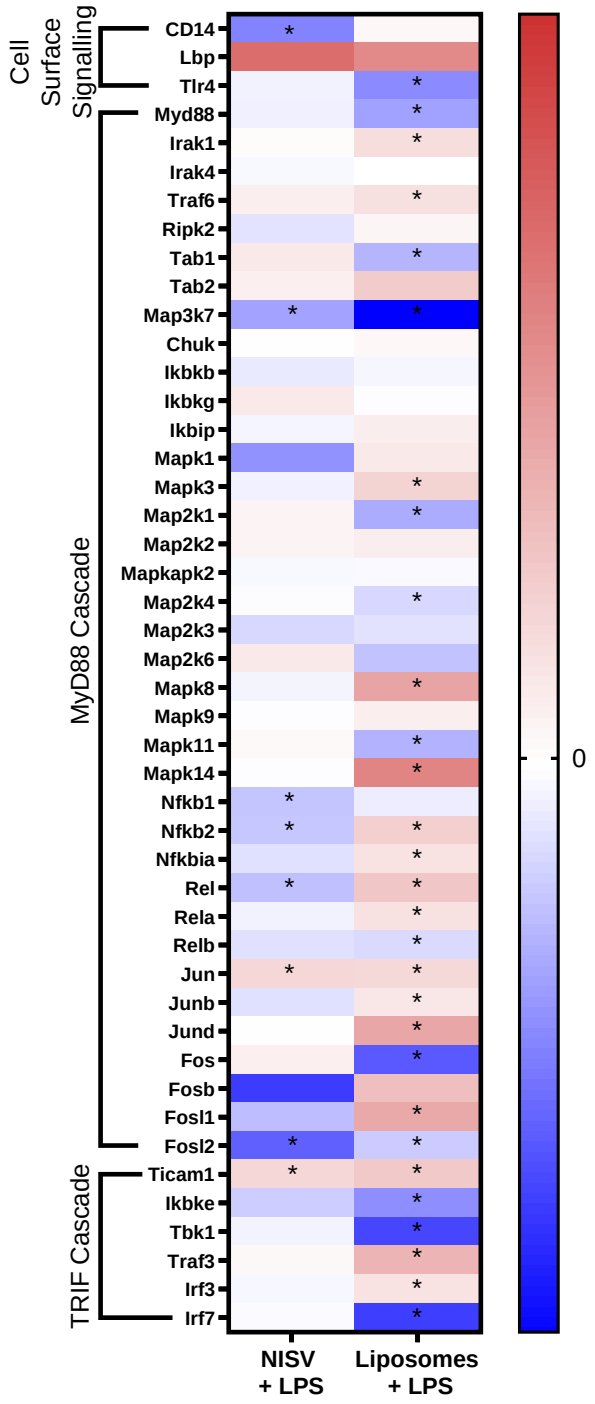


*Figure 4.5.6 NISV Significantly alters NF-κB Expression. Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Heat maps show gene expression involved in LPS signal transduction as they are altered by NISV and liposome treatment in LPS-stimulated cells. Heat maps were made using Prism 8. * indicates significant change. (p<0.05).*

4.5.2 Downstream Effects of NISV LPS Dampening

4.5.2.1 LPS-induced signalling

Seeing the important effects NISV and liposomes have on TLR signalling and particularly NF-κB transcription, investigation of various chemokines, cytokines and receptor expressions was performed to explore the downstream effects of the changes made to TLR4 signalling. Firstly, we investigated the effects on chemokines (Figure 4.5.7). NISV and liposomes were found to affect the expression of chemokines and their receptor subfamilies: CC, CXC, CX3C and XC. Liposomes caused more significant changes than NISV and these changes were more extreme with a Log2 fold change range of -6.78 to 1.25, whereas NISV-induced affects in chemokine expression fell between -0.94 and 0.72. All but one of NISV-induced significant changes were downregulatory. NISV downregulated LPS-induced chemokine expression rather than receptor expression. NISV did significantly upregulate CCR4 transcription in LPS stimulated BMDMs. Liposomes were found to significantly downregulate the expression of 23 chemokines and receptors and significantly upregulate the expression of 4 genes.

4.5.2.2 LPS-induced cytokine and associated receptors and proteins

NISV and liposome effects on LPS-induced interleukin expression are shown on Figure 4.5.8. Liposomes have a great effect on gene expression, when compared to NISV. We see ranges of Log2 fold change from -1.98 to 0.76 with NISV and -4.18 to 1.36 with liposomes. Notably, the genes which NISV significantly downregulated, included IL-1a, IL-1b, IL-6, IL-10, and IL-12, which are typically upregulated in inflammatory diseases. Liposomes, once again, show significant effects on a greater breadth of transcripts in LPS-stimulated BMDMs than NISV. Liposomes affected expression of almost all interleukin transcripts, showing both upregulation (*IL1bos, Il1f6, Il1f9, Il1r1, Il1rn, Il4, Il4i1, Il6, Il7r, Il12b, Il15ra, Il18, Il18bp, Il23r* and *Il27*) and downregulation (*Il1r2, Il6st, Il7, Il10, Il16* and *Il17r*) of specific transcripts.

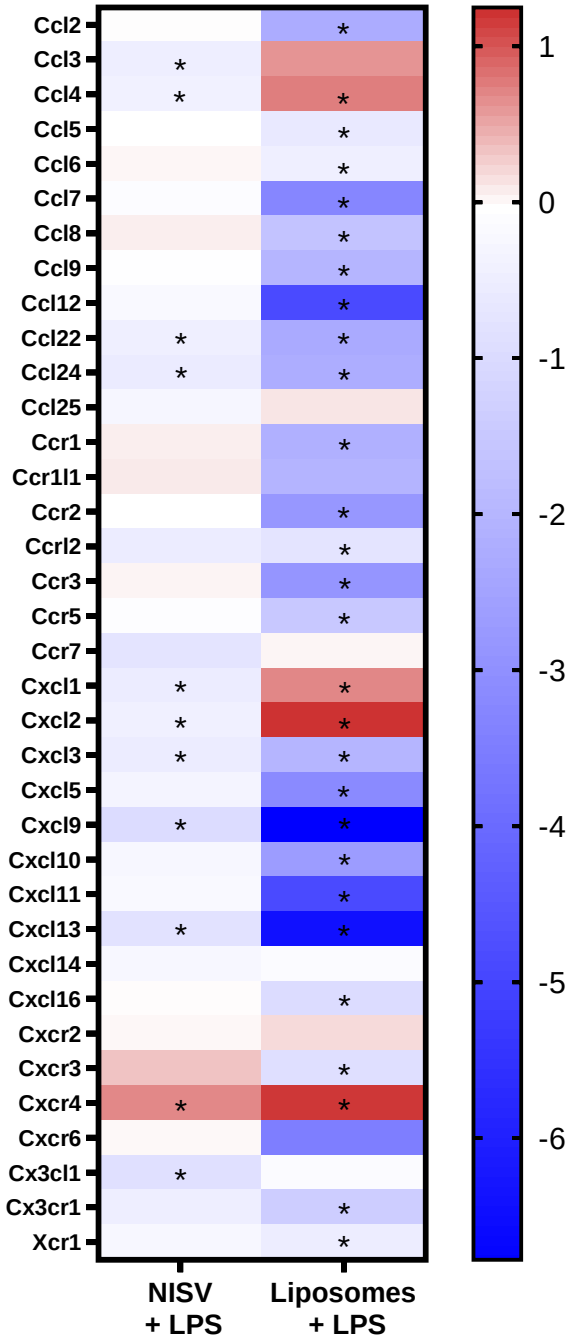


Figure 4.5.7 Chemokines Downregulated by Vesicle Treatments. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Shown are the effects of NISV and liposomes on the genes responsible for chemokine production in LPS-stimulated BMDM. Heat maps were made using Prism 8. * indicates significant change. ($p<0.05$).*

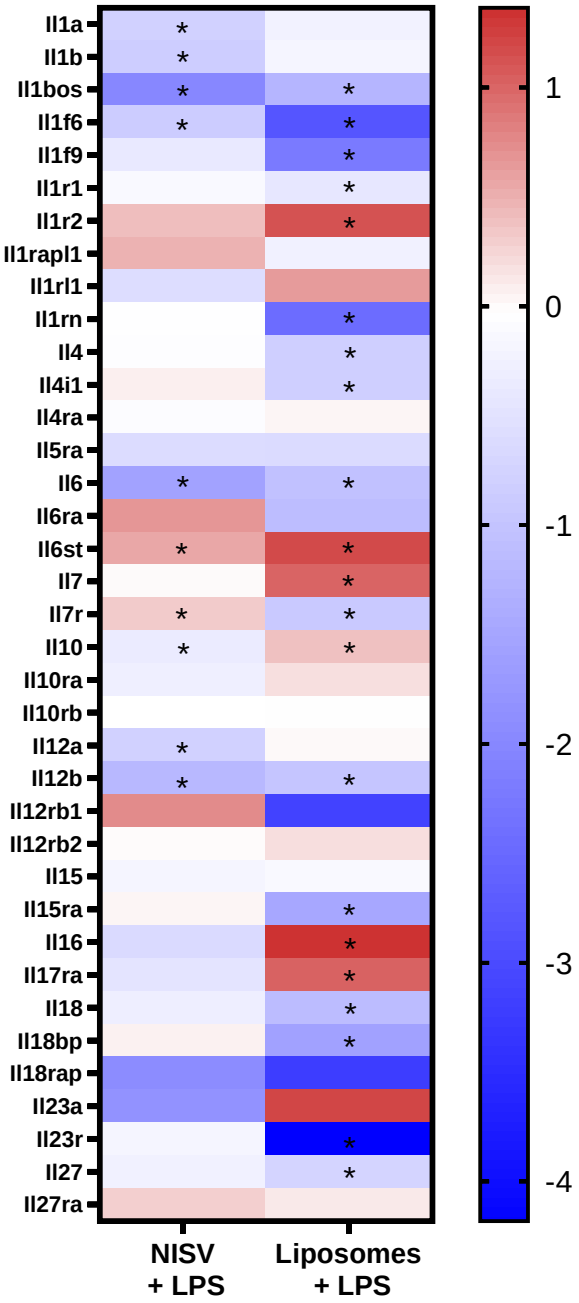


Figure 4.5.8 Vesicle Treatments Differentially alter Interleukin Expression. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Shown are the effects of NISV and liposomes on the genes responsible for interleukin production in LPS-stimulated BMDM. Heat maps were made using Prism 8. * indicates significant change. ($p<0.05$).*

NISV and liposomes were found to affect LPS-induced expression of TNF-α and related genes. These were analysed and shown in Figure 4.5.9. NISV were found to significantly downregulate LPS-induced genes of this family, including *Tnf* (TNF-α), TNF induced protein expression of *Tnfaip2* and *Tnfaip3* and superfamily members *Tnfsf4*, *Tnfsf9* and *Tnfsf15*. Liposomes were mostly pro-inflammatory and upregulated 14 genes of the TNF superfamily and downregulated of 7 genes.

The effect of NISV on other cytokines including growth factors, differentiation effectors and co-stimulatory molecules are shown in Figure 4.5.10. NISV had minimal effects on this group of immune effectors in LPS-stimulated BMDMs and of these, only significantly downregulated CD80 and G-CSF (*Csf3*) expression. Liposomes demonstrated broad effects in transcription of these genes in LPS-stimulated BMDMs where they significantly altered all but two of the measured genes. Specifically, liposomes downregulated G-CSF and its receptor, interferon-γ receptor 2 and TGF-β induced protein (Tgfbi), but upregulated CD80, M-CSF (*Csf1*), GM-CSF (*Csf2*), the interferon-γ receptor 1, TGF-β1 and its receptor, TGF-β3 and TGF-β2 receptor.

The effect NISV and liposomes on toll-like receptors and cell selected surface markers of LPS-stimulated BMDMs are shown in Figure 4.5.11. NISV significantly downregulated TLR2 and upregulated TLR13. Liposome significantly downregulated 9 of the 11 TLR genes detected by transcriptomics.

