1.0 Introduction

The development of an effective treatment against infectious disease requires a good antigen or immune stimulant that is able to stimulate and enhance the immune response and prepare the host to fight potential infections. However, a major challenge for this type of treatment is finding an effective delivery system for the immune stimulating molecule that is able to enhance or boost the immune response against potential infections (Lazoura and Apostolopoulos, 2005; Mishra et al., 2006). Immune stimulating molecules are of increasing interest as antibiotic resistance becomes a greater problem. The effective delivery of these immunotherapeutic molecules has been a major barrier to their commercial development, since these compounds are often very expensive and have a very short half-life; as well, they have been shown to cause adverse physiological effects at a higher effective dose (Choi et al., 2006; Krieg, 2006). An ideal delivery system would be compatible with a wide variety of compounds and would have an excellent safety profile (Heath et al., 2003). This would allow the delivery of various types of immune stimulating molecules without the threat of negatively affecting the host's health. A system that could also significantly enhance the biological activity of the immune stimulating molecule would also reduce the cost of vaccination as lower doses would be just as or more effective than the higher doses of free antigen (Choi et al., 2006; Croyle et al., 1998).

One immune stimulating molecule that has been considered for use in the development of preventative vaccines is unmethylated cytidine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODN). It has been demonstrated in several studies that synthetic CpG ODN are able to stimulate an immune response in vertebrates (Gomis et al., 2007; Mena et al., 2003; Mutwiri et al., 2003). Microbes such as bacteria, viruses and parasites as well as vertebrates posses CpG nucleotides in their DNA but only microbial DNA contains unmethylated CpG nucleotides flanked by specific nucleotides (Krieg, 2006). The immune system of vertebrates uses this difference in CpG nucleotides to recognize the invasion of microbes which stimulates an immune response. The delivery of synthetic CpG ODN is also able to stimulate an innate immune response which prepares the host for an encounter with a pathogen (Chen et al., 2007; Elkins et al., 1999). However, despite the ability to protect the host

from infection CpG ODN are often required at a fairly high dose as the ODN is quickly degraded by 3' exonucleases (Noll et al., 2005). It also has been observed that there are adverse side effects on animal health when a higher concentration of CpG ODN is administered *in vivo* (Mutwiri et al., 2003). To optimize the immune stimulant capability of CpG ODN a system is required that can deliver smaller doses to target cells while keeping the biological activity of CpG ODN intact. One promising candidate to improve the biological activity of CpG ODN are nanoparticles such as carbon nanotubes.

Nanoparticles such as carbon nanotubes (CNT) can be used with a wide variety of immune stimulants including CpG ODN (Bianco et al., 2005). Nanoparticles show great promise for biomedical purposes because of their physicochemical properties such as their hydrophobicity, ordered structure, light weight, and high surface area which also allows them to pass through biological barriers to get to intracellular targets (Lacerda et al., 2006). Broadly CNTs come in two major forms, single walled (SWCNT) and multi walled carbon nanotubes (MWCNT). Free CNTs however have poor solubility in most liquids but with the functionalization of the nanoparticles this problem is overcome. Functionalization also allows the conjugation of the nanoparticles with other molecules such as oligonucleotides. Not only can other molecules be conjugated to CNT but it has also been shown in short term studies these particles have very low toxicity in the host (Lacerda et al., 2006). These characteristics make nanoparticles excellent delivery systems for immune stimulating particles such as CpG ODN. Preliminary studies revealed that by linking CpG ODN to nanoparticles the CpG ODN is perhaps more stable and better protected from degradation. This would allow smaller doses of CNT linked CpG ODN a better opportunity to stimulate an immune response than free CpG ODN.

2.0 BACKGROUND

2.1 The Immune System

All species are constantly interacting with the environment surrounding them. For higher species continuous interaction with foreign microorganisms has the potential to cause serious disease in the host organism. The constant microbe-host interactions can be tolerated by the host due to the evolution and function of the host's immune system. The immune system samples foreign microbes such as bacteria, parasites and fungi as well as viruses and prepares the host to fight potential pathogens before they can cause serious disease and damage (Kumar et al., 2009). The host immune response has evolved to use a wide variety of complex protective mechanisms to recognize and deal with pathogenic microbes. The immune system of most species contains two general systems to fight potentially harmful microbes. These two systems may be considered separate arms of the immune response but they in fact work together to achieve efficient clearance of invading pathogens (Krieg, 2006; Kumar et al., 2009). The innate immune system is the first line of defence against foreign microbes. The innate immune response provides time for the second and more powerful type of immune response to be generated. This second type of response is the adaptive response which provides a more specific attack against the foreign microbes. This system also maintains an immunological memory of the microbes it has encountered to allow a more rapid response the next time the host encounters the microbe (van Duin et al., 2006). Pathogenic microbes that are able to evade the immune system are much more capable of causing disease in the host. The immune system has evolved over time to recognize and clear these microbes but at the same time the microorganisms have also evolved to evade the host's immune response (Bianchi and Manfredi, 2009). Therefore, the innate and adaptive immune response need to work together to recognize foreign microbes before an infection can begin.

2.1.1 General Features of Innate and Adaptive Immunity

The innate immune system contains mechanisms such as the epithelial cell layer, secreted mucus layers, soluble proteins and small molecules such as complement, cytokines and chemokines as well as surface receptors that bind patterns found on the surface of the invading microbes (Chaplin, 2003; Kumar et al., 2009). A wide variety of cells are also part of the innate

immune system including natural killer cells, mast cells, eosinophils, basophils, and phagocytic cells, which include macrophages, neutrophils and dendritic cells (Medzhitov, 2007). All of these cells act to non-specifically remove pathogens from the host system.

Innate immune cells target foreign microbes through use of their pattern recognition receptor (PRR) which target pathogen-associated molecular patterns (PAMPs) that are present on all microorganisms, pathogenic or non-pathogenic (Medzhitov, 2007). There are several different classes of PRR including the Toll-like receptor (TLR) class for which there are several types, each of which have specific invariant microbial targets. For example TLR4 recognizes Lipopolysaccharide (LPS) found on the surface of gram negative bacteria and TLR9 recognizes unmethylated CpG ODN motifs flanked by specific sequences found in bacteria and viruses (Beutler and Rietschel, 2003; O'Neill, 2004; Wickelgren, 2006). Recognition of the PAMPs by PRRs elicits an inflammatory and antimicrobial response which is essential to fight invading microbes. In general, when an innate immune cell recognizes a foreign microbe it is stimulated to generate an inflammatory response by producing pro-inflammatory cytokines including tumor-necrosis factor (TNF), and interleukins (IL) such as IL-1β and IL-6 (Kumar et al., 2009). The production of these cytokines and chemokines can activate the local endothelium to induce vasodilatation to increase the permeability of the blood vessel to allow more immune cells to be recruited to the site of infection (Chaplin, 2003). Some innate immune cells are also phagocytic cells which engulf the invading pathogen and degrade the microbe; these cells also act as antigen presenting cells (APC). The APCs degrade the engulfed pathogen and present select antigens in the context of their own major histocompatibility molecules (MHC) to adaptive immune cells such as T cells, which in turn, activate the adaptive immune response (Chaplin, 2003). So even though the innate immune system and adaptive immune system appear as separate systems there is interaction between the two to generate the most effective response towards potential pathogens.

The adaptive immune system is different from the innate immune system due to differences such as specificity of antigen recognition, diversity of the receptor repertoire, adaptation to the environment, rapid clonal expansion and immunological memory (Alam and Gorska, 2003). The adaptive immune system is based upon the specific recognition of antigens

recognized by the antigen-specific receptors expressed on the surface of T- and B-lymphocytes. Both cell types have their own type of receptor which recognizes specific antigens presented to them by APCs. Because of the specificity of the adaptive immune cells there are often only a select few of the cells in circulation that can recognize a given antigen. Therefore, when the host encounters a specific microorganism it takes time to generate an adequate adaptive response. The specific T- and B-cell populations that can recognize the antigen and mount a response need to be selected and their population needs to be expanded to have enough cells to fight the infection. The adaptive response also has the benefit of immunological memory so that when the same antigen is encountered the specific T- and B- cells can be generated rapidly allowing a specific and more powerful response to be generated in less time.

2.1.2 Cellular Elements of Immune Responses

Key to early microbe recognition and early immune defence are the phagocytic cells such as neutrophils, macrophages and dendritic cells. These cells have several important immune functions including engulfing and destroying foreign biological molecules, production of chemokines and cytokines to stimulate the immune system and presentation of antigens to adaptive immune cells (Chaplin, 2003; Granucci and Ricciardi-Castagnoli, 2003). These phagocytic cells also provide a link between the adaptive and innate immune systems.

Dendritic cells and macrophages are two of the major cells of the innate immune system. Both capture invading microorganisms through phagocytosis as well as recognize a wide variety of PAMPs expressed on the microorganisms. Dendritic cells are initially immature until they come in contact with PAMPs through their PRRs. Upon binding of the microbial ligand the dendritic cell undergoes maturation which allows the cell to establish an inflammatory reaction and stimulate other immune cells (Granucci and Ricciardi-Castagnoli, 2003; Kaisho and Akira, 2003). Macrophages are bone marrow derived cells that develop from monocytes when the cells leave the bone marrow and enter the blood where they circulate throughout the body ending up in tissues. Approximately 24 hours after entering the systemic circulation, the monocytes can migrate into tissues and differentiate into macrophages (Billack, 2006) Upon activation dendritic cells and macrophages produce pro-inflammatory cytokines such IL-1, IL-6 and TNFα. These cytokines can induce production of other chemokines that

promote an inflammatory response which includes redness, swelling, pain and fever. These reactions are essential for fighting infectious microbes. Activation of dendritic cells can also lead to production of type I interferons (IFNs) including IFN-α and IFN-β (Kaisho and Akira, 2003). These type I IFNs are important for anti-viral defences. A key product of macrophage activation is the production of gene encoded inducible nitric-oxide synthase (iNOS) which converts arginine and oxygen into nitric oxide. This nitric oxide plays an important role in defence against intracellular pathogens (Billack, 2006; MacMicking et al., 1997). Macrophages and dendritic cells are important immune cells but alone they cannot contain all infections. By presenting microbial antigen and costimulatory molecules they stimulate the adaptive immune cells, the T- and B-lymphocytes.