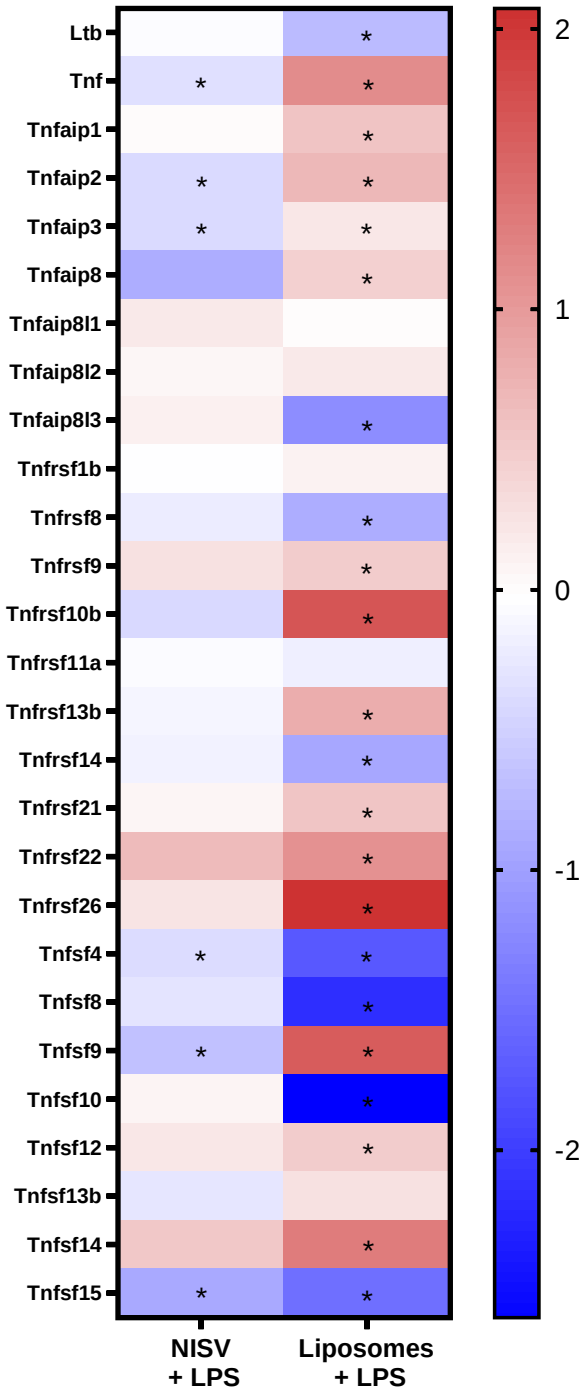


Figure 4.5.9 TNF Expression affected negatively by NISV. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Shown are the effects of NISV and liposomes on the genes responsible for TNF-α and its superfamily production in LPS-stimulated BMDM. Heat maps were made using Prism 8. * indicates significant change. (p<0.05).*

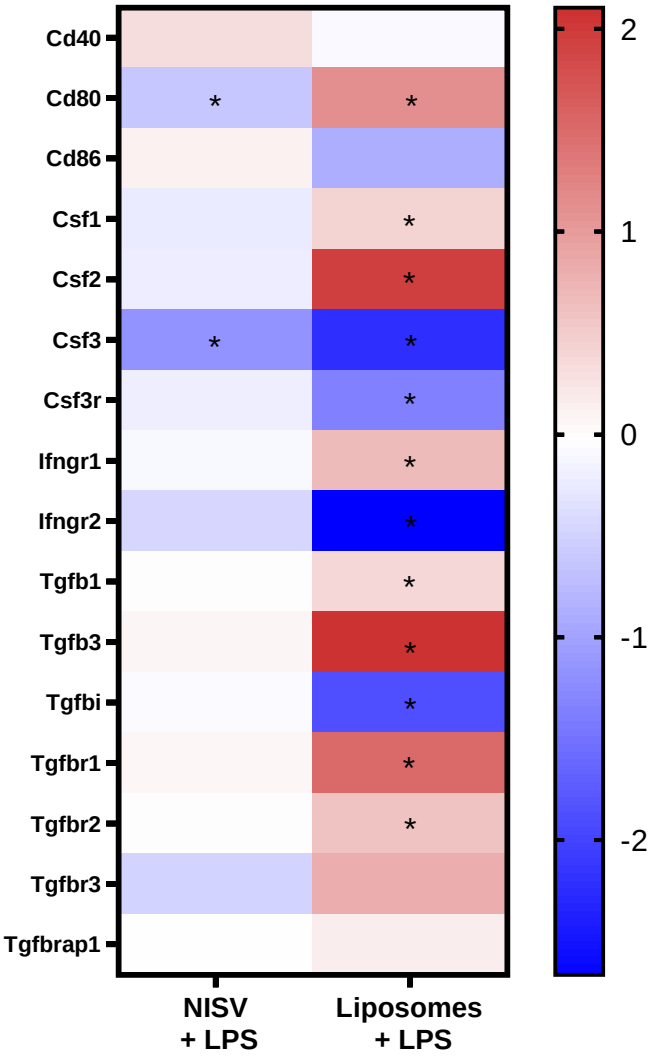


Figure 4.5.10 NISV and Liposomes differentially affect Costimulatory Molecules and Differentiation Factors. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Shown are the effects of NISV and liposomes on the genes responsible for co-stimulatory molecule and differentiation factor production in LPS-stimulated BMDM. Heat maps were made using Prism 8. * indicates significant change. (p<0.05).*

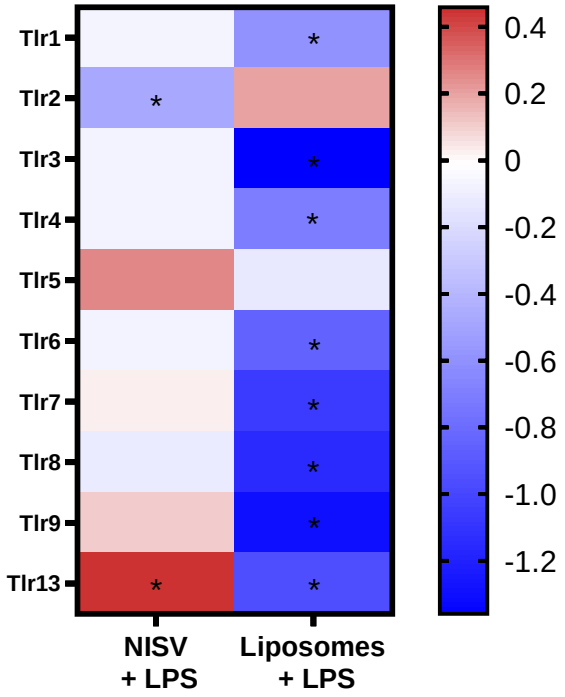


Figure 4.5.11 TLR Expression Significantly Downregulated by Liposomes. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Shown are the effects of NISV and liposomes on the genes responsible for toll-like receptor production in LPS-stimulated BMDM. Heat maps were made using Prism 8. * indicates significant change. ($p<0.05$).*

The effect of NISV on the ‘defense response’ category, as identified by GOrilla was widely covered in the previous figures but the genes that were not discussed are shown in figure 4.5.12. ‘Defense response’ was identified as the group of transcripts most downregulated by NISV treatment of LPS stimulated BMDMs. This is consistent with the expected anti-inflammatory effects of NISV on the LPS-mediated response (and this analysed in the targeted analysis section). However, additional genes in this broad definition were affected by NISV treatment outside of those associated with the LPS-mediated response (Figure 4.5.12). Notably, NISV were found to downregulate NOD and Notch signalling and NLRP3 a component of the inflammasome. NISV also upregulated LPS induced CD180.

This targeted analysis of the immune response gave useful information on wider effects of NISV on the immune system as well as expanding our knowledge of the many effects of liposomes on the transcriptome.

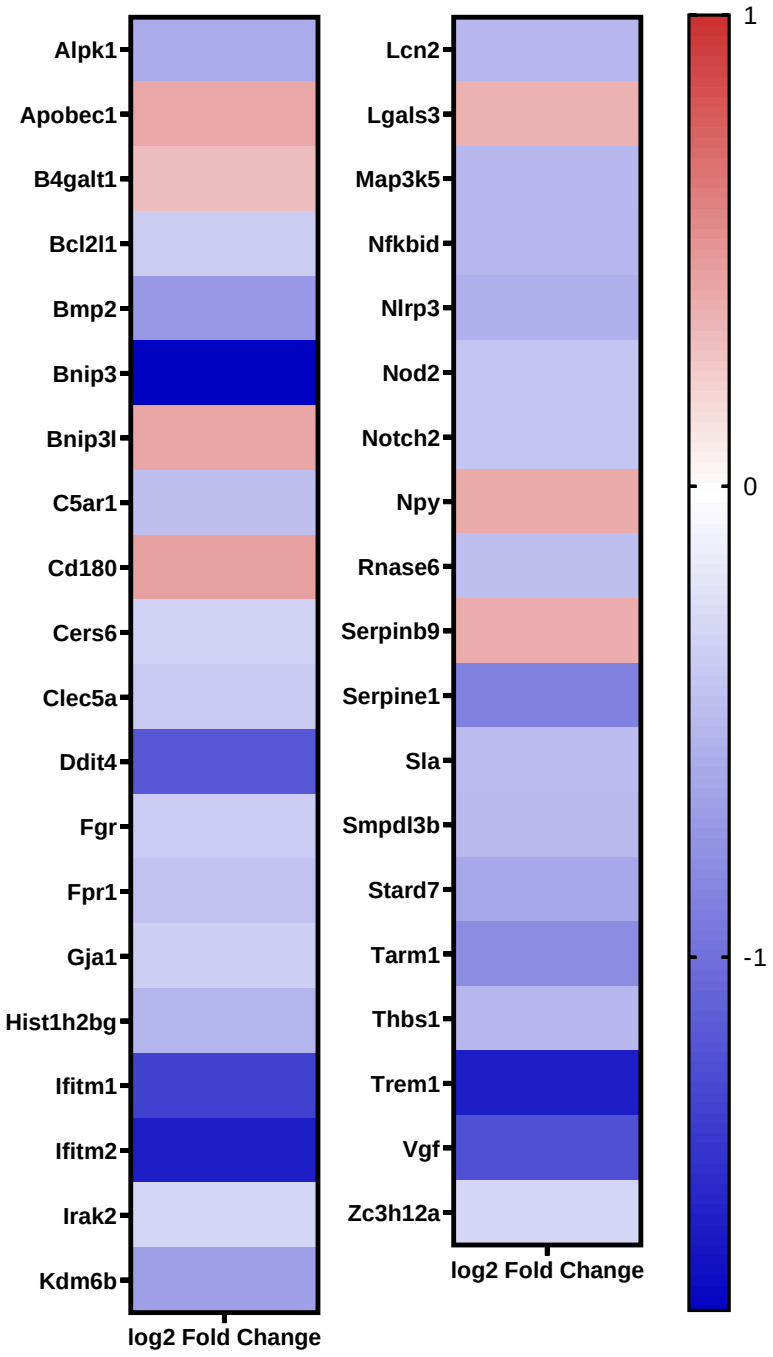


Figure 4.5.12 LPS Response Significantly Downregulated by NISV. Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Heat map produced using Prism 8 shows genes significantly altered by NISV in LPS-stimulated BMDM, in a group identified by GOrilla as defence response. ($p<0.05$).

4.6 NISV affect Sphingosine-1-Phosphate Transcription

Prior to this chapter, in Chapters 3 & 4, NISV have been postulated to effect sphingosine-1-phosphate-related functions due to the structural similarity of MPG with S1P. Using transcriptomics we had the opportunity to delve further into this hypothesis and either support or refute this hypothesis.

With transcriptomic analysis, the effects of NISV on various genes related to sphingosine production and function were examined. In figure 4.7.1, we have previously shown NISV significantly affect *Sgpl1* expression in LPS stimulated cells and wanted to further analyse transcripts related to sphingosine production. NISV caused notable changes in S1P metabolism in unstimulated cells including upregulation of *Cers6, Psap* and *S1pr2* downregulation of *Cers4, S1pr1* and *Sgpp1*. NISV increased levels of *Psap* and *S1pr2* and decreased levels of *Cers6* transcripts in LPS-stimulated cells (Figure 4.6.1).

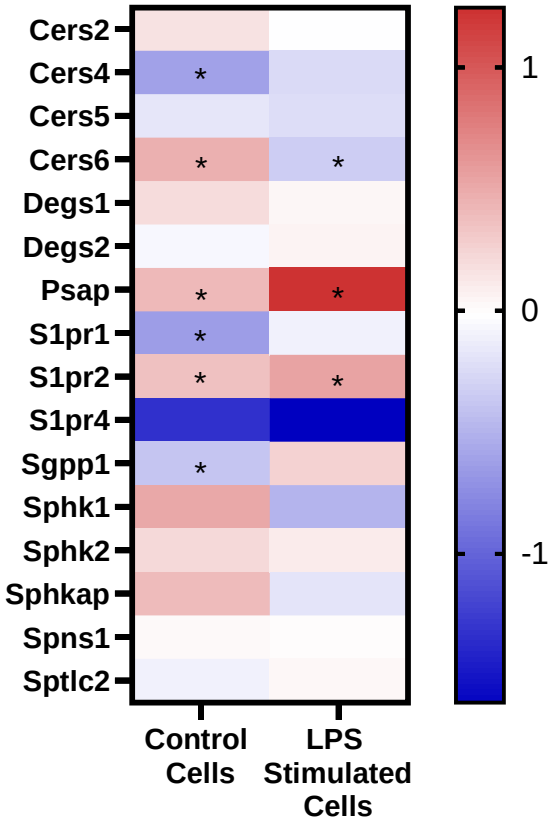


Figure 4.6.1 NISV Significantly alter Expression related to Sphingosine Production. *Transcriptomic analysis was performed on RNA extracted from BMDM stimulated with LPS and treated with 1.5mM vesicle formulations NISV and liposomes, and the relevant controls, unstimulated, NISV alone and liposomes alone. Heat maps, produced using Prism 8, showing genes related to sphingosine production as they are affected by NISV treatment in unstimulated cells and LPS-stimulated cells. * indicates significant change. ($p<0.05$).*

4.7. Discussion

The studies described in this chapter have used transcriptomic analyses to expand upon the effects of NISV on resting BMDMs and BMDMs stimulated with LPS. These data provide mechanistic insight into how NISV mediate anti-inflammatory effects on BMDM, but paradoxically can also function as an adjuvant.

4.7.1 Insight into how NISV mediate their anti-inflammatory effects

Initial studies confirmed the anti-inflammatory effects of NISV on LPS-stimulated BMDM through comparison of the gene products analysed in the cytometric bead array results. NISV were confirmed to downregulate the transcripts for *Ccl22, Csf1, Il6, Il12a* and *Il12b*, all of which products were noted to be downregulated in the cytometric bead array. Transcripts for *Il18*, *Il23a* and *Tgfb1* and their products were not altered by NISV treatment. However, transcripts for IL-1β were downregulated, although the gene product was noted to be upregulated in the cytometric bead array analysis. This phenomenon is likely caused by two factors, the first being the transient nature of RNA and its post-transcription fate, the second being the accumulation of cytokines is not accounted for by the snapshot of cellular expression transcriptomics gives. The combination of these datasets demonstrate the advantages and limitations of a transcriptomic investigation. While transcripts are a good indication of protein production, biological testing must be performed to measure gene products in order to confirm hypotheses derived from the transcriptomic datasets.