The antigen presenting cells play a critical role in the type of adaptive immune response that is generated. The APCs present the antigen to a class of T cells called CD4+ T cells which can differentiate into distinct subsets of helper T cells, such as Th1 and Th2 cells. Th1 cells can secrete IFN-γ which is involved in cellular immunity against bacteria and viruses, while Th2 cells release IL-4 and IL-13 and play crucial roles in humoral immunity. The balance between Th1 and Th2 responses depends upon the antigen presentation by APC. For example the dendritic maturation stage, dendritic cell to T cell ratio or dendritic cell subsets plays a role in the type of Th response generated (Kaisho and Akira, 2003). T cells recognize antigens presented in a complex with either class I or class II MHC proteins located on APC. T cells can also differentiate into several different subsets that have different roles. CD8+ T cells act to kill cells infected with intracellular microbes and are selected by a complex of antigen with MHC I while CD4+ T cells regulate cellular and humoral immune responses and are selected by a complex of antigen with MHC II (Alam and Gorska, 2003; van Duin et al., 2006). Although CD8+ cells major activity is against cells infected with intracellular microbes like viruses or against tumour cells, they also contain regulatory cells that down regulate immune responses (Chaplin, 2003). The CD4+ T cell population act mostly as helper cells to help B cells with humoral immune responses.

The other types of adaptive lymphocytes are the bone marrow derived B lymphocytes. These cells are defined by the production immunoglobin (Ig) antibodies towards foreign

biological materials such as pathogenic microorganisms. The binding of a foreign antigen to the B cell receptor leads to B-cell proliferation, differentiation into an antibody producing cell, memory formation and antigen presentation to T cells (Alam and Gorska, 2003). T cells can modulate B-cell functions by cytokine production such as IL-2, -4, -5, -6 and IFN-γ which enhance B cell proliferation and differentiation into antibody secreting cells (Medzhitov, 2007). Also the physical interaction between T cells and B cells through the CD40L-CD40 co-receptors allows immunoglobulin class switching from IgM and IgD to IgG, IgA or IgE which play important roles in fighting infections by binding pathogens to block adhesion, neutralize toxins and activate complement to lyse the bacteria. The Ig class switch is vital to create the highest affinity for a given antigen (Alam and Gorska, 2003). The adaptive immune cells play vital roles in generating powerful specific immune responses towards potential pathogens, but, the cells and effectors of the innate system also play crucial roles as both immune systems cells interact with each other to generate the most effective response. Therefore the stimulation of the immune system requires the effective activation of both arms of the immune system.

2.2 Immune Stimulation

The stimulation of the immune system prepares the host to fight off potentially destructive infections as well as provides an immunological memory that allows the immune system to quickly act upon re-encountering the same or similar pathogens. This has been the basis for the development of vaccines and immunotherapy as preventative treatment. The use of specific antigens or general stimulates of the innate immune system can prepare a host for contact with a microorganism with the same antigen which would result in a rapid response before the microbe can establish disease. The stimulation of the immune system with specific antigens with or without innate stimulatory adjuvants is based upon the natural immune response to antigens and PAMPs encountered from the environment. Stimulation of an effective response often requires stimulation of the innate immune system as well as the adaptive system as it is known that the innate immune response plays a vital role in the evolution of the adaptive response through antigen presentation (Hoebe et al., 2004; Krieg and Vollmer, 2007). There are various techniques and strategies to stimulate an immune response that is effective against a

particular microbe or a wide variety of microbes. The later often involves the general stimulation of the innate immune response with a PRR ligand.

2.2.1 Benefits of Immune Stimulation

The stimulation of the immune system to recognize potential pathogens and to prevent serious infection has been used successfully for decades ranging in the level of pathogenesis of the microbe and the success of the stimulation. Vaccines have been developed to help fight serious microorganisms responsible for diseases such as small pox and polio. Preventative medicine is constantly looking for better ways to stimulate the immune system to prepare the host against a wide range of pathogenic microorganisms as well as against the host's self in the case of fighting cancer by stimulating the immune system to specifically target tumour cells (Chen et al., 2007). All these methods try to stimulate specific aspects of the immune system, either innate or adaptive, with the use of an antigen or a ligand that will be recognized by immune cells to generate the required immune response to deal with a potential pathogen or disease. To effectively clear harmful microorganisms and build an immunological memory the adaptive immune system needs to be stimulated. This also requires the initial activation of the innate immune system to present the antigen to T-cells which activates B-cells. Therefore, the most effective way to stimulate the immune system requires not only a good antigen to stimulate the adaptive response but a way to stimulate the innate response to get a more robust long-lasting adaptive response (Pashine et al., 2005). This can be achieved by selecting particles that the innate immune cells can recognize with their own less diversified receptors that will stimulate them to generate an immune response (Chen et al., 2007; Diefenbach and Raulet, 2003). The benefit of focusing on the innate immune response is that the response generated can fight a wide variety of microbes non-specifically and is quicker to generate. Another benefit is that the type of response generated can be selected, which might have advantages against particular pathogens. For example, by targeting dendritic cells to respond in a specific way to a particular ligand they can then stimulate a Th1 or Th2 T cell response by the type of cytokines they produce (Granucci and Ricciardi-Castagnoli, 2003). Targeting a specific response is the goal of vaccine development and immunotherapy to effectively fight infections and disease.

2.2.2 Immunotherapy

Immunotherapy is modulation of the immune system to achieve a proper response to treat an allergy, a particular infection or disease. A wide variety of techniques and strategies are available to try and achieve the desired result. There are several ways to stimulate an immune response or in some cases, such as autoimmune diseases, to suppress an immune response. The basis of immunotherapy is to use either cytokines alone or an exogenous antigen that is to be taken up by the APCs which then present it on their surface in the context of their major histocompatibility complex to stimulate adaptive immune cells (Blanks, 2007). The development of an effective immune stimulating therapy such as a vaccine requires a good antigen to stimulate the immune system and an effective delivery vehicle to ensure the antigen reaches its' target (Pashine et al., 2005). A wide variety of antigens have been successfully used or attempted to be used either alone or with the presence of an adjuvant which also stimulates the innate immune response which then further bolsters the immune response (Gupta and Siber, 1995). Attempts have been made to use immunotherapy to treat not only infectious disease by use of key microbial antigens but to treat host derived diseases such as cancer (Chen et al., 2007). There have been a wide variety of attempts to treat cancer with immunotherapy including the specific activation of dendritic cells and T cells to generate a cytotoxic response towards the cancer cells either within the host or *ex vivo* followed by transfer the activated cells back into the host (Dudley et al., 2008). There have also been attempts to develop peptide-based vaccines to treat diseases such as cancer by stimulating T cells to target tumour cells (Lazoura and Apostolopoulos, 2005). The wide variety of attempts to treat the same disease indicates the use of immunotherapy has wide applications and that stimulating the immune system to fight a particular type of disease is not as easy as selecting any antigen to stimulate any type of response.

Currently, a wide variety of antigens and adjuvants have been used in an attempt to stimulate the innate immune response, which in turn, will give rise to a strong adaptive immune response. In general, immune stimulants can stimulate recruitment of inflammatory cells, promote ingestion of microbes, stimulate production and release of pro and anti-inflammatory agents, induce antimicrobial action and aid in tissue repair (Elsbach, 2003). Since there are a

wide variety of receptors that can interact with microbial molecules to elicit a response, there is a wide variety of ligands that are being examined for immunotherapeutic treatments including viral and bacterial molecules and synthetic oligonucleotides. In most cases the recognition of the ligand by the receptor results in an intracellular signalling cascade that leads to the activation of NF-κB or IRF-3 which are transcription factors that give rise to the pro-inflammatory cytokines and chemokines (Beutler and Rietschel, 2003; Colina et al., 2008). Combined with an antigen or on their own these immunotherapeutic treatments could accelerate the generation of a robust immune response, sustain responses longer, induce local immune responses, generate antibodies with increased avidity and neutralization capacity and elicit strong cytotoxic T lymphocyte responses (Pashine et al., 2005). This would be beneficial to everyone, but especially to individuals with weakened immune systems such as the elderly, children or immunocompromised individuals. There would also be benefits to those infected with a bacterial or viral infection that do not respond well to currently available antibiotics (Levy et al., 1999). The more that is understood about the immune system, especially the innate immune system, the more effective immunotherapeutic treatments can be developed to fight potential disease and infection.

2.2.3 Challenges of Immunotherapy

Despite the promises of immunotherapy there are several challenges with its use to treat infection and disease. The immune system is a complex system and its interaction with certain foreign materials and how certain disease can be controlled is almost always never fully understood. Another challenge that has been faced with immunotherapy is moving effective *in vitro* studies *in vivo*, as the molecule may experience different interactions with different molecules inside the host that do not exist in the *in vitro* setting. The molecule needs to remain in its tertiary structure so the delivery vehicle cannot alter the structure of the stimulant or the immune stimulating ability of the molecule could be lost (Pashine et al., 2005). To get an optimal response using antigens the right antigen or immunostimulant needs to be selected that will stimulate the system and provide immune memory. Even when the right immune stimulating molecule is being used, there is often the problem of the delivery of the molecules to the target cells in large enough quantity to stimulate the immune system but not cause any

toxic effects towards the host. One of the biggest issues with use of immunostimulating molecules for immunotherapy has been concerns with possible toxicity and adverse side effects in the form of tissue damage and disrupted cellular functions which can be found with some immunostimulants, especially at higher doses (Choi et al., 2006; Gupta and Siber, 1995; Krieg, 2006). Not that many immunotherapeutic molecules have been approved for clinical use, as regulatory guidelines and toxicity testing are still being formulated (Pashine et al., 2005).