The use of LPS as a stimulant in this study allowed a focused analysis into signalling events in response to TLR4 activation of BMDM and subsequent down-stream events. While NISV were found to generally downregulate transcripts for TLR4 mediated MyD88 dependent signalling, liposomes were found to upregulate many transcripts involved in this pathway. NISV also reduced CD14 significantly, which could have a negative effect on LPS recognition and so reduce the cells reaction to this stimulant (Tsukamoto et al, 2010; Wright et al, 1990; Zanoni & Granucci, 2013). Further down-stream in this signalling cascade, NISV downregulated transcripts for *Fosl2* a critical component of the AP-1 transcription factor. Furthermore there is clear down-regulation of transcripts for many NF-κB subunits in LPS-stimulated BMDM. Together these effects should have profound anti-inflammatory consequences as these transcription factors regulate the expression of many pro-inflammatory systems (Gilmore, 1999; Perkins, 2007). Notably, a reduction in NLRP3 transcript levels was observed following treatment of LPS-stimulated BMDM with NISV. Liposomes have a wide-

ranging effect on this pathway, while they downregulated TLR4 and MyD88 transcripts, they upregulated MAP3K signalling showing an increase in end products ERK1, JNK and p38 as well as an overall upregulation of AP-1 and NF-κB subunits. Increased levels of these transcription factors would be predicted to induce rather than ameliorate inflammation. The TRIF signalling cascade, while unaffected by NISV, was affected variably by liposomes showing eventual upregulation of transcripts for the IRF3, but downregulation of IRF7 transcription factors. Overall liposomes upregulate many aspects of TLR4 signalling inducing a pro-inflammatory response. This is broadly consistent with the ability of liposomes to upregulate the production of many LPS-induced cytokines including IL-10, IL-12 and TNF-α as seen in the cytometric bead array.

Further analysis of various immune effectors encompassing both chemokines and cytokines supports the hypothesis that NISV mediates their effects through modulation of NF-κB and AP-1 transcription factors. In NISV-treated, LPS-stimulated BMDM all significantly affected genes are downregulated other than CXCR4. Transcripts for the macrophage chemoattractant, CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL2, CXCL3 and CX3CL1, are all significantly downregulated. This would indicate a potential negative effect on macrophage recruitment caused by NISV treatment (Cocchi et al., 1987; Smith, Galkina, Ley, & Huo, 2005). Other downregulated chemokine transcripts are related to leukocyte (CCL22), T Cell (CCL24) and neutrophil (CXCL1) attraction and so NISV also show the potential to reduce recruitment of these immune cells and by proxy the immune response (Nakayama et al., 2004; Patel et al., 1997). The vast majority of the chemokines affected are known to be regulated by NF-κB (CCL3, CCL4, CCL22, CXCL1, CXCL3 and CX3CL1) and/or AP-1 (CXCL9) and thus further supports the downstream effect of NISV downregulation of NF-κB and AP-1 signalling.

Liposomes predominantly downregulated chemokine expression in LPS-stimulated BMDM, with the exception of CCL4 (MIP-1β), CXCL1 CXCL2 and CXCR4. These upregulated chemokines are macrophage (CCL4 & CXCL2) and neutrophil chemoattractants (CXCL1) and CXCR4 the receptor for CXCL12 (Blackwell et al, 2001; Wolpe et al., 1989). The upregulation of some of these chemokines and their receptors is consistent with the observation that liposomes increased NF-κB transcripts in LPS-stimulated BMDM. However, the reason for the downregulation of some chemokines and receptors is not clear, but likely indicates at least some of liposome-mediated

effects are outside TLR-4-induced signalling events. Ultimately, further studies that carefully dissect signalling events will be necessary to confirm findings and explore hypotheses generated from the transcriptomic dataset.

As previously mentioned, NISV significantly downregulated transcripts for NF-κB dependent cytokines (*Nfkb1, Nfkb2* and *Rel*) in LPS-stimulated BMDM. Liposomes downregulated transcripts for pro-inflammatory interleukins IL-4, IL-6 and IL-12p40 and upregulated transcripts for IL-16 and the anti-inflammatory IL-10 in LPS-stimulated BMDM. The effects on IL-10 and IL-12p40 were consistent with protein levels measured in the cytometric bead array. However, IL-6 was upregulated by liposomes in the bead array and ELISA. Importantly, TNF-α protein levels were upregulated in LPS-stimulated BMDM treated with liposomes (consistent with transcript modulation). However, TNF-a protein levels were unaffected in NISV treated LPS-stimulated BMDM although a decrease in their transcripts was observed. These data demonstrate that modulation of transcript levels is not always consistent with protein levels and shows the importance of measuring actual cytokine production. As previously stated, liposome effects on the transcription factors induced by TLR4 signalling were inconsistent and this data on chemokines and interleukins strengthens the hypothesis that liposome have effects outwith TLR4 signalling.

In keeping with the cytometric bead array results that demonstrated NISV induced a significant decrease of G-CSF, transcripts for its gene *Csf3* were downregulated in NISV-treated, LPS-stimulated cells. G-CSF is important in the production of granulocytes and the proliferation and differentiation of neutrophils, consequently a reduction of this cytokine would inhibit these effects reducing the inflammatory capabilities of granulocytes and neutrophils (Roberts., 2005). In comparison to the bead arrays results, liposomes effects on transcription of growth factors does not line up with actual protein production. Liposomes downregulated *Csf3* transcripts, but upregulated its gene product G-CSF in the cytometric bead array in LPS-stimulated BMDM. Liposomes also upregulated of *Tgfb1* transcripts but downregulated TGF-β1 protein production in LPS-stimulated BMDM. This again emphasises the importance of confirming transcriptomics results by protein measurement. The transcripts of these growth and differentiation mediators indicate a mixture of pro and anti-inflammatory effects caused by liposomes the consequences of which cannot be deduced without further experimentation.

In TLR transcription, NISV were found to downregulated TLR2 only, which is regulated by NF-κB (Johnson & Tapping, 2007). This effect would reduce the response to Gram-positive organisms adding to the effects found in TLR4 signalling.

In chapter 3 liposomes were shown to significantly increase BMDM cytokine production stimulated by LPS or PolyI:C, but paradoxically transcripts levels of all TLRs, with the exceptions of TLR2 and 5, were reduced by liposome treatment. Discrepancies like these are likely a shortcoming of the transient nature of RNA which is often measured at a single time point. A solution to this and many problems of transcriptomics would be to perform a time series and collate the results, giving a better picture of the physical phenotype the cells take.

4.7.2 Insight into how NISV Function as Adjuvants

Evidence indicating NISV modify the immune status of unstimulated BMDM was identified using GOrilla. This analysis highlighted immune-stimulatory effects of NISV in unstimulated cells. GOrilla almost exclusively identified immune related groupings, with defence response encompassing the majority of groups. The effects of NISV on defence response in unstimulated cells were stimulatory, in direct opposition to their previously discussed anti-inflammatory effects. However, the specific nature of the effects and their magnitude were distinct and less profound than noted in LPS-stimulated BMDM. For example, upregulation of MHC class I (proteasome, cathepsins, TAP transporters) and class II (subunit, activator) related genes showed NISV pro-inflammatory effects were noted in NISV treated BMDM. In direct contrast to their effects on LPS-stimulated cells, NISV induced an upregulation of NF-κB subunit transcripts as well as many inflammatory mediators governed by this transcription factor including IL-1α, IL-1β and TNF-α genes as well as the genes of many chemokines. The costimulatory molecule, CD40, was also found to be upregulated in unstimulated cells treated with NISV. Overall these effects provide evidence supporting previous studies that have used NISV as vaccine adjuvants, despite their apparent anti-inflammatory effects within LPS-stimulated cells (Brewer & Alexander, 1992; Hassan et al, 1996).

NISV induced significant changes in Golgi and sorting nexin transcripts, indicating uptake could be controlled by sorting endosomes and Golgi trafficking but confirmation would require further experimentation.

4.7.3 NISV Effects on Sphingosine Transcription

Finally, as previous results have led to the hypothesis that NISV exert their anti-inflammatory effects through interfering with sphingosine-1-phosphate synthesis, signalling and/or transport, we investigated levels of transcript for the genes involved. Specifically, we wish to explore the hypotheses that: (i) MPG is an antagonist of the S1P receptors, or (ii) MPG is a sphingosine kinase inhibitor or subversive substrate. In unstimulated conditions, NISV upregulated *Cers6* transcripts but downregulated *Cers4* ceramide synthases, but had no obvious effect on *Cers2* and *Cers5*, making it difficult to draw any immediate conclusion of the likely consequences. NISV also affected transcript levels of sphingosine-1-phosphate receptors, with upregulation of S1PR2 transcripts, which is involved in macrophage and dendritic cell activation and downregulation of S1PR1 transcripts which is involved in immune trafficking in macrophages. These results are largely consistent with the adjuvant properties of NISV. NISV also downregulated transcripts for sphingosine-1-phosphate phosphatase (*Sgpp1*) which is involved in S1P degradation. This would result in maintenance of S1P levels. Prosaposin (*Psap*) transcripts were raised in NISV treated BMDM (irrespective of whether they were stimulated with LPS or not). This is a pro-enzyme which is cleaved into saposins, enzymes related to lysozyme function where they catabolise glycosphingolipids (Morimoto et al, 1990). This protein has been hypothesised to sequester lipids and make them more accessible for degradation. Due to the structural similarity of MPG with sphingosine, a likely function of these increased levels of saposins would be the sequestering of MPG for eventual degradation (Ponting, 1994). Also in LPS-stimulated cells we see downregulation of *Cer6* (ceramide synthase 6) indicating a possible reduction in certain types of ceramide synthesis (Lahiri et al., 2007). As in unstimulated cells, NISV induced upregulation in *S1pr2* transcripts. This gene encodes the receptor S1PR2 which, when phosphorylated, activates a cascade culminating in NF-κB inducing a pro-inflammatory response. Its upregulation is contradictory to our previous finding where NF-κB expression was downregulated following NISV treatment of LPS stimulated BMDM. This might support the hypothesis that MPG is an antagonist of the S1P receptors, rather than a sphingosine kinase inhibitor or subversive substrate. These results remain inconclusive neither confirming NISV involvement in S1P synthesis, signalling and/or transport nor ruling it out. Future functional studies will be required to further explore these hypotheses remains.

4.7.4 In Conclusion

Overall, these studies further support the data presented in chapter 3 that demonstrates the anti-inflammatory effects of NISV are not a general property of all vesicular systems. Moreover, the data illustrate that NISV have profound effects on TLR4-mediated signalling events culminating in downregulation of NF-kB and AP-1 transcripts and subsequent gene products. Although liposomes do not share these properties, they do have multifarious effects on cell transcription and immune mediators. The data also provide mechanistic insight into how NISV can function as an adjuvant despite their anti-inflammatory effects in LPS stimulated cells. Notably they upregulate transcripts for MHC 1and 2 processing, the CD40 costimulatory molecule and NF-κB as well as a number of down-stream cytokines important in the development of adaptive immunity. Confirmatory studies will be necessary to substantiate and further mechanistically dissect the hypotheses formed from this transcriptomic data.

5. NISV Effects on Cellular Metabolism

5.1 Abstract

Recent studies have demonstrated that LPS stimulation of macrophages not only induces pro-inflammatory cytokine production, but also induces metabolic changes. Some of these changes in metabolism, notably increased glycolysis and reduced oxidative phosphorylation are believed to facilitate the metabolic demands of the immune response including cytokine production, but others including changes to arginine and tryptophan metabolism are known to have direct immunological functions. In the following studies the effects of NISV consisting of MPG, DCP and cholesterol on BMDM and LPS-stimulated BMDM are examined. NISV had no profound effect on the energy metabolism of BMDM, but induced production of itaconate, creatine and taurine. Importantly, itaconate can have anti-inflammatory effects. In LPS-stimulated macrophages, NISV increased the rate of glycolysis as determined by a reduction in the intermediate glyceraldehyde-3-phosphate, and an increase in pyruvate and lactate. Commensurate with these observations increases in transcripts for key glycolysis-related enzymes were observed. NISV ameliorated LPS-induced production of succinate and itaconate. NISV reduced LPS-induced argino-succinate and Nos2 transcript levels in BMDM. These studies demonstrate that NISV interfere with LPS-induced changes to the metabolome of BMDM. Many of the observed effects of NISV on LPS-stimulated BMDM have the potential to reduce inflammation. Whether these effects mediate the anti-inflammatory actions of NISV or are merely a consequence is not known.

5.2 Introduction

Previous chapters identified immunomodulatory effects of NISV on BMDM utilising a combination of ELISAs, cytometric bead arrays and RNAseq methods. The following studies were performed to characterise the effects of NISV on the metabolism of resting BMDM and LPS-stimulated BMDM. LPS stimulation of macrophages is known to induce a number of changes to their metabolism, some of which are discussed below.

5.2.1 The Warburg Effect

The Warburg effect was first described by, its namesake, Otto Warburg in the 1920s in tumour cells. In simple terms the Warburg effect is defined by high glucose uptake, aerobic glycolysis and lactate secretion as well as reduced oxidative phosphorylation (OXPHOS) (Warburg, 1925). The inflammatory profiles of immune cells has been

studied since the discovery that in inflammatory situations, neutrophils depend on the “Warburg effect” (Racker, 1972). Similar observations have now been documented in a number of other cell types including macrophages and dendritic cells (Hargrave et al., 2019).

Aerobic glycolysis is relatively inefficient compared to OXPHOS in the generation of ATP but, as it is accompanied with changes to the TCA cycle, it ultimately results in increased production of a number of biosynthetic intermediates, which are essential for cell proliferation, cytokine production and the inflammatory response (Krebs & Johnson, 1980; Vander Heiden et al, 2009). In a pro-inflammatory M1 macrophage, glycolysis provides substrates for the pentose phosphate pathway (PPP) and TCA cycle (Viola et al., 2019). Glucose-6-phosphate is directed to the PPP where it is converted to ribose-6-phosphate, which is used to reduce $NADP^+$ to NADPH this is then used in the generation of reactive oxygen species (ROS). Glycolysis also provides pyruvate for the TCA cycle, but in a pro-inflammatory reaction the TCA cycle is modified to produce and promote pro-inflammatory mediators.

The alterations to the TCA cycle disables its energy output instead producing three mediators: citrate, itaconate and succinate. In the canonical TCA cycle citrate is sequentially converted through many forms, as shown on figure 5.2.1, producing energy for cellular function and some intermediates for biosynthetic processes. However, in activated M1 macrophages, where oxidative glycolysis is induced, and the TCA cycle remodelled, citrate can be exported into the cytosol. In the cytosol citrate is essential for three inflammatory response mechanisms, nitric oxide (NO), reactive oxygen species (ROS) and prostaglandin E2 production. This is because citrate is required for conversion into acetyl CoA, feeding prostaglandin synthesis, and oxaloacetate, feeding NADPH production which is necessary for ROS and NO production (Infantino et al, 2013). Cis-aconitate is converted into itaconate rather than isocitrate in M1 activated macrophage. Itaconate has a number of immune functions. During the pro-inflammatory response, itaconate is produced for its bactericidal effects against gram-negative organisms as it has been shown to inhibit bacterial isocitrate lyase. Itaconate has also been shown to have anti-inflammatory effects on macrophages (Berg, Filatova, & Ivanovsky, 2002). Itaconate inhibits nuclear factor erythroid 2-related factor 2 (NRF2), through degradating of Kelch-like ECH-associated protein 1 (KEAP1) which would normally signal NRF2 for degradation itself. In this way itaconate increases NRF2 levels, which upon translocation to the nucleus induces transcription

of genes involved in protection against apoptosis and oxidative stress. Itaconate is also an inhibitor of succinate dehydrogenase (SHD) which leads to accumulation of succinate (O'Neill & Artyomov, 2019). High levels of succinate increases binding to the succinate receptor SUCNR1, beginning a signalling cascade culminating in up-regulation of IL-1β and maintaining the pro-inflammatory phenotype. Within the cell succinate functions as a stabilizing element for hypoxia induced factor 1 alpha (HIF1α) by inhibiting prolyl hydroxylases (PHD), this transcription factor regulates glycolytic enzymes and pro-inflammatory mediators including; IL-1β, IL-6 and TNF-α. HIF1α's function supports glycolysis and promotes the inflammatory phenotype of the M1 macrophage (Tannahill et al., 2013).

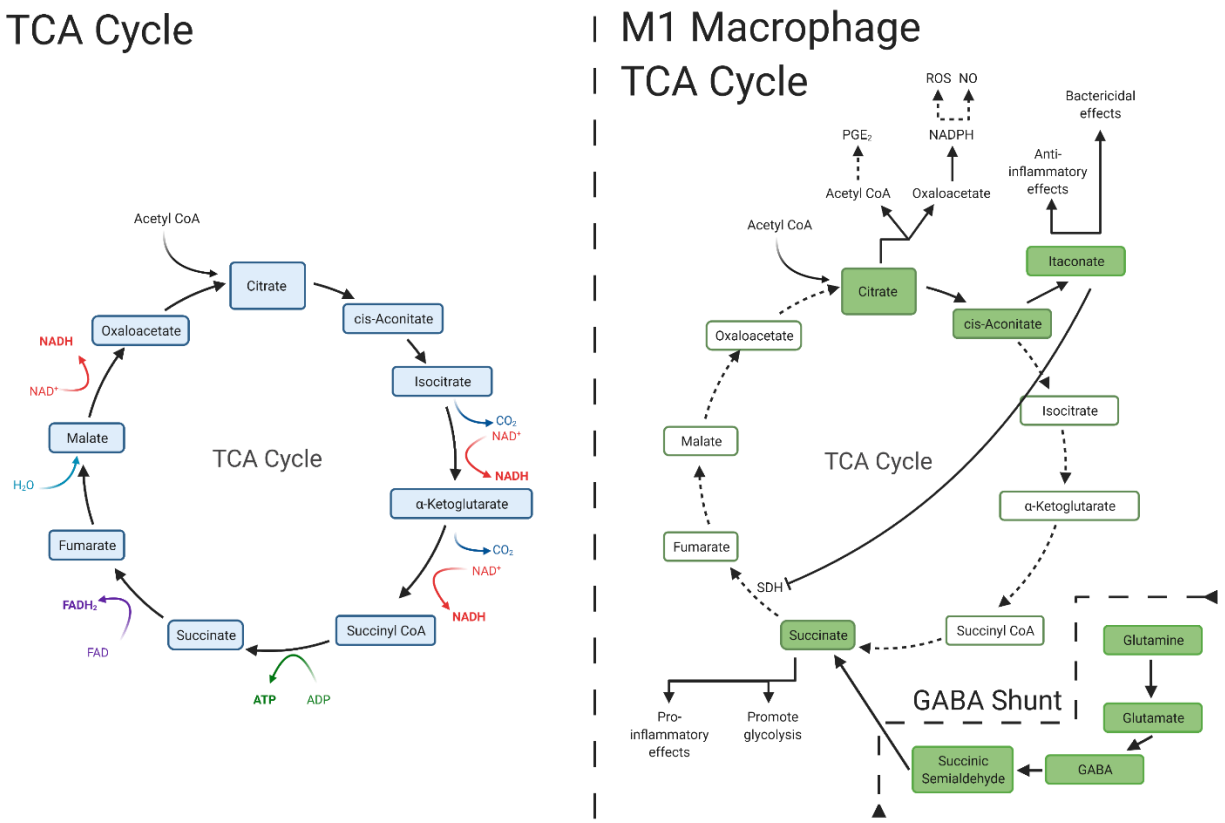


Figure 5.2.1 The Traditional TCA Cycle & the Activated Macrophage's TCA Cycle. *Shown is the normal function of the TCA cycle (left) producing energy for cellular use and the TCA cycle of the M1 Macrophage (right) in which the TCA cycle is seized to produce the bioactive metabolites; citrate, itaconate and succinate. Created with BioRender.com.*

5.2.2 Amino Acid Metabolism & Inflammation

Inflammation is greatly affected by glycolysis and the TCA cycle, but these two pathways also provide precursors and have the potential to metabolise a number of amino acids. Some of these amino acids or their metabolites sustain the inflammatory response providing precursors or even regulate the immune response. Others are manipulated by the immune response to result in antimicrobial effects.

The amino acid glutamine is essential in the activated macrophage, sustaining the alternate TCA cycle function, the 'GABA' shunt (Figure 5.2.1). Due to the activities of citrate and cis-aconitate in M1 macrophages the TCA cycle is stunted and because of this glutamine is funnelled into the TCA cycle via the GABA shunt. In this process, glutamine is converted into glutamate, then γ-aminobutyric acid (GABA), succinic semialdehyde and finally succinate. This allows succinate production despite the dysfunctional TCA cycle, meaning succinate can exert is pro-inflammatory mediation. In M2 macrophages glutamine supplements normal TCA function being converted into α-ketoglutarate. With this, α-ketoglutarate promotes prolyl hydroxylases (PHD) function inhibiting HIF1α expression, in direct opposition of succinates function in M1 macrophages (Tannahill et al., 2013).

Nitric oxide production is controlled by the fate of arginine metabolism, which can be directed in two ways depending on the immune response and can have direct effects on macrophage polarisation. In pro-inflammatory M1 macrophages, arginine metabolism is directed into citrulline and NO production by iNOS, boosting the antimicrobial abilities of the macrophage. NO also prevents M1 macrophages from repolarising into the M2 phenotype by blocking OXPHOS (Van den Bossche et al., 2016). NO is produced when arginine is converted into citrulline by iNOS, but citrulline is then converted into arginosuccinate which is broken down into arginine, forming the nitric oxide cycle, allowing constant recycling of arginine and thus sustained NO production. M2 macrophages direct arginine to be converted into ornithine through the overexpression of arginase 1 (ARG1). Ornithine is then directed into polyamine synthesis, promoting tissue repair and controlling cell growth (Viola et al., 2019). Notably, the availability of arginine has been shown to affect immune cell proliferation and the growth of certain microbes such as *Toxoplasma gondii* (Werner et al., 2017).

Metabolism of tryptophan can deprive auxotrophic microbes of this essential amino acid and has been reported to limit T cell proliferation in some circumstances (Edinger & Thompson, 2002). Furthermore, metabolism can also dampen the pro-inflammatory

response by upregulating kynurenine production. Indoleamine 2,3-dioxygenase (IDO) converts tryptophan into kynurenine and in macrophages IDO overexpression is induced by pro-inflammatory mediators such as IFN-γ and TNF-α. Kynurenine acts as an anti-inflammatory regulator of T cells and dendritic cells (DCs) by acting upon the aryl hydrocarbon receptor (AHR) (Opitz et al., 2011). In T_{reg} cells AHR is responsible for the downregulation of pro-inflammatory transcription factor HIF1α and upregulation of the anti-inflammatory cytokine, IL-10. In the pro-inflammatory T_H17 cells AHR induces the differentiation of the cell into the immune supressing T_{reg} cells. DCs too undergo an inflammatory transformation, with ARH down-regulating cytokines, IL-6 and IL-12, as well as the receptors, CD40 and MHC class II (Opitz et al., 2011; Rothhammer & Quintana, 2019).

5.2.3 The Effects of Metabolism in Disease

With the complexity of immunometabolism, its dysfunction can lead to major illness and infectious agents and cancerous cells can further exacerbate this dysfunction.

In cancers, tumour associated macrophages (TAMs), M2d macrophages, which express a number of metabolites and cytokines which control the inflammatory environment in the associated area. To manipulate the metabolism of the local macrophage population, TAMs overexpress and export IDO leading to upregulation of kynurenine in the non-tumour associated macrophages which, in turn, causes the suppression of T cell activation and imposing an anti-inflammatory profile on dendritic cells. Another anti-inflammatory measure taken by TAMs, and bacterial agents, is the overexpression of ARG1 (Zhou et al., 2020). This leads to the conversion of arginine into ornithine and urea, as opposed to citrulline and NO, promoting further the anti-inflammatory phenotype in the tumour-surrounding environment protecting the tumour from the immune system.

The ability to control macrophage metabolism is useful in many ways. Understanding the effect NISV have on these complex processes is important if they are to be further developed into a therapeutic, but could also provide insight into their mechanisms.

5.2.4 Aims & Hypothesis

The aims of this chapter are to investigate the effects of NISV comprised of MPG, DCP and cholesterol on the metabolism of BMDMs and determine how NISV alter the immunometabolomic changes induced by LPS in these cells. This will be achieved through the use of LCMS to detect metabolites and cross-interrogation of the RNAseq data obtained in the previous chapter to determine key enzyme transcript levels. Specifically, studies will:

i. Use a targeted approach to determine the effects of NISV on metabolic processes known to be important in immunometabolism including, glycolysis and the TCA cycle, arginine, tryptophan, and glutamine metabolism.
ii. Use a non-targeted approach to identify unique effects of NISV on BMDM metabolism and analyse their function within or external to the spectrum of the immune system. This will be done utilising broad system analyses to identify systems of interest. After which their metabolic pathways will be analysed. This should potentially provide insight into the effects of NISV on novel aspects of immunometabolism and effects they have other than those linked directly to immunity.

5.3 Metabolomics Analysis

Following confirmation inflammatory modulation was achieved in the samples (through use of an ELISA for IL-6 and a cytometric bead array), metabolite extracts were sent to Glasgow PolyOmics for LCMS/MS analysis. The raw data was processed using Glasgow Polyomics PiMP pipeline. The samples metabolic profiles were then compared using SIMCA 14.1. This analysis was performed to find if the samples were significantly different and to find the metabolites which contribute most to their differences.

Principle component analysis (PCA) was performed, figure 5.3.1, and shows the variation within and between the groups tested. Surprisingly the largest variation within groups was in the control, which did not show close grouping in PCA. The unstimulated groups with NISV treatment shows tight grouping, as did the LPS stimulated group. NISV are most closely related to NISV+LPS. The LPS stimulated cells show a distinct separation from all other groupings.

Following PCA of all groups, focussed comparisons between specific groups using orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out. With this analysis distinct groups were found between the test groups LPS and NISV+LPS and were able to identify the metabolites which contribute most to differences between these groups using a variable importance in projection (VIP) plot. The OPLS-DA of LPS and NISV + LPS further demonstrates the separation between these groups and from this OPSL-DA a VIP plot was produced showing the metabolites contributing most to the difference between these groups (figure 5.3.2.A). The most notable of the metabolites identified on the VIP table are involved in the TCA cycle (itaconate), and arginase metabolism (glutamine, proline, arginine, ornithine). Other important metabolites identified as contributors to the difference between LPS stimulated cells and NISV treated stimulated cells are creatine, taurine, phenylalanine and pyridoxine (vitamin B6). Following this global analysis of the metabolome, a more targeted analysis was performed.