The effective delivery of these immunotherapeutic molecules has been a major barrier to their commercial development since these compounds are often very expensive and have a very short half-life (Choi et al., 2006; Krieg, 2006). The selection of the proper immune stimulant is only half of the problem of achieving effective immunotherapy. The other half is the effective delivery of the molecule to target cells. An ideal delivery system would be compatible with a wide variety of compounds and have an excellent safety profile (Heath et al., 2003). A system that could also significantly enhance the biological activity of the immune stimulating molecule would also reduce the cost of vaccination as lower doses would be just as, or more effective then the higher doses of free antigen (Choi et al., 2006; Croyle et al., 1998). The route of delivery is also something that needs to be considered in immunotherapy, as the target cells and their location play a critical role in an optimal immune response. There are some challenges in the development and use of an effective immune stimulating molecule for use in immunotherapy, but with the correct selection of immune stimulant and technique to deliver the molecule the immune system can be stimulated adequately to provide the host with protection against certain types of infection and disease.

The use of immune stimulating molecules to prevent infections does have some potential drawbacks. The innate immune response generally does not confuse self molecules with non-self molecules as their PRR recognize molecules that are unique to foreign microorganisms such as components of the bacterial cell wall like lipopolysaccharides (LPS) or nucleic acids like unmethylated CpG motifs (Kaisho and Akira, 2003). In most cases the adaptive immune response does not mount a response against self antigens due to self-nonself discrimination. Some of the adaptive immune cells will recognize self-antigens. These autoreactive cells are eliminated during early development which will prevent reaction against

self-cells (Sprent and Cho, 2008). However, autoimmune diseases can develop if PRRs are stimulated inappropriately (Matzinger, 2002). In autoimmune diseases like systemic lupus erythamatosus, where the immune system is reacting against RNA and DNA associated antigens, it is possible that Toll-like activation may advance the disease in some cases (Krieg and Vollmer, 2007). This has to be considered in using immune stimulation in certain patients that are at greater risk of autoimmune disease. In most cases inflammatory immune responses are down regulated after their job is complete. This is accomplished by unwanted cells undergoing apoptosis which is signaled by molecules of the TNF family signaling through the type I TNF receptor or through Fas, an apoptosis-inducing receptor (Ayala et al., 2003; Chaplin, 2003).

2.3 Immune Stimulatory and Immunotherapeutic Molecules

To stimulate the immune system to provide the host with a protective response the correct type of immune stimulating molecule needs to be used. There is a wide variety of these types of molecules that can be used based upon what type of response needs to be generated. Various types of antigens from microorganisms are often used to stimulate the adaptive immune response to generate a specific response against a particular pathogen. These antigens can be whole inactivated or attenuated organisms or isolated and purified proteins, glycoproteins and carbohydrates (Pashine et al., 2005). Generally, the use of these molecules focuses on the stimulation of an adaptive response which can be directly stimulated in some cases. However, immunotherapy also focuses on the stimulation of the innate immune response as well by using microbial molecules that are recognized by the PRRs of the innate immune cells. These immune potentiators include bacterial products, toxins, lipids, nucleic acids, peptidoglycans, cytokines, hormones, carbohydrates and other small molecules (Pashine et al., 2005). The purpose of using these molecules is to stimulate the immune system in a general way to either prepare the host to fight a wider variety of microbes or to be administered at the same time as a specific antigen to enhance the both the innate and adaptive response to lead to a stronger response to a particular microbe (Krishnamachari and Salem, 2009; Wagner, 2009). Either by themselves or coupled with an antigen the innate immune stimulating molecules can

provide a strong stimulus to the immune system that could otherwise take multiple treatments to achieve the same type of general protection.

2.3.1 CpG ODN – an example of an immune stimulant

There are a wide variety of immunotherapeutic molecules that can stimulate the innate and adaptive immune systems. A major focus in immunotherapy is stimulating the innate system through use of molecules that stimulate cells through recognition of the cell's TLRs and other innate immune cell receptors. The TLR family are phylogenetically conserved mediators of innate immunity essential for the recognition of microbes (Hemmi et al., 2000). There are currently eleven known TLRs in the mammalian system that have specificity towards different types of ligands (Gupta and Cooper, 2008). For example TLR3 recognizes and binds viral double-stranded RNA, TLR4 binds bacterial LPS, TLR5 binds bacterial flagellin, and TLR9 binds unmethylated CpG oligodeoxynulceotides (Gewirtz, 2003). Upon interaction with their ligand a signalling cascade is stimulated which may vary for the receptor but the end result is the up-regulation of gene transcription factors such as NF-κB and IRF-3 which leads to inflammatory cytokine production (Beutler and Rietschel, 2003; van Duin et al., 2006). There are differences between the TLRs, namely the specific ligands that bind to them and stimulate the pathway which results immune activation. These receptors also differ in their location and abundance. Receptors can be located on the cell surface or inside the cell. The ability of the immunostimulating molecule to reach the target receptor is important to its ability to stimulate a response. As a response at the appropriate level is required; too little of a response will not be effective in controlling potential infections whereas too much of a response or a high dose of ligand can cause damage to host tissue. Immunostimulants like LPS can easily reach their target receptor, TLR4, which is located on the surface of most cells but it can sometimes stimulate very strong immune responses (Beutler and Rietschel, 2003). One immunotherapeutic molecule that is getting a lot of attention due to its ability to effectively stimulate a proper immune response against a wide range of microorganisms are CpG motifs isolated from bacterial microbes as well as synthetic CpG ODN.

CpG ODN is recognized by the intracellularly located TLR9 which is a type I membrane protein containing an extra-endosomal domain with leucine-rich repeats and a cysteine-rich domain (Krieg, 2006). As well, TLR9 contains a transmembrane domain and an intra-endosomal domain which, in turn, contains a region homologous to a Toll/interleukin 1 receptor (Ishii and Akira, 2006). This TLR is located at the endoplasmic reticulum and is then redistributed to endosomes upon stimulation where it interacts with molecules that have been internalized before they are either degraded or sent elsewhere in the cell (Latz et al., 2004). Not all immune cell types express TLR9 but key antigen presenting cells like macrophages and dendritic cells do. It has also been shown that there is an abundance of TLR9 expression in key immune sites such as the spleen and in intestinal epithelium (Gewirtz, 2003; Hemmi et al., 2000).

CpG motifs are not unique to bacterial and viral microbes as CpG motifs are found in vertebrate DNA as well. However, there are structural differences between microbial CpG motifs and vertebrate CpG motifs that allow the immune system to recognize microbial CpG as foreign and mount an immune response against it. Both bacteria and vertebrates posses CpG nucleotides in their DNA, however, bacterial CpG nucleotides are 20 times more abundant compared to vertebrate CpG nucleotides and only bacterial DNA contains unmethylated CpG nucleotides which are flanked by specific nucleotides (Krieg, 2006; Mutwiri et al., 2003). Structurally CpG ODNs are single-stranded olgionucleotides (ssODN) of about twenty-four base pairs in length containing palindromic GC-rich sequences that can be synthesized with a nuclease-resistant phosphorothioate backbone to increase their half life (Ishii and Akira, 2006). It is the unmethylated CpG dinucleotides flanked by specific sequences to the 5’ and 3’ end that make up the CpG motif responsible for immune stimulation (Krieg, 2006). The sequence and structure as well as the number of motifs in the sequence determine the immunostimualtory activity of the ODN. Usually the optimal structure contains two to four motifs with spacing between the motifs of at least two intervening bases, preferably thymine residues, as well as the presence of a poly-G sequence or other flanking sequences in ODN (Krieg, 2006). There is also an enhancement in stimulatory activity if there is a TpC dinucleotide at the 5’ end and a pyrmidine rich 3’ end (Hartmann et al., 2000). Synthetic CpG ODNs can be made which

contain the optimal sequence and structure and still be recognized by TLR9 to generate a strong immune response.

There are three classes of CpG ODN which can generate different types of immune responses. Each class is structurally different and can stimulate a slightly different immune response. The A-class (also known as D-type) include a poly G motifs at the 5' and 3' ends which allows them to form very stable and complex structures called G-tetrads with a central phosphodiester region containing CpG motifs. This class can induce strong IFNα secretion from dendritic cells and moderately induce dendritic cell maturation but this class is very poor at inducing B lymphocyte proliferation (Ishii and Akira, 2006; Krieg, 2006). The B class of CpG ODN (also known as the K type) have a complete phosphorothioate backbone and typically do not form any higher order structures and are typically linear. This class poorly induces dendritic cell IFNα production but strongly induces B lymphocyte proliferation and dendritic cell maturation (Ishii and Akira, 2006; Krieg, 2006). However, if class B CpG ODN is forced into higher-order structures they have the same immune profile as the class A CpG ODN (Gursel et al., 2006). The third class of CpG ODN is the C class which can form duplexes due to palindromic 3' ends. The type of immune response elicited by C class molecules is somewhere between that of the class A and class B CpG ODNs (Krieg, 2006). The different types of responses toward the different forms of CpG ODN indicate that a particular form of CpG ODN can be designed to get an optimal response upon binding to TLR9.

In order to bind to TLR9, CpG ODN needs to be internalized by target cells to interact with the receptor located in the endosome. Natural CpG and synthetic CpG ODN are taken up by cells via receptor-mediated endocytosis (Hacker et al., 1998). CpG nucleotides bind to cell surface receptors but there is no evidence of any CpG sequence specificity in the cell surface binding as non-CpG DNA is able to bind and be taken up by cells at the same rate as CpG ODN (Krieg et al., 2000). Once CpG is taken up by the cell it enters the endosome. Endosome maturation then occurs by acidification of the endosome which leads to a signalling cascade stimulated by TLR and CpG ODN interaction that eventually leads to up-regulation of inflammatory molecules. It has been demonstrated that target cells, including tumour cells, can internalize CpG ODN where it then localizes in the cytoplasm. This localization is in the

endosome where CpG ODN then binds to proteins essential for RNA processing and transport (Zhang et al., 2005). Some of the difficulty using CpG ODN for immunotherapy is the delivery of the molecule to target cells. DNase-I which is found in host serum can eliminate free CpG ODN which would not allow CpG ODN to reach its' target cell and even when it does reach intracellular targets, CpG ODN is still prone to degradation (Ishii and Akira, 2006; Krishnamachari and Salem, 2009). The route of delivery also plays a critical role in ensuring the CpG ODN reaches the target cells. Better protection appears to be generated when the CpG ODN is delivered subcutaneously rather than inhaled as the molecules are slowly absorbed from the injection site and enter the draining lymph nodes where they then enter the systemic circulation and are rapidly cleared into tissues including the spleen (Blanks, 2007; Krieg, 2006). The structure of the CpG ODN molecule dictates the stability and the type of response generated. Therefore, a designed sequence of synthetic CpG ODN which targets a specific immune response in a specific species would be ideal, as not all species respond to specific structure of CpG ODN in the same manner.

The use of CpG ODN to treat and prevent disease shows great promise but there are still concerns about the safety of using this immunostimulant. Since CpG ODN is rapidly degraded a high dose is often required. At very high doses it has been seen in mice that there can be serious side effects such as extramedullary hemopoesis, impaired cross-presentation of antigens, lethal cytokine storms that damage tissue, and lymphoid follicle destruction with repeated administration of CpG ODN (Wagner, 2009). There is also a concern that stimulation of the innate immune system and generation of a pro-inflammatory response could impact the development or exacerbation of autoimmune diseases such as lupus, multiple sclerosis and arthritis by enhancing immunogenicity of self-proteins (Blanks, 2007). CpG ODN has been observed to be rapidly cleared from circulation into the liver, kidney, spleen and bone marrow where mononuclear cell infiltration in these organs was observed in chronically dosed mice but this was not observed in humans. As of yet, there has been no induction of autoimmune disease or adverse renal function in patients treated with CpG ODN (Krieg, 2006). It has even been observed in some animal models, such as the colitis model in mice that treatment with CpG ODN can prevent autoimmune disease (Katakura et al., 2005). In most species it appears that at

least with short term treatment at low doses there are no serious health issues with CpG ODN treatment.

2.3.2 Host Responses to CpG ODN

The target of CpG ODN is the endosomally located TLR9 that, when engaged to CpG ODN generates a signalling cascade. The initiation of this cascade begins when there is interaction between ligand and receptor which recruits, binds and activates the adaptor molecule myeloid differentiation factor 88 (MyD88) which then recruits signal transducers interleukin-1 receptor-associated kinase-1 (IRAK1), interferon regulatory factor-7 (IRF7) and tumour-necrosis factor-α receptor activated factor-6 (TRAF6) (Akira and Takeda, 2004)(Hacker et al., 2006). The interaction between the adapter molecules results in a rapid signalling cascade and activation of several mitogen-activated protein kinases such as extracellular receptor kinase (ERK), p38 and Jun N-terminal kinase. Necrosis-factor-kappa-beta (NF-κB) and activator protein-1 (AP-1) are then activated through the IKK complex and the MAP kinase pathway (Akira and Takeda, 2004; Ishii and Akira, 2006). Depending on the class of CpG ODN and the cell type the type of chemokines and cytokines produced by the activation of gene transcription varies slightly. For example, when plasmacytoid dendritic cells (pDC) are treated with class A CpG ODN a second signalling pathway is stimulated which results in production of type I IFN while myeloid dendritic cells (mDC) do not respond to class A CpG ODN by production of type I IFN (Ishii and Akira, 2006; Kaisho and Akira, 2003). The production of IFN-α by stimulated pDC drives the migration of other pDC in the marginal zone and outer T-cell areas of the lymph node where they are to able to stimulate the adaptive immune response better (Krieg, 2006). In general, the treatment of CpG ODN can affect the host immune response in a number of ways including activation of natural killer cells and antigen presenting cells such as macrophages and dendritic cells. Activation of APC results in their increased migration and upregulation of MHC class II and costimulatory molecules CD40, CD80 and CD86, production of interferons and interleukins that promote Th1 responses and enhance cell-mediated immunity (Blanks, 2007). Macrophages responding to CpG ODN stimulus also produce inflammatory cytokines and are stimulated to produce nitric oxide that aids in intracellular killing of microbes (Hemmi et al., 2000). Besides activating the adaptive

response through activation of the innate immune response, CpG ODN can also directly activate B lymphocytes which results in B-cell proliferation and secretion of antibodies (Blanks, 2007). The effectiveness of this B cell activation depends upon the class of CpG ODN being used to stimulate the host's immune system.

Not only does the class of CpG ODN affect the type of response but there has also been species specificity observed regarding the sequence of the CpG ODN used to stimulate the immune system. It has been demonstrated through lymphocyte proliferation that cattle, sheep, goats, pigs, dogs, cats, horses and chickens respond better to the CpG ODN motif of GTCGTT while rabbits and mice respond better to the motif of GACGTT (Mutwiri et al., 2003). As seen with the different classes of CpG ODN the different sequences can result in cytokine production but no B cell proliferation or vice versa or even result in different cytokine production in the same species (Krieg, 2006; Mutwiri et al., 2003). It has also been demonstrated that CpG ODN can stimulate lymphocyte proliferation in peripheral blood mononuclear cells, spleen and lymph node but not in the blood which suggests cells can even respond differently depending upon their immune compartment (Wernette et al., 2002). In general most species are stimulated by CpG ODN to produce a wide range of cytokines including IL-1β, IL-6, IL-10, IL-12, IFN-α, IFN-γ and TNF-α (Krieg, 2006). This response could be useful in treating particular diseases in most species.

The type of response generated by CpG ODN makes it ideal for prevention and treatment of infectious disease. Since the stimulation of the immune system with CpG ODN provides protective immunity against intracellular pathogens CpG ODN treatment could be used against a wide variety of intracellular infectious challenges and the elimination of chronic infections. There has been a wide array of studies in various species using CpG ODN to treat a vast variety of infectious diseases with varying results. For some examples, in mice protection has been generated against lethal challenges of several intracellular bacteria including challenges with *Listeria monocytogenes* (Krieg *et al* 1998), and *Franciciella tularensis* (Elkins et al., 1999). The length of protection can vary depending on various parameters. For example protection in mice against *Listeria monocytogenes* or *Franciciella tularensis* lasted 2 weeks with only a single dose of CpG ODN (Elkins et al., 1999; Krieg et al., 1998), whereas

protection against herpes simplex virus only lasted for a day. Post-exposure therapy is often ineffective against rapidly progressive acute infectious agents (Krieg, 2006). As of yet there has not been many studies to indicate strong protection with CpG ODN treatment against lethal viral challenges but it is still possible that CpG ODN can induce cytokines that can disrupt viral replication and activate natural killer cells to lyse the virally infected cells to limit the disease (Mutwiri et al., 2003). The mechanism of protection against viral infections needs to be determined before an effective protective response can be targeted. CpG ODN has also been shown to be effective in stimulating a protective response towards extracellular bacteria in chickens likely due to the rapid infiltration of leukocytes into the site of CpG ODN injection resulting in cellular activation (Gomis et al., 2003). CpG ODN is able to stimulate a protective response in a wide variety of species against a variety of infectious disease due to the Th1 type of immune response it can generate and with further understanding of the mechanism of disease and immune response a CpG ODN treatment can be fined tuned to provide strong protection against lethal infection.

2.3.2.1 Chicken Immune Response and CpG ODN

One species that has been shown to be very responsive to CpG ODN treatment is the chicken. Although chickens react to treatment with CpG ODN there was no evidence of a TLR9 orthologue in avian species (Boyd et al., 2007; He et al., 2006). However, it has been recently discovered that TLR21 in chickens acts as a functional homologue to TLR9 and recognizes CpG ODN (Brownlie et al., 2009). Both *in vitro* and *in vivo* chicken studies have found a strong stimulation of the immune response with CpG ODN treatment. Several *in vitro* studies have been conducted using the chicken macrophage cell HD11 where it has been demonstrated that treatment with CpG ODN led to greatly increased nitric oxide production of the macrophage cells and aided in intracellular killing of *Salmonella enteriditis* which is observed when macrophages are activated (He et al., 2003; Xie et al., 2003). It has been demonstrated that CpG ODN treatments are able to provide protection against lethal challenges of bacteria in chickens. In one study it was observed that birds treated with 50 or 10 µg of CpG ODN were effectively protected from a lethal challenge of the extracellular bacteria *Escherichia coli* which can cause cellulitis and colibacillosis (Gomis et al., 2003). CpG ODN

as an adjuvant has also been shown to protect chickens against infections including protection against Newcastle disease virus (Linghua et al., 2007). Treatment with CpG ODN alone has been effective at providing a protective response in chickens to a wide variety of infectious disease both intracellular and extracellular, such as *S enteriditis* and *E. Coli* (Gomis et al., 2003; Mackinnon et al., 2009). This is due to the type of response generated. Neonatal chickens treated with CpG ODN were effectively protected from a lethal challenge of *E. coli* and it was observed through polymerase chain reaction (PCR) studies that there was an increase in the RNA of key inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-18 and IFNγ from cells isolated from the spleen of CpG ODN treated birds (Patel et al., 2008). This observation indicates that CpG ODN stimulates a Th1 response which is effective in dealing with certain bacterial infections in chickens as well as other species.