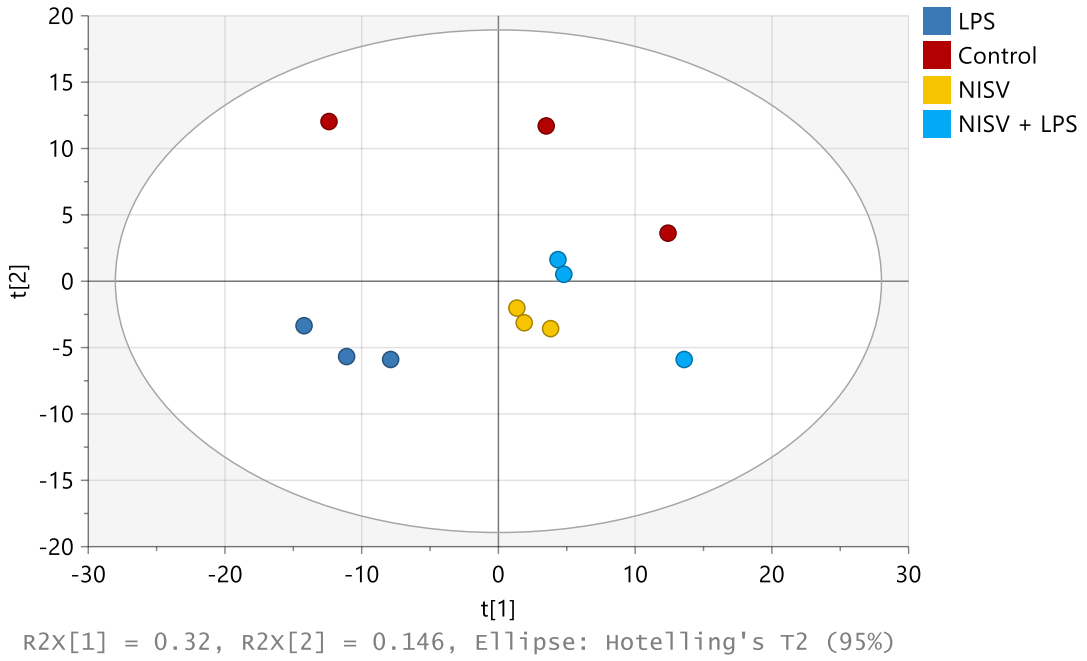
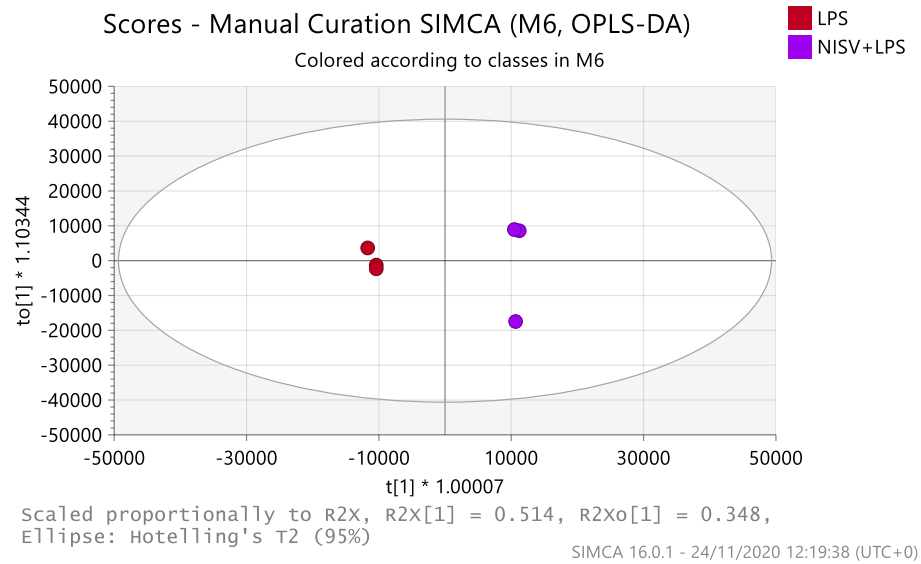


Figure 5.3.1 Principle Component Analysis of Metabolic changes shows grouping based on Treatment but not Stimulation. *100,000 BMDM were plated per well in triplicate and were stimulated using 3µg/ml LPS or left unstimulated; these were then treated with 1.5mM NISV. After 24 hours supernatants were removed and cells were subjected to metabolite extraction, metabolites were then quantified by LCMS/MS. The data was analysed using SIMCA and a PCA plot was produced.*

A.



B.

Metabolite	Log2(fold change)	P value
L-Glutamine	0.285582	0.347058
sn-glycero-3-Phosphocholine	-2.59386	0.000712
Creatinine	0.786498	0.004804
L-Proline	0.468663	0.013492
L-Arginine	0.336757	0.030306
Creatine	0.213624	0.0223
5-Oxoproline	0.485047	0.018098
Betaine	0.386035	0.016770
Hippuric acid	3.735264	0.000217
Orthophosphate	-0.09978	0.574692
Taurine	-0.65785	0.005122
(S)-Lactate	0.263793	0.0073
Choline phosphate	-2.85697	0.000438
L-Leucine	0.395989	0.157751
Phenylalanine	0.445063	0.074868
Pyridoxine	0.664145	0.054054
L-Lysine	0.693138	0.020736
L-Histidine	0.321468	0.006410
Itaconate	-0.91859	0.003931
Formylanthranilate	-0.56266	0.101175
O-Acetylcarnitine	-0.65985	0.030627
L-Valine	0.493785	0.019321
L-Glutamate 5-semialdehyde	0.254943	0.159076
L-Ornithine	0.434059	0.033718
Hypoxanthine	1.162561	0.001015

Figure 5.3.2 NISV Alter LPS Stimulated Macrophages Metabolism. *100,000 BMDM were plated per well in triplicate and were stimulated using 3µg/ml LPS or left unstimulated, these were then treated with 1.5mM NISV. After 24 hours supernatants were removed and cells were objected to metabolite extraction, metabolites were then quantified by LCMS/MS. The data was analysed using SIMCA and an OPLS-DA & VIP plot were produced.*

5.4 Vesicle Treatment & the Warburg Effect

A targeted analysis on specific pathways related to the immune system was performed. Glycolysis and the TCA cycle are essential to macrophage polarisation and by extension the inflammatory profile of a macrophage. The effects of NISV on these processes were analysed to find if NISV induce changes to the Warburg effect in LPS stimulated cells and to investigate the changes cause in unstimulated cells. This analysis used metabolomics data in tandem with transcriptomics data to define the changes treatments made to both the metabolites and the enzymes which catalyse reactions in the pathways.

5.4.1 Glycolysis

The effects NISV on glycolysis are shown on figure 5.4.1, part A shows all the detected metabolites and transcripts related to glycolysis and part B shows those that were significantly affected by treatment with NISV. Levels of the metabolite D-glyceraldehyde 3-phosphate are significantly ($p<0.05$) upregulated in LPS stimulated BMDM consistent with the ability of LPS to induce the Warburg effect. NISV and liposomes had little effect on D-glyceraldehyde 3-phosphate levels administered to BMDMs, but ameliorated LPS-induced levels reducing relative intensity from 520853 to 197948 and 216507. Pyruvate levels were also significantly increased by NISV in both unstimulated and stimulated cells, from a relative intensity of 930236 to 1502884 and 669416 to 1056341. NISV increased lactate levels, but only in LPS-stimulated cells (relative intensity of 34926857 to 41934274), but not in unstimulated cells.

Enzyme transcripts were also affected by NISV treatment. NISV increased transcription of *Aldoart1* in both stimulated (from FKPMs of 775 to 1110), and unstimulated cells (from 966 to 1487). *Aldoa* transcription was increased in LPS-stimulated cells by NISV (FKPM from 0.13 to 255). Triosephosphate isomerase, Tpi1, is increased by NISV in both stimulated (from FKPM 381 to 1189), and unstimulated cells (from 631 to 1186). Pgam1 shows this same phenotype with NISV increasing transcription, from FKPMs of 298 and 235 to 432 and 385 in unstimulated and stimulated cells respectively.

5.4.2 The TCA Cycle

Continuing from glycolysis analysis, analysis of the effect of vesicle treatments on the macrophage TCA cycle through analysis of the metabolites and transcripts related to its processes.

Figure 5.4.2.A shows the detected metabolites and transcripts associated with the TCA cycle and the alternate M1 macrophage TCA cycle. Part B shows the metabolites and transcripts which were significantly affected by vesicle treatment. NISV reduced succinate and itaconate metabolites significantly in LPS-stimulated cells, reducing the relative intensity of succinate from 146453 to 85161 and itaconate from 7017459 to 3712409. In unstimulated cells NISV increased itaconate levels from relative intensities of 139779 to 2670894. Transcription of citrate synthase, *Cs*, was significantly up-regulated by NISV in unstimulated cells, from an FKPM of 55 to 60. Fh1 transcription was reduced in NISV treated unstimulated cells (FKPM of 44 to 33), but not affected in LPS-stimulated cells. Malate dehydrogenase, Mhd2, transcripts were increased by NISV in unstimulated cells (from FKPM from 72 to 78).

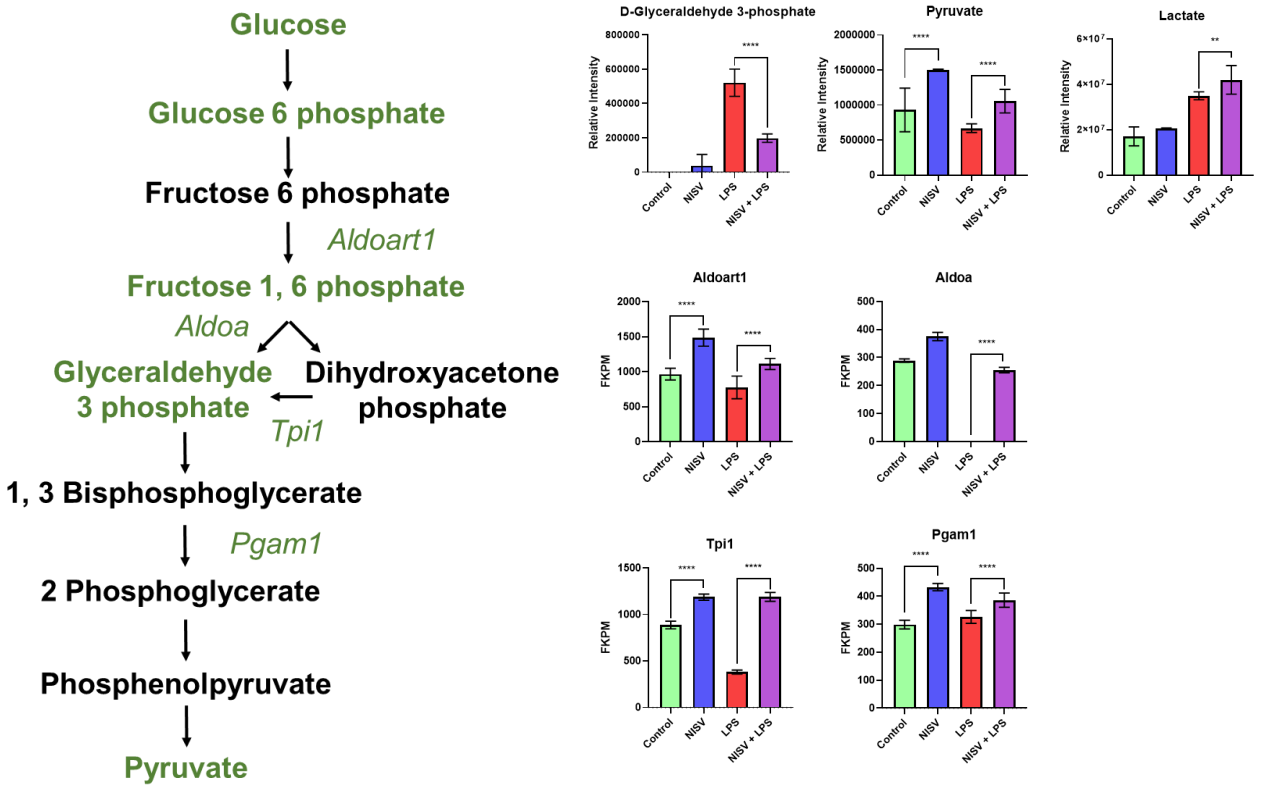


Figure 5.4.1 Vesicles effects on Glycolysis. *100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05).*

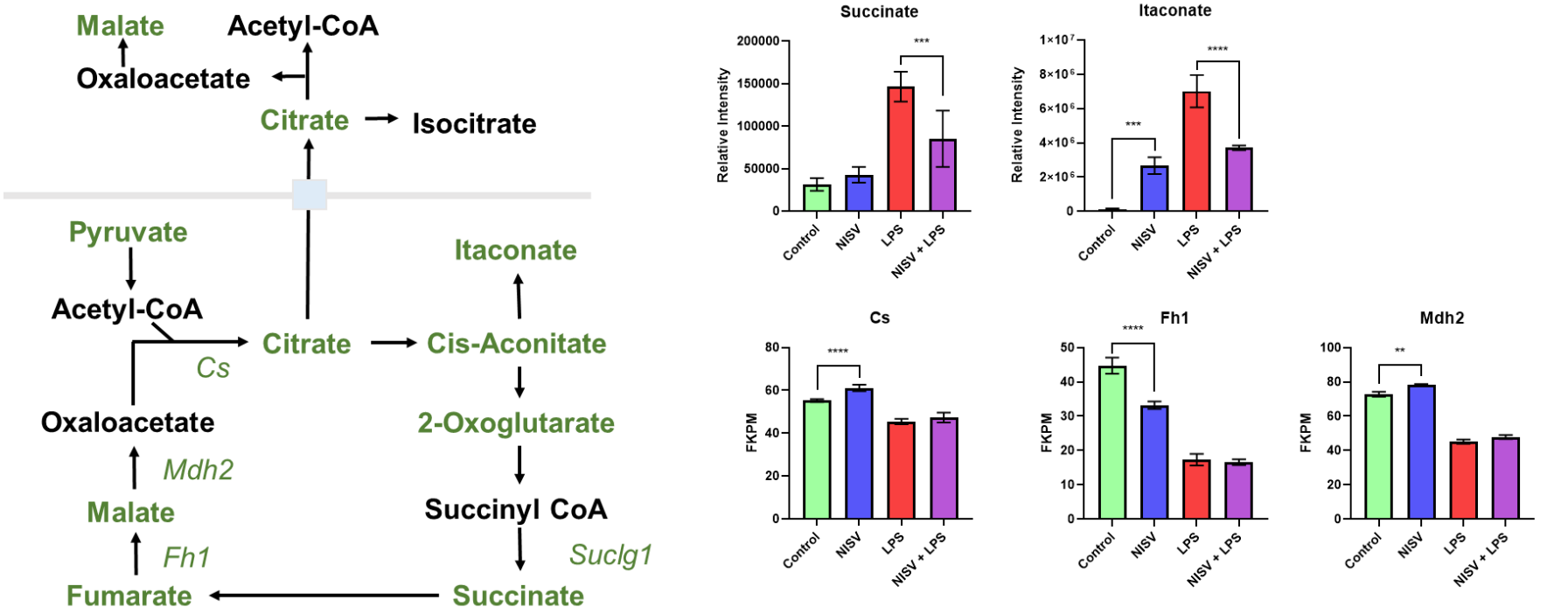


Figure 5.4.2 NISV effects on the TCA cycle. *100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance ($p<0.05$).*

5.5 Amino Acid Metabolism

5.5.1 Arginine Metabolism

In M1 macrophages arginine metabolism is directed to citrulline production in order to induce nitric oxide production. Whereas, in anti-inflammatory M2 macrophages production is driven towards ornithine rather than citrulline, to be converted into spermine and putrescine pushing the cell toward proliferation and growth. The effects of NISV of these processes was investigated in resting and LPS-stimulated BMDM.