2.4 Nanotechnology

Nanotechnology involves the study and control of materials and systems at the nanoscale. Nanotechnology generally deals with materials 0.1 to 100 nm in size as well as the manipulation of single atoms and molecules. The results are structures with novel and improved physical, chemical and biological properties due to their small size (Riehemann et al., 2009). Nanotechnology is very diverse and covers many disciplines including physics, chemistry and biology. There are also several different applications of nanotechnology to create new materials and control matter at an atomic level.

2.4.1 Status of Nanotechnology

Nanotechnology and nanoscience are rapidly expanding and being applied to several fields including computers and other electronics, medicine, energy production and packaging in the food industry to name a few. There are two approaches to nanotechnology, the “top down” approach which is based on seeking smaller devices using larger devices to direct their assembly. The second approach to nanotechnology is the “bottom up” approach which aims to arrange smaller components into more complex assemblies. This is based on the idea of self assembly and self organization derived from biology and chemistry (Sozer and Kokini, 2009).

New products containing nanoparticles are reaching consumers daily even if they are not aware of it. In cases where nanotechnology is used in computers and other electronics most people are not concerned about their interaction with the nanosized devices however, nanoparticles are becoming involved more and more in medicine in the form of sensors and drug delivery vehicles as well as in all aspects of the food sector. As people become aware of how much they come in direct contact with these nanoparticles there is a growing concern with the safety of these molecules and if they are actually beneficial.

Nanotechnology has shown great improvements in the fields of medicine and food production and packaging (Riehemann et al., 2009; Sozer and Kokini, 2009). But as of yet in the food industry there are no worldwide accepted rules or regulations even though there are over 200 manufacturers in the USA alone that market products as nanoproducts (Sozer and Kokini, 2009). These manufacturers still follow regulations on food preparation but there are social and ethical issues using nanotechnology in the food sector and in medicine. A lot of the concerns with nanotechnology come from the public perception that could be generated by the lack of information for the public on nanotechnology and its benefits in drug delivery and other areas.

Nanoparticles are currently being used in medicine and the food sector as delivery vehicles of various therapeutics, proteins and enzymes as well they are also used as bio-sensors for disease and bacteria (Bouwmeester et al., 2009; Riehemann et al., 2009). Nanoparticles used as delivery vehicles encompasses a wide range of particles being used for delivery of a wide variety of molecules. Some particles being used include nano-encapsulators that are lipid-based and polymer-based and nanoparticles such as nanotubes (Bouwmeester et al., 2009; Pastorin, 2009). The use of nanoparticles in animal and human systems is relatively new and long term effects of their presence in the biological system are not fully understood. This in part leads to knowledge gaps in assessing their risk in the fields of medicine and food. Some studies suggest that nanoparticles toxicity may deviate from the toxicity profile of the chemicals that they are comprised of due to their size and function (Nel et al., 2006). Some consider that the toxicity studies do not fully observe the natural exposure of the nanoparticles on the host as tests are often done at high concentrations of nanoparticles under artificial conditions (Bouwmeester et

al., 2009). *In vitro* toxicity studies provide relevant observations of how nanoparticles can cause toxicity at the cellular level which can give some insight on how they will behave *in vivo*. It has been found in some studies that certain nanoparticles can trigger the release of reactive oxygen species which then causes oxidative stress and inflammation by interaction between the reactive oxygen species and the reticulo-endothelial system (Nel et al., 2006). These types of studies give an initial insight of the toxicity threat of the nanoparticles and an idea of what dose can be harmful. Nevertheless, long term *in vivo* studies need to be conducted to see how the biological system responds to exposure to nanoproducts over time. There is concern that nanoparticles could act in a similar manner as asbestos due to their needle-like structure and cause tissue damage with long exposure as had been seen in mice organs at very high concentrations of nanoparticles (Poland et al., 2008). Currently there is limited long term toxicity information available regarding exposure to nanoparticles. With the advancing field of nanotechnology both humans and animals are coming in contact with nanoparticles on a more regular basis. The use of these particles have led to advances in drug delivery and food preservation but there still remains the ethical side of the use of nanotechnology in the biological system. All nanoproducts go through intense safety testing but due to lack of information there still remains the perception that in the long term heavy exposure to these products could lead to harmful effects which could outweigh the benefits.

2.4.2 Promise of Nanotechnology and Medicinal Application

The field that nanotechnology shows the most promise for significant improvement to current applications is in medicine. Nanomedicine uses nanoparticles for a wide variety of applications including using them to target certain cells to either destroy or repair them. Nanoparticles can also be used as nano-biosensors or to deliver or aid in the delivery of drugs to target cells. The size and site specific properties of the nanotechniques in medicine could lead to development of personalized medicine that can be modified to treat each individual at the proper time and site which would be ideal for cancer and infectious disease treatments (Riehemann et al., 2009). Various uses of nanoparticles have been found in medicine including their use in medical diagnostics which leads to more effective early diagnostics to prevent and effectively treat disease. Both *in vitro* and *in vivo* diagnostic techniques have incorporated the

use of nanoparticles including nanoarrays for DNA analysis and nanocantilever arrays that use resonant frequencies that result from binding to biomarker proteins to detect specific diseases (Riehemann et al., 2009). *In vivo* imaging can use nanoparticles and their properties to target specific cells such as cancer cells. Nanoparticles have a high surface area to volume ratio that allows the attachment of other molecules that can bind to specific markers on cancer cells. The injection of these functionalized nanoparticles into a patient would result in the molecules binding to tumour cells which then can be imaged with the use of labels on the nanoparticles (Nie et al., 2007). Diagnostic applications are very useful for preventing disease and early detection and proper treatment. Another promising use of nanoparticles in medicine is in drug delivery.

Nanoparticles that are able to be attached to other molecules are very useful as nanovectors to deliver their cargo to target cells. These nanovectors have the ability to pass through biological barriers and localize in target tissue (Sakamoto et al., 2007). There are a wide variety of types of nanoparticles being attempted for use as drug delivery vehicles. There are lipid based molecules that can be used to carry drugs however they can have the problem of not being able to effectively enter the cell to deliver their drug directly (Riehemann et al., 2009). There are also natural polymers used as delivery vehicles such as proteins or polysaccharides which are internalized and rapidly degraded allowing release of their cargo drug or gene but there have been some severe side effects with their use (Riehemann et al., 2009). Another promising candidate for nanoparticle drug delivery is carbon nanotubes which can be attached to a wide variety of proteins and nucleic acids (Bianco et al., 2005; Lacerda et al., 2006). These various nanoparticles show great promise for use as a drug delivery vehicle, each with their own benefits and challenges. With more studies delivery vehicles can be developed to carry specific cargos to specific cells and even release the drug a specific time points to get more effective treatments. The biology of the potential immune response to these particles and the limitations of the molecules need to be evaluated to give a full perspective of their clinical use.

2.4.3 Nanobiotechnology

To improve the application of nanoparticles for use in biological systems such as the use of nanoparticles as drug delivery vehicles the biological and biochemical nature of the particles needs to be studied as well as the molecules with which they interact. This lies in the field of nanobiotechnology which is similar to nanomedicine and nanotechnology but differs slightly as nanobiotechnology encompasses the basic research at the nanoscale level on biological systems and uses existing elements of nature to develop new products. An example is the linking of an immune stimulating molecule such as CpG ODN to nanoparticles such as carbon nanotubes to improve the immune stimulating capabilities of the CpG ODN molecule. The process of linking the two molecules effectively requires knowledge of the biology and chemistry of the two molecules.

Carbon nanotubes are composed exclusively of carbon atoms and belong to the family of fullerenes which is an allotropic form of carbon. They are arranged in a condensed polyaromatic surface rolled-up into a tubular structure with closed ends. In general there are two main forms of carbon nanotube, the single walled form, with a single cylindrical graphene sheet and the multiwalled form which is made of several concentric graphene sheets spaced approximately 0.34 nm apart (Bianco et al., 2005). The problem exists with carbon nanotubes in that they have poor solubility in almost all types of solvents. However, this problem can be overcome with the covalent or non-covalent functionalization of the carbon nanotubes which not only makes them more soluble but also allows effective attachment of a wide variety of proteins and other molecules to their surface (Bianco et al., 2005; Pastorin, 2009). It has been demonstrated that functionalized carbon nanotubes are taken up by cells and are less harmful nanovectors (Pastorin, 2009). There is also no direct correlation between the type of functionalization and their ability to be internalized indicating that numerous different chemical procedures could be used to functionalize the carbon nanotube (Kostarelos et al., 2007). There are still safety concerns with use of carbon nanotubes in the biological system as there is with most, if not all, nanoparticles. Currently there is no long term data to confirm or deny the threat of toxicity after long time exposure to carbon nanotubes either directly or through diet of animals that have been exposed to carbon nanotubes or other nanoparticles. The promise of

improving delivery to target cells and improvement of immune stimulating molecules by protection makes the use of nanoparticles as drug delivery vehicles an exciting possibility.

3.0 Hypothesis

It has been demonstrated in other studies that CpG ODN are able to stimulate a protective immune response in different species either alone or as an adjuvant (Mutwiri et al., 2003). In chickens, CpG ODN has been found to stimulate a protective immune response on its' own against a lethal bacteria challenge. The problem exists that CpG ODN are often required at a high dose to elicit an effective immune response. CpG ODN is a very expensive molecule and high doses raise the cost of using CpG ODN as an immunotherapeutic drug. A means to lower the effective dose of CpG ODN would be ideal for its use to prevent infections. It has been demonstrated that a wide variety of molecules can be linked to carbon nanotubes, including nucleic acids such as CpG ODN.

It is my hypothesis that carbon nanoparticles can be used to maintain the biological function of CpG ODN while enhancing the biological activity of CpG ODN without significantly compromising the host immune response and safety in the chicken model.

4.0 Objectives and specific aims

My goals are to:

- Enhance the biological activity of CpG ODN using MWCNTs without altering CpG ODN's biological function.
- Understand mechanism of actions of MWCNT linked CpG ODN as compared to to free CpG-ODN.

Specific aims:

In order to achieve the goals mentioned in the preceding section, I compared the activity of free and bound CpG ODN with MWCNT. My specific aims are

1. Selection of the best MWCNT bound CpG ODN in regards of immune stimulating capabilities.
2. Measurements of uptake of MWCNT-CpG ODN by viable cells.
3. Evaluation of toxicity of MWCNT, CpG ODN, and MWCNT-CpG ODN in cells and the comparative immune stimulation of these cells by these molecules.
4. Evaluation of the efficacy enhancement of CpG ODN by MWCNT by ability to protect chickens against bacterial infection.
5. Initiate the exploration of the molecular mechanism of immune stimulation by MWCNT linked CpG ODN.

5.0 Materials and Methods

5.1 Cell Culture

5.1.1 Splenic Lymphocytes

Chickens were humanly euthanized at the age of 2-4 weeks with halothane. The spleens were removed aseptically and separately placed in 10 mLs of Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, Canada). Spleens were macerated and passed through a 70 µM mesh strainer (BD Biosciences, USA) with a syringe plunger to obtain a single-cell suspension. Lymphocytes were washed through the mesh with 10 mLs of HBSS. Single-cell lymphocyte solution was kept on ice for 5 minutes and tissue that settled on the bottom of the tube was removed. Lymphocyte suspension was then overlaid onto 15 mLs of HISTOPAQUE®-1077 density gradient medium (Sigma-Aldrich, Canada). The density gradient was then centrifuged at 700 x g for 20 minutes at room temperature. Lymphocytes at the interface were then collected and washed three times with HBSS. Lymphocytes were then suspended in complete Dulbecco Modified Eagles Medium (DMEM, Sigma Aldrich, Canada) with 2 mM L-glutamine (Sigma-Aldrich, Canada), 1mM sodium pyruvate (Sigma-Aldrich, Canada), 10% Fetal Bovine Serum (FBS) (Lonza Walkersville, Inc. USA), 100 U/mL penicillin (Invitrogen, Canada), 100 µg/mL streptomycin (Invitrogen, Canada), and 2 x 10^{-6} M 2-mercaptoethanol (Sigma-Aldrich, Canada). Viable cells were counted by trypan blue dye exclusion assay (Miyamoto *et al.* 2002).

5.1.2 Chicken macrophage cell line HD11

The avian MC29 virus-transformed chicken macrophage cell (HD11) were obtained from Dr. Reno Pontarollo (Genome Prairie, Canada). Cells were cultured in 75cm^2 flasks (Corning CELLBIND® Surface, USA) at 37°C in a 5% CO_2 incubator. Cells were maintained in RPMI 1640 media (GIBCO®, USA) with 10% FBS (Lonza Walkersville, Inc. USA) and 50 µg/mL of gentamicin (Sigma, USA). Viable cells were counted by trypan blue dye exclusion assay.

5.2 Linkages of Carbon Nanotube to CpG ODN

5.2.1 Linking via Adsorption

5.2.1.1 Adsorption chemistry with a linker (MWCNT-PySE-CpG ODN)(MWCNT-1-CpG)

MWCNTs were purchased from Cheap Tubes Inc. (USA) and were used without any further purification. MWCNTs at 5 mg/mL were suspended in N,N-Dimethylformamide (DMF) (Sigma-Aldrich, Canada). Separately 1-pyrenebutanoic acid, succinimidyl ester (PySE) (Invitrogen, Canada) was also suspended in DMF at a concentration of 5 mg/mL. The MWCNT suspension was then mixed with the PySE suspension in DMF at a 1:2 ratio with a final concentration of 1 mg/mL MWCNT and 2 mg/mL PySE. This reaction mixture was then incubated in the dark at room temperature for 4 hours. Under these conditions PySE is absorbed on the hydrophobic surface of MWCNT (Chen et al., 2001). CpG ODN was then added to the MWCNT-PySE solution at a 1:1 ratio. The reaction was continued overnight at room temperature in the dark. In the aqueous environment the succinimidyl ester group is hydrolyzed and single stranded CpG ODN is thought to be non-covalently absorbed on the aromatic surface of MWCNT-PySE (Kam et al., 2005).

5.2.1.2 Direct Adsorption Chemistry (MWCNT-CpG ODN)(MWCNT-2-CpG)

CpG ODN was mixed with MWCNT at a 1:1 ratio for a final concentration of 1 mg/mL in PBS (20 mM, pH 7.2, made in house) and shaken overnight in the dark at room temperature.

5.2.2 Linking Via Electrostatic Interactions (MWCNT-PEI-CpG ODN)(MWCNT-3-CpG)

The MWCNT-COCL was made by first measuring 10 mg of the MWCNT-COOH, purchased from Cheap Tubes Inc. (USA), and dispersed them in 500 µL of N, N-Dimethylformamide (DMF, Sigma-Aldrich, Canada). The 5 mg/mL sample was sonicated using a pulse sonicator 1/3” probe for 5 minutes at 5 second intervals. 250 µL of Thionyl Chloride ($SOCL_2$, Sigma-Aldrich, Canada) was slowly mixed with 250 µL of Dichloromethane (DCM, Sigma-Aldrich, Canada). The mixture was then added to the sonicated MWCNT-COOH in DMF drop by drop and allowed to reflux on a heat block for 1hr at 65ºC. The tube was vortexed occasionally during the hour. After that time, 250 µL of the solution was put into

two clear eppendorf tubes each. The excess $SOCL_2$, DCM and DMF were removed using a Savant SC11OA SPEEDVAC ® (Thermo Scientific, USA) for 30 minutes. The final products contain a concentrated amount of 2.5 mg of MWCNT-COCL in each tube.

The conjugation of branched Polyethyleneimine (PEI) to MWCNT-COCL was done by mixing 5 µL of PEI, which is dissolved in 1-Methyl-2-pyrrolidinone, (NMP, Sigma-Aldrich, Canada) at 10 mg/mL, to each 2.5 mg of MWCNT-COCL then topped with an additional 225 µL of NMP. The mixture, now at 0.21 mg/mL of PEI and 11 mg/mL of MWCNT-COCL, was pulse sonicated for 5 minutes at 5 second intervals then put on a shaker in the dark at room temperature overnight. The next day an additional 10 µL of PEI was added and sonicated as above then placed on the same shaker in the dark for 2 hrs. Another aliquot of 10 µL of PEI was added to the mixture and sonicated again for 5 minutes at 5 second intervals. The resulting reaction, now two tubes of 1 mg/mL of PEI and 11 mg/mL of MWCNT-COCL in 250 µL of NMP solution was again put on the shaker overnight at room temperature in the dark.

The next day each tube was dried of excess NMP and PEI using Savant SC11OA SPEEDVAC (Thermo Scientific, USA) concentrate the MWCNT-COCL-PEI as our final product. 500 µL of PBS was added to the tube. The final product, now at 0.5 mg/mL of PEI and 5.5 mg/mL of MWCNT-COCL. CpG ODN was added to MWCNT-PEI at a 1:1 ratio and shaken overnight in the dark

5.2.3 Dialysis of MWCNT-CpG ODN

To purify the MWCNT-CpG ODN dialysis was performed. Excess reagents (PySE, PEI and CpG ODN) were removed by dialysis for 24 hours at 4^oC in PBS (20mM, pH 7.2, made in house) using a SLIDE-A-LYZER ® Dialysis Cassette (extra strength) with a 10 kDA molecular weight cutoff (Pierce Biotechnology, USA). The PBS dialysis solution was changed three times to remove free CpG ODN, PysE or PEI from the dialysis solution. The final concentration of the MWCNT-CpG ODN conjugate was determined by measuring the nucleic acid concentration of the MWCNT-CpG ODN remaining in the dialysis cassette. CpG ODN concentration was measured at 260 nm using a NANODROP ® spectrophotometer ND-1000 (Nanodrop Technologies LLC, USA).

5.2.4 CpG ODN

CpG ODN was a gift from Merial Limited (USA). The CpG-ODN used was CpG-ODN 2007 which is 22 bases in length with the sequence of t**cg**t**cg**ttgt**cg**ttttgt**cg**tt. **cg** sequences are the CpG dinucleotides which stimulate an immune response (Mena et al., 2003).

5.2.5 Biophysical Characterization of the Formulation

5.2.5.1 Raman Spectroscopy

Raman spectroscopy was done with MWCNT-PySE CpG ODN and controls using a Renishaw ® 2000 Raman microscope operating with a 785 nm exicitation laser line (7mW at the laser head) and a 1200 lines/mm grating. Dry solids were placed on a Au-plated Si wafer, and the microscope was focused onto the surface of the solids using a 50X long working distance objective (NA=0.50). Raman measurements were collected using a 10s detector response time, and the number of accumulations varied from sample to sample.

5.2.5.2 Fluorescence Measurements

Fluorometric emission spectroscopic measurements were performed on various samples such as CNT, CNT-PySE, CpG-PySE and CNT-PySE-CpG using Hitachi F-2500 (Japan). Emission spectra were recorded between 370 nm and 500 nm by exciting the samples at 340 nm with 5 nm bandwidth at both excitation and emission windows.

5.3 Cellular Proliferation

To observe the ability of MWCNT-CpG ODN and CpG ODN to stimulate a proliferative B cell response lymphocytes were isolated from the spleen of chickens as described earlier. Lymphocytes were plated in a flat bottom 96 well plate at a concentration of 2.5×10^4 cells/well. The cells were treated with various concentrations of the three MWCNT-CpG ODN conjugates or free CpG ODN ranging from 5 µg/mL to 0.005 µg/mL. Cells were also left untreated as a negative control. For a positive control cells were treated with 5 µg/mL of Concanavalin A (Con A). Each treatment was done in triplicate. After treatment cells were incubated for 48 hours at in a 5% CO_2 incubator at 41^{o}C. Proliferation was measured using the

Quick cell proliferation kit II (BioVision, USA). Proliferation was measured by the ability of viable cells to cleave the tetrazolium salt WST. After the incubation time cells were treated with 10 µL WST per well and incubated another 4 hours. The optical density at 440 nm was then measured. Fold Change of cells was calculated by comparing the A_{440} of treated cells to the A_{440} of media treated control cells. Results from the proliferation studies helped to select the best conjugate for further studies.