Figure 5.5.1.A shows us the metabolites and transcripts detected in our samples related to arginase metabolism. Part B of this figure shows the significantly affected metabolites and transcripts related to this pathway. NISV had no effect on proline levels in unstimulated cells, but significantly upregulated this amino acid in LPS stimulated cells, increasing the relative intensity from 54541745 to 75476330. N-(L-arginino) succinate was significantly reduced in LPS-stimulated cells treated with NISV, from 146453 to 85161. In LPS-stimulated cells NISV increased ornithine levels but not to a significant degree. Transcription of *Nos2* was significantly downregulated by NISV in LPS stimulated cells, from FKPMs of 574 to 492.

5.5.2 Tryptophan metabolism

Kynurenine is important in the maturation of T cells and DCs and is produced from the amino acid tryptophan. Figure 5.5.2.A shows the metabolites and transcripts detected within the tryptophan degradation pathway, with part B showing those that are significantly changed by vesicle treatment. NISV significantly increased tryptophan in LPS-stimulated cells, from a relative intensity of 1050395 to 1514004. Nicotonic acid mononucleotide adenylyltransferase 1, Nmnat, transcription was significantly reduced from 7.1 to 1.7) by LPS stimulation of BMDM, but this effect was ameliorated by NISV treatment of LPS-stimulated BMDM (rising to 8.5).

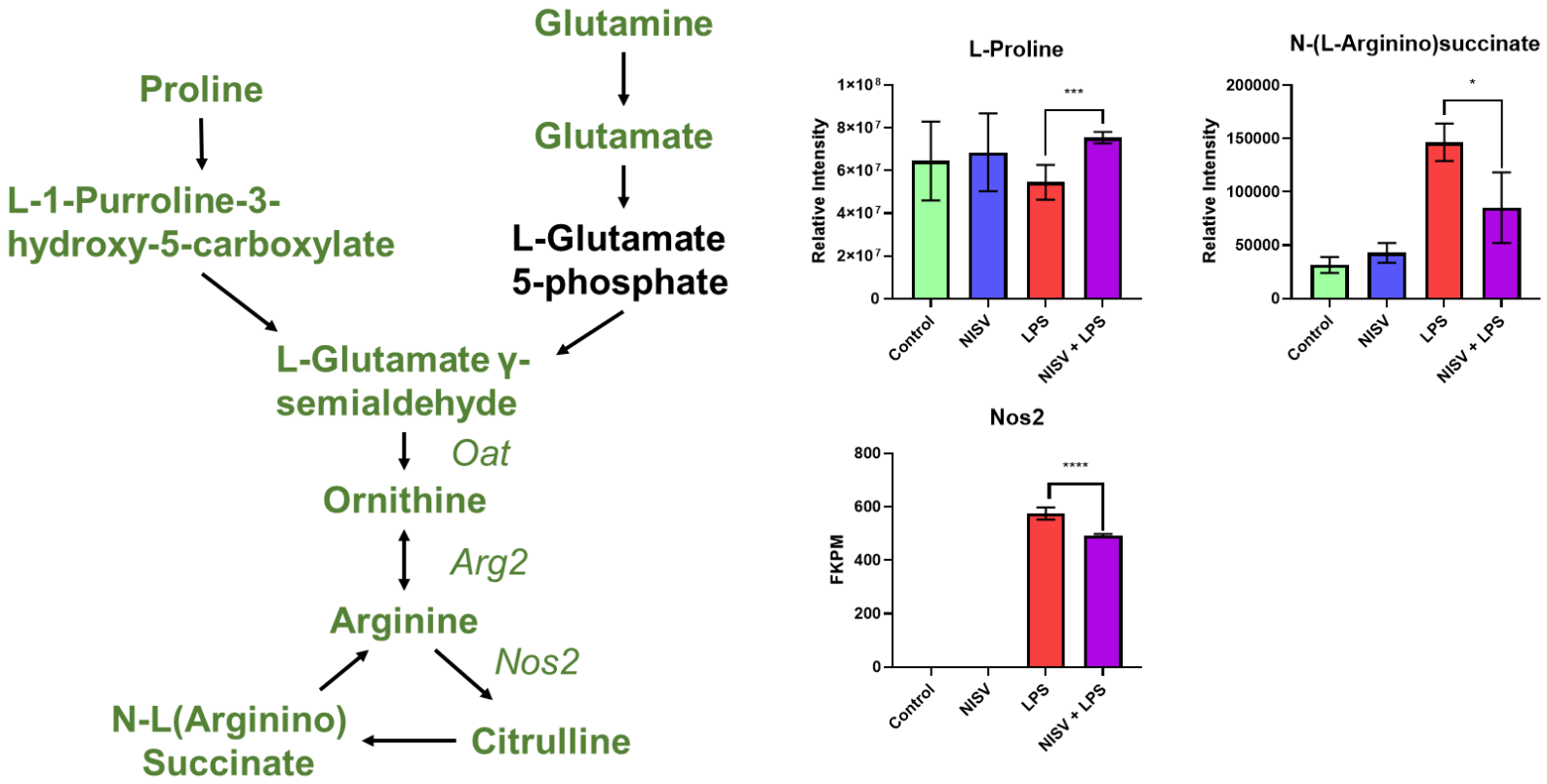


*Figure 5.5.1 Vesicle effects on Arginine Metabolism. 100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05).*

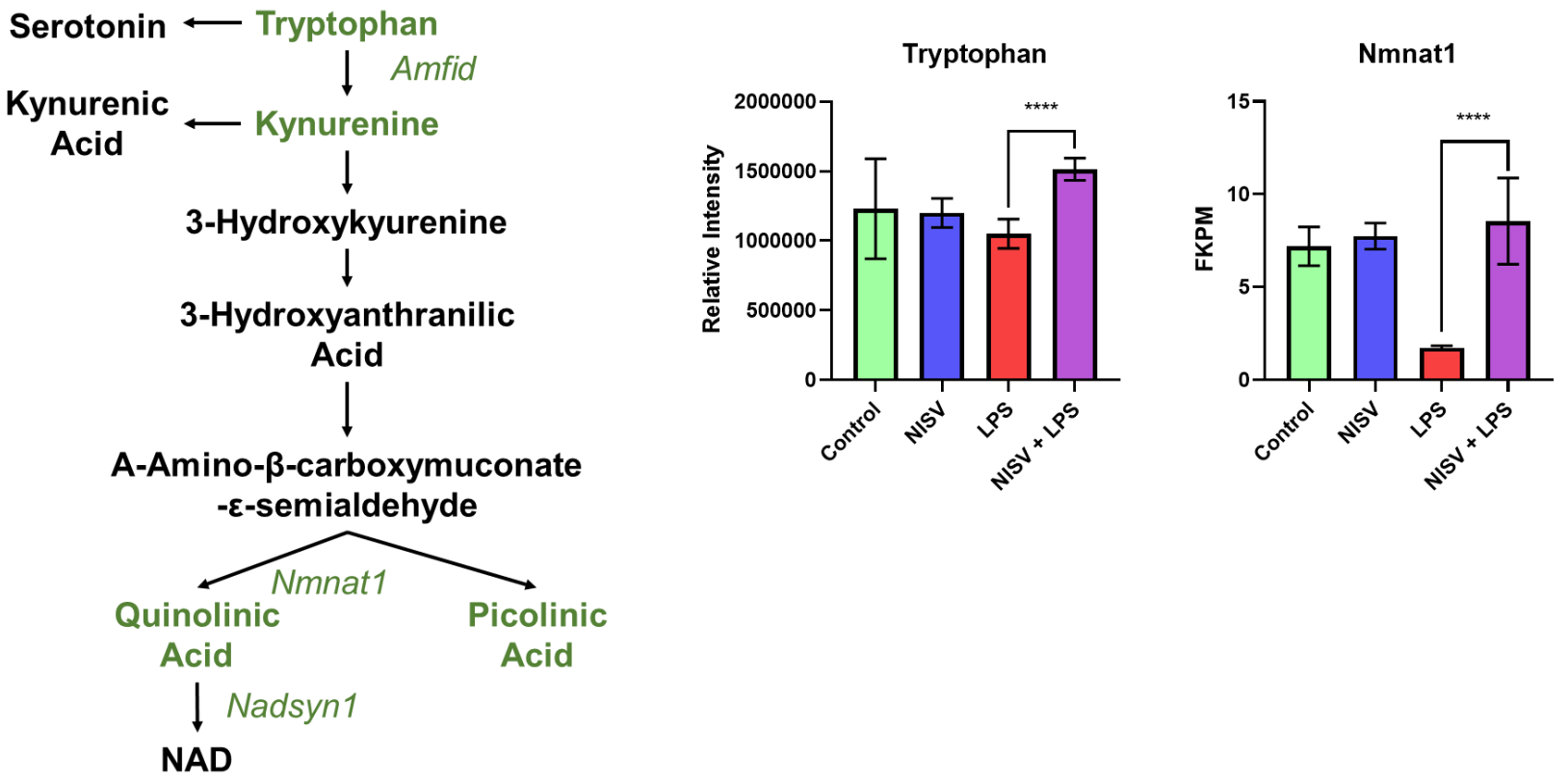


*Figure 5.5.2 The effects of Vesicle treatment on Tryptophan Metabolism. 100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05).*

5.6 Alternate Inflammatory Effectors

Following up the OPLS-DA analysis of NISV, three more pathways of interest were identified, creatine biosynthesis, pyridoxine (vitamin B_6) metabolism and taurine metabolism.

5.6.1 Creatine biosynthesis

NISV were found to increase creatinine biosynthesis in LPS stimulated BMDM. Creatine levels were significantly increased by NISV treatment in non-stimulated cells, from a relative intensity of 66085782 to 153741320, this trend was seen in LPS-stimulated cells, although not statistically significant. Creatine-phosphate was significantly increased by NISV in unstimulated cells, from 630160 to 334634, but decreased in LPS-stimulated cells, (from relative intensities of 2012437 to 582601) treated with NISV. In LPS stimulated cells, NISV significantly upregulated creatinine production from 30031680 to 51801101. Glycine aminotransferase*, Gatm,* transcription was reduced by NISV in unstimulated BMDM, from FKPMs of 134 to 96, but not significantly changed in LPS-stimulated cells. Transcription of creatine kinase B-type, *Ckb* was reduced by NISV in non-stimulated BMDM (from 338 to 226).

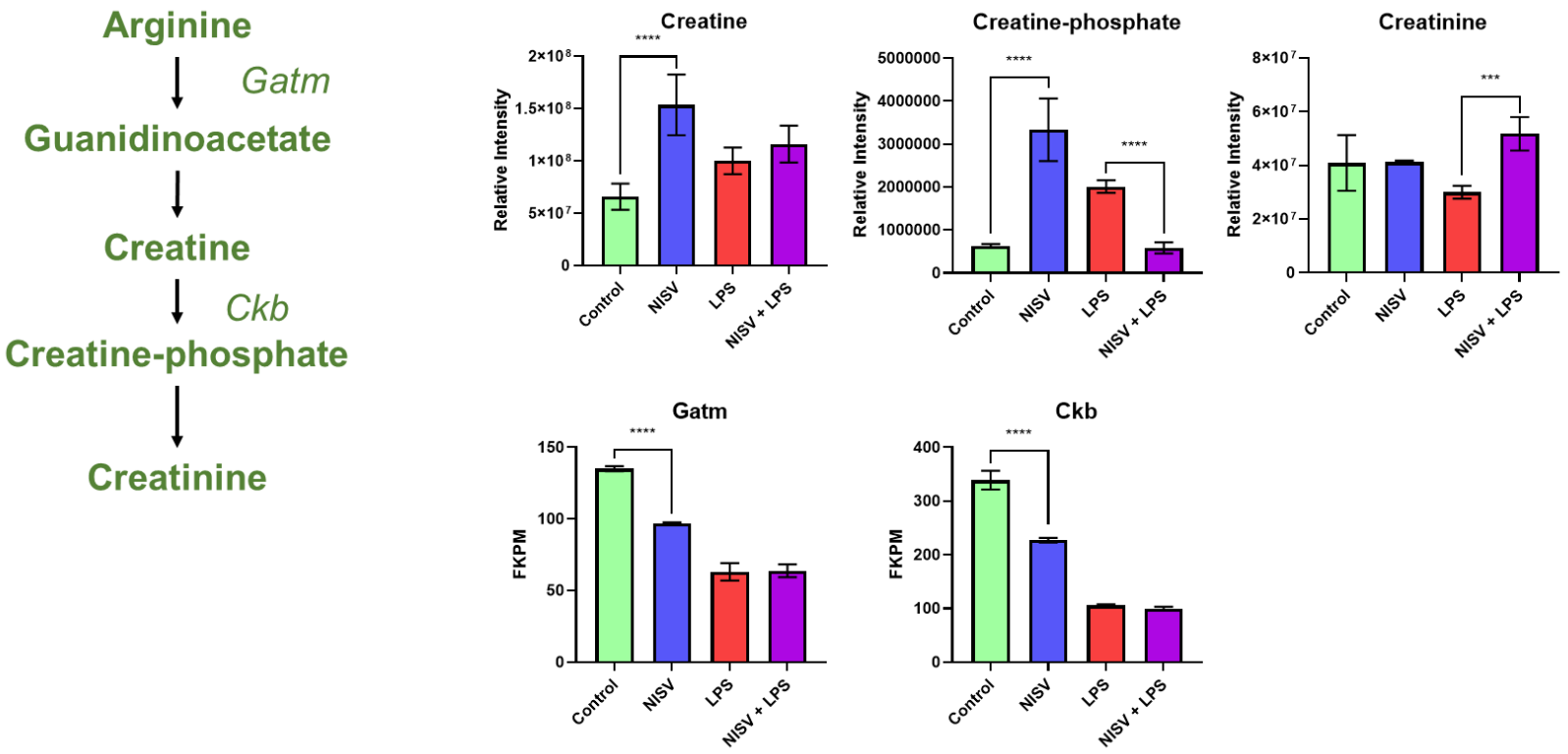


Figure 5.6.1 Creatine metabolism altered by vesicle treatment. *100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05).*

5.6.2 Pyridoxine Metabolism

NISV were shown to significantly increase pyridoxine, from a relative intensity of 7894298 to 12509533 in LPS-stimulated BMDM (figure 5.6.2).