5.4. Comparative Cellular Uptake

5.4.1 Labeling of CpG ODN and MWCNT-CpG ODN

To make the same pure conjugate with a label for confocal use, CpG ODN 2007 was purchased with Cy3 dye (Integrated DNA Technologies, USA) attached. The sequence of the CpG ODN was the same as the sequence used in all other studies. Ethanol precipitation was performed to purify the oligodeoxynucleotide by removing any free dye. MWCNT-PySE was conjugated to the labeled CpG ODN using the same techniques as described previously. Dialysis was performed as described previously with PBS being changed until no visible dye was left in the dialysis solution over a 2-3 day period. The optical density was measured for the purified labeled MWCNT-PySE-CpG ODN conjugate using a NANODROP spectrophotometer (Nanodrop Technologies LLC, USA). The concentration of CpG ODN (µg/mL) was measured at 260 nm while the Cy3 concentration (µM) was calculated using the Beer-Lambert law ($A = \varepsilon / c \cdot l$) using the absorbance at 550 nm.

5.4.2 Confocal microscopy

A 24 well cell culture plate (COSTAR ® Corning Incorporated, USA) received 1.0 x 10^6 cells/ well of HD11 cells along with the appropriate media and treatment to equal a total of 500 µL per well. Treatments included Cy3 alone (Amersham Biosciences, UK), CpG ODN-Cy3 and MWNT-PySE-CpG ODN-Cy3 at a final concentration of 3µM of Cy3 for each treatment. A time course was performed at the time points of 30, 60, 120, 240 minutes. All cells were also treated with Hoechst 33342, (Invitrogen, USA) for one hour to visualize the cell's nucleus.

Cells were observed live using glass bottom culture dishes (MatTek Corp., Ashland, MA, USA) on a Leica ® 1-photon confocal microscope (DMI 6000 B inverted, TCS SP5, Germany).

A live cell chamber was attached to the motorized scanning stage. Diode laser was used for Hoechst (excitation 405 nm) and the HeNe 543nm laser was used for Cy3 (excitation 550nm). The conventional fluorescence filters were: Analyzer, Dapi, Green, and Red. The objective used was the 40x/1.25-0.75 oil (Leica Plan NEOFLUAR ®). The tunable dichroic mirrors used for all lasers were AOBS. The detector consists of the following; two channel, all cooled PMTs, all spectral and transmitted light detectors. The computer software used was HP ® xw6400 workstation Intel XEON ® CPU, 4 GB RAM, Dual hard- drives, CD/DVD burners, MICROSOFT WINDOWS XP ® Professional SP2 and LEICA LAS AF 1.8.2.

5.5 Toxicity Assays

5.5.1 Cell Viability

In vitro toxicity of MWCNT-COOH and MWCNT-PySE-CpG ODN was measured using a CELLQUANTI-MTT^{TM} cell viability assay kit (Bioassay Systems, USA) following the manufacturer's protocol. The HD11 cells were cultured at a concentration of 5.0 x 10^4 cells/ well in a 96 well flat bottom plate (VWR, Canada). Cells were treated with a 10-fold dose titration of MWCNT-COOH, CpG ODN or MWCNT-PySE-CpG ODN starting at 100 µg/mL and ending at 0.001 µg/mL. HD11 cells were incubated at 41^{o}C in a 5% CO_2 incubator for 1,3, and 7 days. Cells treated with media alone acted as a negative control. Cells treated with 0.1% Saponin (FLUKA ® Analytical, USA) acted as a positive control as Saponin rapidly destroys cellular membranes resulting in cell death. Cell viability was measured by the ability of the living cell to convert MTT, a tetrazolium salt, to formazan. The optical density (OD) of the product was measured at 570 nm on a BENCHMARK^{TM} microplate reader (Bio-Rad Laboratories Inc., USA). All treatments were done in triplicate.

5.5.2 Measurement of Nitric Oxide and Peroxide Production

In vitro nitrite production was measured using a nitric oxide assay kit (Biomedical research service, USA). Nitrite represents the levels of nitric oxide (NO) as it is the more stable metabolite (He et al., 2003). Chicken macrophage cells (HD11) were cultured in a 96 well

round bottom plate (VWR, Canada) at a concentration of 5.0 x 10^5 cells/well. Cells were treated with a 10-fold dose titration of 100 µg/mL to 0.001 µg/mL of MWCNT-COOH, CpG ODN or MWCNT-PySE-CpG ODN and then were incubated at 41°C for 1, 3 or 7 days in a 5% CO_2 incubator. For a negative control cells were treated with media alone which was RPMI 1640 with Gentamicin reagent solution and 10 % Fetal Bovine Serum (FBS). For a positive control cells were treated with 1ug/mL of LPS (Sigma-Aldrich, Canada) which stimulates NO production in HD11 cells (Crippen et al., 2003). After incubation cells were pelleted by centrifugation at 400 x g for 4 minutes and 100 µL for each well was removed and placed in a new flat bottom 96 well plate. Nitrite standards were prepared from fresh sodium nitrite and 100 µL of nitric oxide assay solution was added to each well both standard solution and assay solution were provided by the Nitric Oxide Assay Kit (Biomedical Research Service Center, USA). The optical density of the samples was read at 540 nm on a Benchmark microplate reader (Bio-rad laboratories, USA) and the concentrations of nitrite were calculated based on the standard curve generated from the nitrite standards. The nitrite content in cell-free medium was subtracted from the values obtained with cells. All treatments were done in triplicate.

In vitro peroxide production was measured using a peroxide assay kit (Biomedical Research Service Inc., USA). HD11 cells were plated at a concentration of 5 x 10^5 cells/well and were treated with a 10-fold dose titration of 100 µg/mL to 0.001 µg/mL of MWCNT-1-CpG ODN, MWCNT, or CpG ODN an incubated for 24 hours in a 5 % CO_2 incubator at 41^{o}C. Media treated cells were used as a negative control. Media used was RPMI 1640 with Gentamicin reagent solution and 10 % Fetal Bovine Serum (FBS). Cells were pelleted by centrifugation by spinning the cells at 400 x g for 4 minutes. Media was removed and cells were lysed with 1 mL of ice cold double distilled water and vortexing. The lysate was transferred to fresh tubes and spun to confirm lysate. Standards of peroxide were prepared from the kit by diluting stock peroxide solution from 4 mM to 20 µM in double distilled water and performing serial dilutions to obtain concentrations of 2.5 µM, 5 µM,10 µM and 20 µM. 40 µL of double distilled water, lysates and standards were transferred to a new 96 well plate. 200 µL of working solution provided in the kit was added to each well. The absorbance was read with a BENCHMARK^{TM} microplate reader (Bio-Rad Laboratories Inc., USA) at 595 nm. Concentrations of peroxide from the treated cells were calculated using the O.D.'s from the

lysates of the treated cells and the standard curve generated from the peroxide standards. All treatments were done in triplicate.

5.6 Comparative Analysis of Inhibition of Nitric Oxide Production

5.6.1 Endocytosis and Endosomal Maturation

HD11 cells were seeded in a 96 well plate (VWR, Canada) at 2 x 10^5 cells/well and grown overnight (18 hours). The cells were pelleted as explained previously and the media was replaced with fresh media containing various concentrations of monodansylcadaverine (MDC) (Sigma-Aldrich, Canada) or chloroquine (Sigma-Aldrich, Canada). The cells were then pre-incubated for 1 hour in the presence of the inhibitors. Cells were then treated with CpG ODN 2007 (1 µg/mL), MWCNT-PySE-CpG ODN (1 µg/mL) or LPS (1 ug/mL) for a total volume of 200 µL/well. The treated cells were then incubated for an additional 24 hours (He and Kogut, 2003). Nitrite levels were measured as described previously. All treatments were done in triplicate.

5.6.2 Inhibition of MAPK Pathway Signal Transduction

HD11 cells were seeded in a 96 well plate (VWR, Canada) at 2 x 10^5 cells/well and grown overnight (18 hours). The cells were pelleted as explained previously and the media was replaced with fresh media containing various concentrations of PKC inhibitor (Ro 32-0432), p38 MAPK inhibitor (SB-203580), MAPK kinase (MEK) inhibitor (U-0126), IκB phosphorylation inhibitor (Bay 11-7086) or iNOS inhibitor (1400W) (Biomol, USA) for 1 hour. Cells were then treated with either CpG ODN (1 µg/mL), MWCNT-PySE- CpG ODN (1 µg/mL) or LPS (1 µg/mL) for an additional 24 hours (He and Kogut, 2003). Nitrite levels were measured as described previously. All treatments were done in triplicate.