5.6.3 Taurine Biosynthesis

NISV were found to augment biosynthesis of taurine (from 6967365 to 17350480) and its precursor hypotaurine (from 180737 to 514911) in resting BMDM. These metabolites were also increased in LPS-stimulated BMDM (to 15451744.67 and 579849.5867, respectively). Treatment of LPS-stimulated BMDM with NISV ameliorated these effects (decreasing to 9793652 and 275679, respectively).

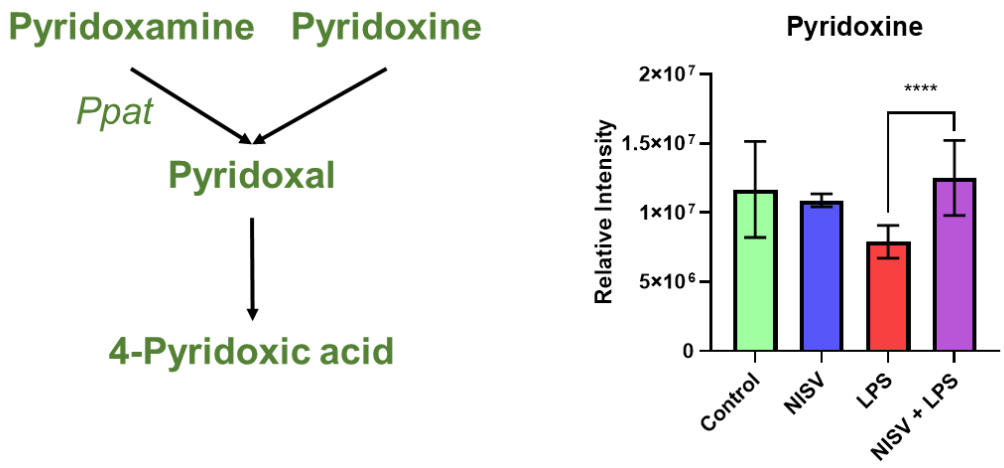


Figure 5.6.2 NISV increase Pyridoxine expression in LPS-stimulated cells. *100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05).*

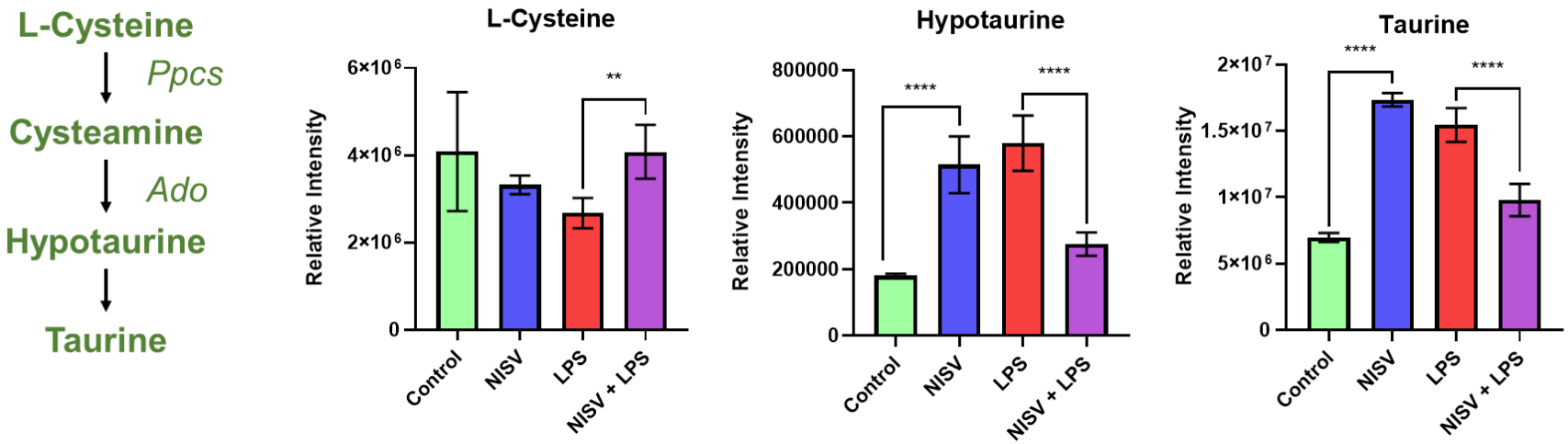


Figure 5.6.3 NISV reduce Taurine Metabolism in LPS-stimulation. *100,000 BMDM were plated per well as 10 replicates and stimulated with LPS at 3µg/ml or media, in controls, followed by treatment with NISV. Samples were pooled to make 1,000,000 cells/sample. Cells then underwent metabolite extraction and were sent for metabolomics analysis. Pathway diagrams indicated metabolite detection in green and non-detection in black. Statistical analysis was performed on prism 8 using two-way ANOVA and Tukey's multiple comparisons test. * Indicates statistical significance (p<0.05)*

5.7 Discussion

In the previous chapter, NISV were found to not have obvious polarising effects on the phenotype of macrophages. However, NISV treatment of resting and LPS-stimulated BMDM is found to induce changes to certain aspects of their metabolome, some of which are known to differ between M1 and M2 macrophages. In general, NISV had relatively modest effects on the BMDM metabolic aspects examined. However, their ability to modulate LPS-induced changes to metabolism was clearly evident.

5.7.1 NISV Dampen Pro-Inflammatory Changes of the Warburg Effect

NISV caused important changes in glycolysis in LPS-stimulated BMDM by augmenting the Warburg effect with increased pyruvate and lactate production. Remodelling of the TCA cycle as previously reported was found to be induced by LPS. Thus, BMDM stimulated with LPS were found to have increased succinate and itaconate levels. NISV treatment of these cells reduced these two metabolites, but not to the levels of resting macrophages. Itaconate is known to be produced as a result of inflammation and functions both as an antimicrobial and anti-inflammatory mediator. Its down-regulation by NISV in LPS-stimulated cells could be negative feedback due to its function no longer being necessary in the presence of NISV (O'Neill & Artyomov, 2019). Succinate, which is also known to be augmented in macrophages stimulated with LPS and responsible for increased HIF1a stability and downstream cytokine production was downregulated by NISV (Tannahill et al., 2013).

5.7.2 NISV Effects on Amino Acid Metabolism

NISV were found to disrupt one particular aspect of arginine metabolism, the citrulline-nitric oxide cycle. NISV did not affect arginine or citrulline levels; however the third component of the cycle, arginosuccinate, was significantly down-regulated by NISV, concomitant with reduced *Nos2* transcripts. The down-regulation of arginosuccinate indicates a reduced capacity for NO production as does the reduction in *Nos2*, which is often tested for as a proxy for NO production. Testing nitric oxide production (by measuring nitrite levels) from cells treated with NISV was unreliable due to opacity of NISV producing a false positive in the NO assay. It is likely that NISV reduce the nitric oxide expression based on this data but otherwise do not affect arginine metabolism in activated macrophages.

Tryptophan metabolism is vital to T cell regulation producing kynurenine, which acts as an anti-inflammatory mediator this induces differentiation of Th_{17} cells into T_{reg} cells (Opitz et al., 2011). The only metabolite significantly altered by NISV treatment in this

pathway was tryptophan, in which NISV induced upregulation in LPS stimulated cells. With no change in kynurenine levels there is no evidence that the NISV affect this pathway and any downstream immunomodulatory pathways in macrophages. However, as tryptophan can be metabolised by other immune cells, such as T cells, this upregulation could have a greater effect on the surrounding immune response.

5.7.3 OPLS-DA Identified Pathways

With the identification of several metabolites that possess inflammatory effects but were not involved in the processes previously described, analysis of their pathways was performed.

Creatine is a commonly available and widely used dietary supplement due to its ability to enhance athletic performance and decrease muscle inflammation. Its impact on the immune system is not fully understood, but some of its effects have been investigated. In vitro studies demonstrate that supplementation of the media used to culture macrophages with creatine monohydrate, creatine ethyl ester or creatinine have differential effects on the macrophages TLR expression. Creatine monohydrate and creatinine were found to downregulate expression of TLRs; 2, 3, 4 and 7 in macrophage whereas, creatine ethyl ester was found to increase expression of these receptors (Leland et al, 2011). With the contradiction between different forms of creatine it is hard to say how pure creatine would contribute to TLR expression in macrophages, but creatinine's effects are comparable to the results reported previously. Creatine has also been shown to affect the expression of T cells in which it acts as an energy reserve for T cells actions and when absent impairs proper T cell function (Di Biase et al., 2019). In the current analysis, NISV were found to significantly alter creatine metabolism in both stimulated and unstimulated cells. In unstimulated cells increased creatine and creatine-phosphate levels reduced expression of Gatm and Ckb, glycine amidinotransferase and creatine kinase B-type respectively. The upregulation of creatine and creatine-phosphate is unlikely to be an immune response to the NISV, but potentially an induction of creatine as a conservation of energy. In stimulated cells, creatine-phosphate is reduced and creatinine is increased indicating the NISV have encouraged the conversion of one to the other (Leland et al., 2011). This response could lead to anti-inflammatory effects in the macrophage by downregulating TLR4.

Taurine, like creatine, is a widely available and well-used supplement in fitness. However, its therapeutic effects are currently being investigated and it has been identified

as an anti-inflammatory mediator. In immune cells, taurine is essential in the protection of cells from oxidative stress (Marcinkiewicz & Kontny, 2014). It reacts with the potent oxidants hypochlorous acid and hypobromous acid, neutralising them, and forms taurine chloramide and taurine bromamine, which are stable and non-toxic (Marcinkiewicz, 2009; Marcinkiewicz & Kontny, 2014). Taurine has recently been shown to have anti-inflammatory effects in mice when challenged by a *Streptococcus uberism,* where it reduced glycolysis and OXPHOS (Lan et al., 2021). These anti-oxidant effects show the taurine pathway's importance in cell health including that of macrophages. In LPS-stimulated cells, vesicle treatment reduced taurine and its precursor hypotaurine, whereas in unstimulated cells NISV significantly increased these compounds. The reduction of these molecules on the one hand could be interpreted as a negative aspect of NISV as this would result in reduced protection of the macrophage from oxidants. On the other hand, the reduced levels of taurine could be due to NISV downregulating the oxidants which taurine neutralises. The levels of ROS were not measured in this experiment, but it would be useful to identify if NISV reduce or increase protection against ROS.

In regards to sphingosine-1-phosphate metabolism pyridoxine again acts as an enzyme co-factor for serine palmitoyltransferase (SPT) and S1P lyase (SPL). SPT is responsible for the conversion of palmitoyl CoA and serine into 3-ketosphingamine which is then converted into ceramide, the precursor to sphingosine. (MacEyka & Spiegel, 2014). SPL is responsible for the irreversible conversion of sphingosine-1-phosphate into trans-2-hexadecenal and phosphoethanolamine (Aguilar & Saba, 2012). NISV down-regulated the gene encoding SPT, *Sptlc1*, in control cells and had no effect on LPS-stimulated cells. This could indicate a downregulation of the overall pathway in non-stimulated cells, possibly due to the MPG mimicking sphingosine. NISV also induced up-regulation of SPL transcript *Sgpl1* in LPS-stimulated cells, this has the potential to reduce S1P levels as upregulation of SPL and its co-factor pyridoxine could cause increased conversion to trans-2-hexadecenal and phosphoethanolamine. This data furthers the link between NISV treatment and disruption of traditional sphingosine-1-phosphate signalling.

5.7.4 In Conclusion

The effects NISV have on the basal metabolism of resting BMDM were found to be relatively small which is encouraging in terms of their lack of toxicity and unwanted perturbation of cellular processes. However, NISV were found to augment aspects of

the Warburg effect such as potentiating oxidative glycolysis in LPS-stimulated cells. Perhaps most interesting are the effects of NISV on the TCA cycle in LPS stimulated cells, where they alter the production of succinate and itaconate. NISV were also found to downregulate *Nos2* transcripts and arginosuccinate levels which is consistent with their ability to downregulate NFkB as suggested in the previous chapter. NISV also induced metabolism of creatine towards creatinine which is known to be anti-inflammatory. The increase in pyridoxine production could also have implications for many pathways as it is a widely used co-factor. Thus, the results obtained are largely consistent with the ability of NISV to be anti-inflammatory as noted in the previous chapters. At least some of the effects can be directly linked to the ability of NISV to modulate NF-kB signalling events, while others might be indirect consequences of this proposed mechanism. The relatively mild effects of NISV supports their low toxicity and provides further confidence in their potential use as a drug.

6. Discussion

The data contained within this thesis have identified many aspects of vesicle-based immunomodulatory effects, contributing evidence to NISV proven use as vaccine adjuvants, expanding their sparsely researched anti-inflammatory characteristics and identifying potential mechanisms of action through which these effects are exerted. The following will expand upon and combine the findings raised in the previous chapters, philosophically discussing their place within the literature and their greater potential in the medical field.

6.1 Adjuvant Properties of NISV and Liposomes.

The basis of this project was to elucidate apparent contradictions in the literature. NISV have historically and successfully been used as vaccine adjuvants, meaning they heighten the immune response allowing successful vaccination. The identification of apparent anti-inflammatory effects of NISV is in stark contrast to their previous use. However, throughout this project we have discovered plentiful evidence of both pro-inflammatory and anti-inflammatory effects caused by NISV treatment. The data has shown that treatment of unstimulated BMDM with NISV induce immune changes consistent with their adjuvant activity and also often associated with inflammation. These changes are not as potent as the pro-inflammatory effects of LPS stimulation. The NISV induced changes in key macrophage pro-inflammatory cytokines and transcripts that are associated with “defense response”. This pro-inflammatory effect ties in with previous literature, giving insight into how NISV induce their adjuvant effects and can still be used as potent vaccine adjuvants (Brewer & Alexander, 1992). The studies provide some insight into the mechanism of action of NISV on a cellular level. Specifically, the data demonstrate that NISV upregulate transcripts of NF-κB subunits and downstream products including an array of cytokines, chemokines (evident in cytometric bead array and transcriptomic data) and potentially surface co-stimulatory markers as evident in the transcriptomic data. The molecular mechanisms that NISV induce to initiate these events remain to be determined.

Liposomes were used as a comparator for NISV and were found to have more profound inflammatory effects. Identification of these pro-inflammatory effects provide novel insight into how they mediate their beneficial effects in current medicines. For example, their pro-inflammatory effect in unstimulated cells potentially contributes to their successful use as anti-cancer drug carriers. Tumours manipulate their microenvironment into an anti-inflammatory state in order to reduce the ability of the immune

system to clear the cancerous cells. One of the main effectors of this microenvironment are Md macrophages (Zhou et al., 2020). The observed effects of liposomes on BMDM, if replicated *in vivo* would counteract this microenvironment allowing a more pro-inflammatory state to persist and increase immune based clearing as well as delivering the anti-cancer drug cargo to the area. Vaccination against COVID-19 has utilised novel mRNA vaccines. The mRNA is inherently unstable and the vaccines are given stability by a solid-lipid nanoparticle formulation utilising the same composition of liposomes used with this project (DSPC & cholesterol). Arunachalam *et al* have found data indicating the vaccine induces a heightened innate immune response in addition to the expected adaptive immune response (Arunachalam et al., 2021). While this is seen as a beneficial effect in these circumstances, it might not always be desirable in other therapeutics.

Notably, liposomes in contrast with NISV were pro-inflammatory even following LPS-stimulation. This might not be a desirable effect in some settings. In septic patients, fatality is caused by the overreaction of the immune system rather than the infection itself, in such a reaction the liposomes could heighten the pro-inflammatory response and accelerate morbidity. Furthermore, these data demonstrate that NISV have unique anti-inflammatory properties that are not shared with liposomes.

How these results translate into adjuvant effects *in vivo* will require further testing.

6.2 Anti-Inflammatory effects of NISV in LPS-Stimulated BMDM

Despite the evidence provided towards NISV adjuvant effects, this project was undertaken primarily to further characterise the anti-inflammatory effects if NISV and to obtain mechanistic insight into their mode of action in this respect. The anti-inflammatory effects of NISV have been proven and expanded upon by this series of experiments. The data found using the cytometric bead array generally concurred with the findings of Roberts *et al* and was expanded to include important immune mediators. Transcriptomics allowed further expansion into the effects of NISV at a single time-point. This also provided a potential mechanism behind NISV effects. Specifically, NISV were found to significantly down-regulate NF-κB subunits, as well as many downstream changes that would be expected upon alteration of this transcription factor. Although the down-regulation of NF-κB explains many of the effects caused by NISV in LPS-stimulated macrophages, the molecular mechanism responsible remains to be determined.

One potential hypothesis for how NISV might initiate their effects is their primary component's (MPG), structural similarity to an important immune regulator, Sphingosine-1-phosphate (S1P). S1Ps effects have been previously discussed, and interruption of this pathway would show effects similar to those shown by NISV. It is thought that MPG could affect the S1P pathway in multiple ways including, as indicated in Figure 6.2.1, interruption of phosphorylation and/or agonistic/antagonistic effects on the many S1P receptors. MPG when administered to BMDM as a single component did not mimic the anti-inflammatory effects of NISV. However, due to MPG being a surfactant it disrupts cell membranes at high concentrations when unformulated. Furthermore, formulation also provides a means to transport MPG into the cell. These hypotheses and those identified previously require further testing and experimentation to confirm, and some examples of tests that could answer the questions raised are identified in the following section.

The potential of NISV to be used as a medicine requires greatly expanded testing, specifically using *in vivo* models to confirm the effects shown in this experiment are replicated. If the effects found in BMDM translate to the *in vivo* model its uses as a medicine could become a real possibility. Some interesting findings within the experiment carried out here could indicate potential for NISV use in some illnesses, specifically sepsis. In sepsis caused by a TLR4 or TLR3 agonist (*E. coli* or rotavirus, respectively), NISV could prove a powerful anti-inflammatory and, in combination with antibiotics/antivirals, could allow clearing of the infection with minimal harm to the patient. However, if NISV were used therapeutically against a TLR2 agonist, such as *S. aureus,* NISV may not prove effective as pam3csk4-stimulated BMDM were not modulated to the same degree. Furthermore, liposomes were found to have some anti-inflammatory effects upon pam3csk4 stimulation, meaning they could potentially be used to treat sepsis of a Gram-positive microorganism, to mirror the hypothetical NISV therapy. These differential effects indicate the potential to create a spectrum of immunomodulatory drugs using different vesicle formulations. If the anti-inflammatory effects of NISV and pro-inflammatory effects of liposomes could be tightly controlled, the possibility for tailorable inflammatory effects could be possible, greatly expanding the potential uses of vesicular formulations as inflammatory mediators. The effects of formulation and composition on immunomodulation caused by vesicles warrants further investigation.

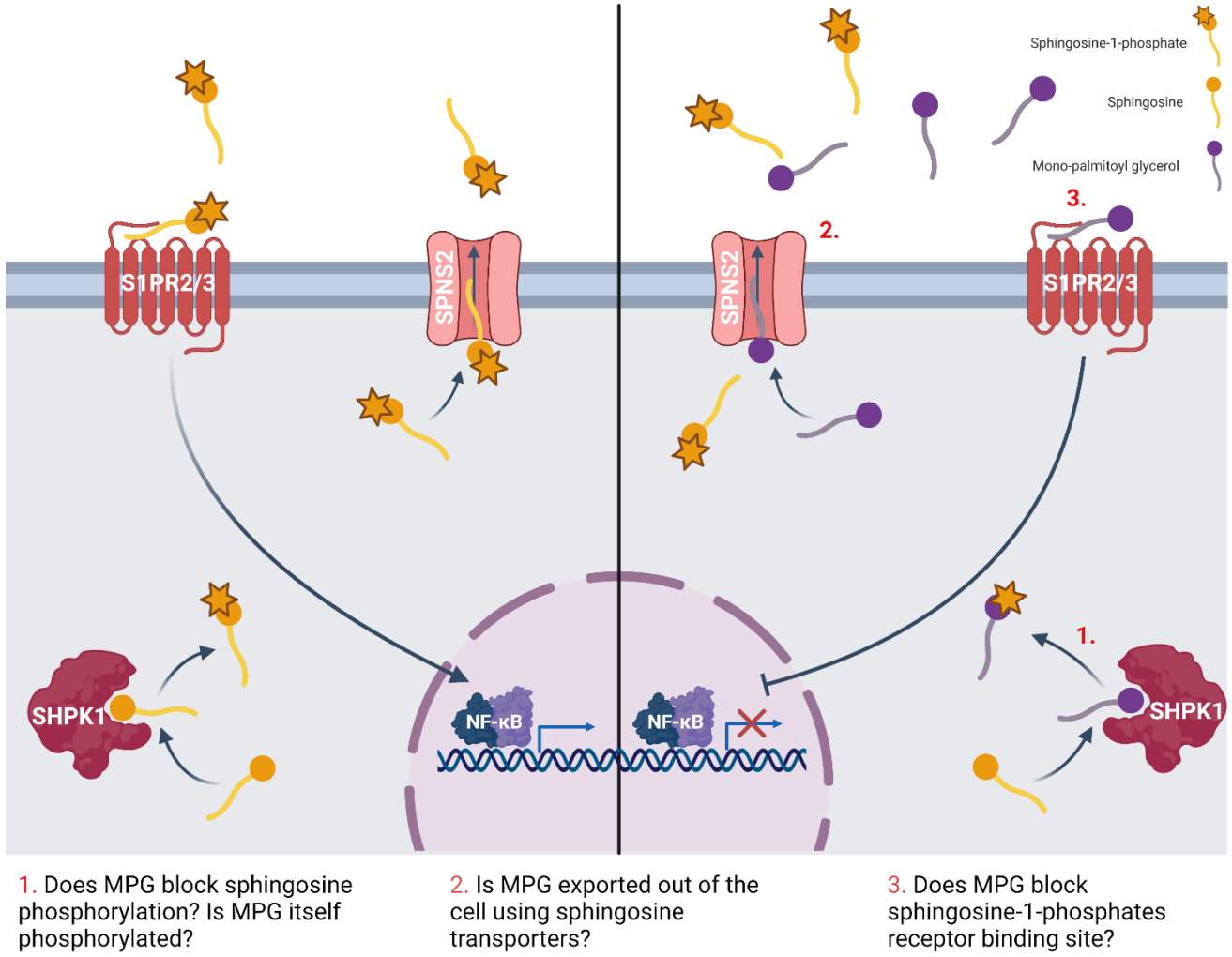


Figure 6.2.1 Potential Interactions of MPG with S1P signalling. *On the left traditional S1P signalling is shown and on the right mechanism through which MPG may interrupt S1P signalling are shown. Those mechanisms are identified at points (1.), (2.) and (3.), which are then expanded upon in the diagram above.*

6.3 Future Work

This project has answered many questions but leaves many unanswered and requiring further investigation. To prove or disprove the hypotheses made various experiments are proposed.

To test the hypothesis that NISV interfere with S1P signalling in macrophages experiments with a range of complexity have been designed. A simple test to indicate if NISV are antagonistic or agonistic of S1P signalling, would be to use a S1P agonist and antagonist in parallel with NISV. This would quickly identify and shared effects found between either NISV and the agonist or antagonist. Assuming the findings of this previous experiment are positive, investigation into NISV interaction with S1P kinases and receptors would be pertinent, using enzyme kinetics and receptor interaction tests.

Further experiments into the various hypotheses formed using the transcriptomics data should be performed to confirm their validity. As previous discussed, transcriptomics are a snapshot of the cells RNA production, rather than a look into actual protein production, so it gives valuable insight into what may be happening but is not definitive. To confirm the major findings from this experiment, investigation into NF-κB subunit levels and signalling events would confirm one hypothesis. Following that expansion to use different forms of the cytometric bead array would further expand the known repertoire of NISV effects and identify any inconsistencies with the transcriptomics data. A definitive experiment for confirming these effects would be a time-series transcriptomics and proteomics experiment carried out in tandem, this would allow expansive data covering both changes over time and confirmation of changes through protein measurement, but such an experiment would be restrictive in cost at this time.

Further expansion upon NISV effects would be desirable, as would narrowing down NISV potency. Testing of NISV effects using different TLR agonists, ideally TLR7/8 mimicking COVID-19 infection, would be useful in knowing the extent of the effects NISV induce. So too, testing NISV anti-inflammatory effects on other immune cells would indicate the span of NISV effects and further elucidate potential mechanisms of action. Furthermore, comparison of NISV anti-inflammatory effects to a currently used medicine, such as dexamethasone or cortisol, is important in identifying the potency of NISV. In this respect, NISV could potentially have less side effects than the

hormone based drugs. Investigation of NISV effects on NF-κB could prove invaluable using RT-PCR or p65 accumulation measurement.

Finally, expansion of NISV immunomodulation testing into *in vivo* models would solidify its potential. Mimicking the previous experiments *in vivo* would provide ample evidence towards NISV use as an anti-inflammatory medicine. Investigation in to the effects of NISV on different immune cells *in vivo* would also provide great insight in to the breadth of effects NISV hold. Some *in vivo* evidence may already exist in a study by D'Elia *et al*. While investigating the use of bilosomes, an NISV modified to incorporate bile salts allowing greater oral delivery, to increase efficacy of antibiotics, it was found that encapsulation of the antibiotics decreased antibiotic-induced weight loss. This study initially tested the gut microbiota, deducing that encapsulation of an antibiotic had no effect on its composition leaving its effects unknown (D'Elia et al., 2019). The protection against antibiotic-induced weight loss could have been due to anti-inflammatory effects of bilosomes interacting with the gut and protecting the mice. In addition, this protection been shown against *Toxoplasma* infection in the original source of NISV anti-inflamatory effects, Roberts et al's patent where they showed NISV could reduce weight loss in *Toxoplasma* induced cachexia in mice (Roberts et al., 1997)

6.4 Conclusions

The work in this thesis provides insight into the adjuvant and anti-inflammatory effects of NISV using BMDM. The data demonstrate that NISV anti-inflammatory effects are unique and not shared by liposomes. NISV exert their effects through modulation of NF-κB and consequently affect a broad array of effects on immune products.

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