5.7 Protection of Chickens against *Salmonella* Infection

Broiler chicken eggs were obtained from a local hatchery. Upon hatching birds were placed randomly into twelve groups (n=25/group) were treated sub-cutaneously (SQ) with CpG ODN or MWCNT-PySE-CpG ODN at 50, 10, 1, 0.1 and 0.01 µg/injection. Control groups were injected with PBS or MWCNT-COOH for each challenge dose on day 0. Groups were

then placed in separate isolation rooms. Room temperature was maintained at 20 to 22°C. Each room was ventilated with filtered, nonrecirculated air at a rate of 10 to 12 changes/hour. Air pressure differentials and strict sanitation were maintained in this isolation facility. Groups were then challenged with *Salmonella typhimurium* ST5 at 10^8 cfu/chicken on day 3. The *Salmonella typhimurium* used was a field isolate from a 25 week old broiler chicken. Aliquots were stored a -70^{o}C in 50% Brain-heart infusion broth (Difco, USA) supplemented with 25% (w/v) glycerol (VWR Scientific Inc., Canada). Bacteria used for challenges were cultured on triple-sugar iron agar (TSIA) with 5% sheep blood (Becton Dickinson, USA) for 18-24 hours at 37^{o}C. Two to three colonies from the plates were then added to 200 mL Luria Broth (Miller, BDH, UK) and grown at 37^{o}C for 18-24 hours with shaking at 200 rpm. The cultures were diluted in saline to the challenge dose (Taghavi et al., 2008). The survival of the birds was monitored for the next 7 days following the challenge. Clinical, bacteriological and gross pathological evaluations were conducted as described before (Gomis et al., 2003). This procedure was done with the approval of the University of Saskatchewan Committee on Animal Care.

5.8 *In vivo* Cell recruitment

Day old chickens were placed into 7 groups (n=12 per group) with 4 subgroups (Day 1, Day2, Day3 and Day 7 after injection) (n=3 per subgroup). The birds were treated SQ with PBS (negative control), MWCNT (10 µg), CpG ODN (10, 1 or 0.1 µg) or MWCNT-1-CpG ODN (1 or 0.1 µg). Birds were euthanized based on subgroup and the injection site was removed with a 10mm punch biopsy and stored in 10% buffered formalin. Histological slides were prepared and the tissue was scored on cell infiltration 1+ = mild, 2+ = moderate, 3+ = moderate-severe, 4+ = severe.

5.9 Characterization of Surface Antigen Expression

HD11 cells were treated for 24 hours with either media alone (RPMI with gentamicin and 10% FBS), a 10-fold dose titration of CpG ODN (5 µg/mL – 0.05 µg/mL) or MWCNT-CpG ODN (5 µg/mL – 0.5 µg/mL). Cells were washed with a flow cytometry solution (PBSA pH 7.3, sodium azide (0.03%), Gelatin (0.2%) made in house) twice by spinning down cells

and resuspending them in 15 mL flow cytometry solution. Cells were resupended at a concentration of 20 x 10^6 cells/mL in flow cytometry solution. 50 µL of cell suspension was added to wells of a 96 well plate. All treatments and monoclonal antibodies were done in triplicate. For each treatment there was a group of cells without antibody treatment, one group with the isotype control (mouse-anti human IgG1), one group with mouse-anti chicken MHC I, and one group mouse-anti-chicken MHC II. Antibodies were purchased from SouthernBiotech, USA. The cells were treated with 1 µg/well of primary antibody. Cells were put on ice for 30 minutes. Cells were then washed 3 times with flow cytometry solution. Cells were then treated with the secondary antibody FITC conjugated goat anti-mouse IgG (SouthernBiotech, USA) for 30 minutes on ice in the dark. Cells were washed three times. Cells were then suspended in 2% Formaldehyde (Sigma) and analyzed by FACS with 10,000 cells analyzed per sample. This protocol was adapted from (Xie et al., 2003).

5.10 Innate Immune Gene Expression

The HD11 cell line was treated with various doses of CpG ODN or MWCNT-PySE-CpG ODN for 4 hours or 24 hours at 41°C in a 5% CO_2 incubator. Cells were also treated with a RPMI 1640 media control as well as a negative control of 5 µg/mL of MWCNT. RNA was isolated using an RNEASY ® isolation kit (Qiagen,USA) following the manufacturer's protocol. cDNA was then transcribed from isolated RNA using an iscript CDNA synthesis kit (Bio- Rad Laboratories Inc., USA) following manufacturer's protocol. cDNA was used to test the expression profile of select innate immune genes by use of quantitative real-time polymerase chain reaction (qRT-PCR) as described before (Aich et al., 2007). Expression of select innate immune genes is expressed as fold change values which is the amount of expression in treated cells compared to gene expression to media treated control cells. An average of triplicate measurements was presented and all fold changes were normalized against GAPDH as a house keeping gene. A list of genes examined is shown in Table 5.1.

TABLE 5.1- Primers for Chicken Genes

GENE ID	Accession #	Gene Name	Sequence
GAPDH	U_60320	Glyceraldehyde-3-phosphate dehydrogenase	TGGGCACGCCATCACTATCTTC
			AACACGCTTAGCACCACCCTTC
IFNα	NM_205427	Interferon alpha	TCACGCTCCTTCTGAAAGCTCTCG
			GCAGGCGCTGTAATCGTTGTCTTG
IFNβ	NC_006127.2	Interferon beta	CCACCTTCTCCTGCAACCATCTTC
			TGGTCCCAGGTACAAGCACTGTAG
IFNγ	NM_205149	Interferon gamma	TCCCGATGAACGACTTGAGAATCC
			TGCTTAGAGCTGAGCAGGTATGAG
IL12	NM_213588	Interleukin 12	CACAACCTGGCCAAGGGACTCAAC
			TCTGCAGTGAGGGCACTCAGGTAG
IL1β	NM_204524	Interleukin 1 beta	GCATCAAGGGCTACAAGCTCTAC
			TAGAAGATGAAGCGGGTCAGCTC
IL6	NM_204628	Interleukin 6	GACGAGGAGAAATGCCTGACGAA
			CGCCAGGTGCTTTGTGCTGTA
IL8	NC_000004.10	Interleukin 8	CCTGGGTACAGCTGATCGTAAAGG
			GTCTGACTGAAGTGGCTTCCAAGG

IRF5	NM_001031587	Interleukin regulatory factor 5	CTCCTGGAGCATGGAGTCCCCAAT
			CCGCTGGAGGCTGAACACTTGGAT
IRF7	NM_205372	Interleukin regulatory factor 7	GAATGTGCCATCCCTGCACCATCC
			CCTCCAGCAAGTCCTCAGTGTAGC
MyD88	NM_001030962	Myleoid differentiation factor 88	GGTAGACAGCAGCGTGCCAAAGAC
			CTTGACCGGAATCAGCCGCTTGAG
NF-κB1	NM_205134	Necrosis Factor kappa beta 1	GCAGAAGCTCATGCCAAACTTCTC
			CAGCATCCTCACATCTCCAGTTAC
p65	NC_006088	Protein 65	GTCCTCTCCCAGCCATCTATGAC
			CGTTTGCGCTTCTCCTCAATGCAC
TICAM1	NM_00108156	Toll-like receptor adaptor molecule 1	CTGGTCCTGCCTGCATACCCATAG
			GCCAGTTGGAGAGGGAGATGAGTG
TLR15	NC_006090.2	Toll-like receptor 15	GTTCTCTCTCCCAGTTTTGTAAATAGC
			GTGGTTCATTGGTTGTTTTTAGGAC
TLR3	NC_006091.2	Toll-like receptor 3	GACAAAGGCAGGAGAAACACAGTC
			GATTCCTTGGCCTTCTGCTCATAG
TLR4	NC_006104.2	Toll-like receptor 4	ATACAAGCCACTCCAAGCCCTCAC
			TACACCCACTGAGCAGCACCAATG

TLR7	NC_006088.2	Toll-like receptor 7	ACGCCCCATATCCTTGTACTCCCT
			AGTCCCAAGATCCAACACCGTCAG
TRAF3	NC_006092	Tumour-necrosis factor-α receptor activated factor 3	ATAAGGTGGCCTTGCTCCAGAATG
			CAACACTTTCCAGCTCAGTCACTC
TRAF6	XM_001235884	Tumour-necrosis factor-α receptor activated factor 6	GTGGAGGCCGAGACTTGGATAGTG
			GCTTCCCGAAGGGCCATCAAACAG

6.0 Results

6.1 Optimization of the Carbon Nanotube linked CpG ODN molecules

6.1.1 Cell Proliferation

To select the chemical linkage to link CpG ODN to MWCNTs, three types of MWCNT-CpG ODN were compared in their ability to stimulate immune response. The first test of the immune stimulating capabilities of the conjugates was to measure the ability to stimulate splenocyte proliferation. Chicken lymphocytes isolated from chicken spleens were treated with the three MWCNT-CpG ODNs, free CpG ODN, or free MWCNT. Cells were also treated with Con A (5 µg/mL) as a positive control, as this mitogen stimulates lymphocyte proliferation. Untreated cells were used as a negative control. Proliferation was measured 48 hours post treatment. It was observed that the stimulating ability of CpG ODN decreased as the dose concentration decreased (Fig. 6.1). The decrease in proliferation stimulating ability was also observed with MWCNT-2-CpG ODN and MWCNT-3-CpG ODN, which used the direct adsorption and linkage with the electrostatic linker PEI respectively (Fig. 6.1). Treatment with MWCNT-1-CpG ODN, which uses adsorption with the linker PySE, was able to stimulate proliferation at all doses as low as 0.05 µg/mL. Figure 6.1 indicates the increased proliferation compared to the media treated control cells for cells receiving 5, 0.5 or 0.05 µg/mL of CpG ODN and the three MWCNT-CpG ODN conjugates as well as 5µg/mL of MWCNT or Con A. Cells treated with MWCNT-1-CpG ODN had significantly higher levels of cellular proliferation compared to MWCNT, CpG ODN, MWCNT-2-CpG ODN and MWCNT-3-CpG ODN treated cells. The level of proliferation of MWCNT-1-CpG ODN treated cells is comparable to the Con A treated control cells.

6.1.2 Gene Expression

To further examine the three techniques used to link CpG ODN to MWCNT, gene expression of the chicken macrophage cell line HD11 treated with various doses of the three MWCNT-CpG ODNs, CpG ODN or MWCNT were examined to see if there is a significant difference in the stimulation pattern of innate immune cells between the three conjugates. After 4 hours post stimulation there was an increase in selected key innate immune genes that

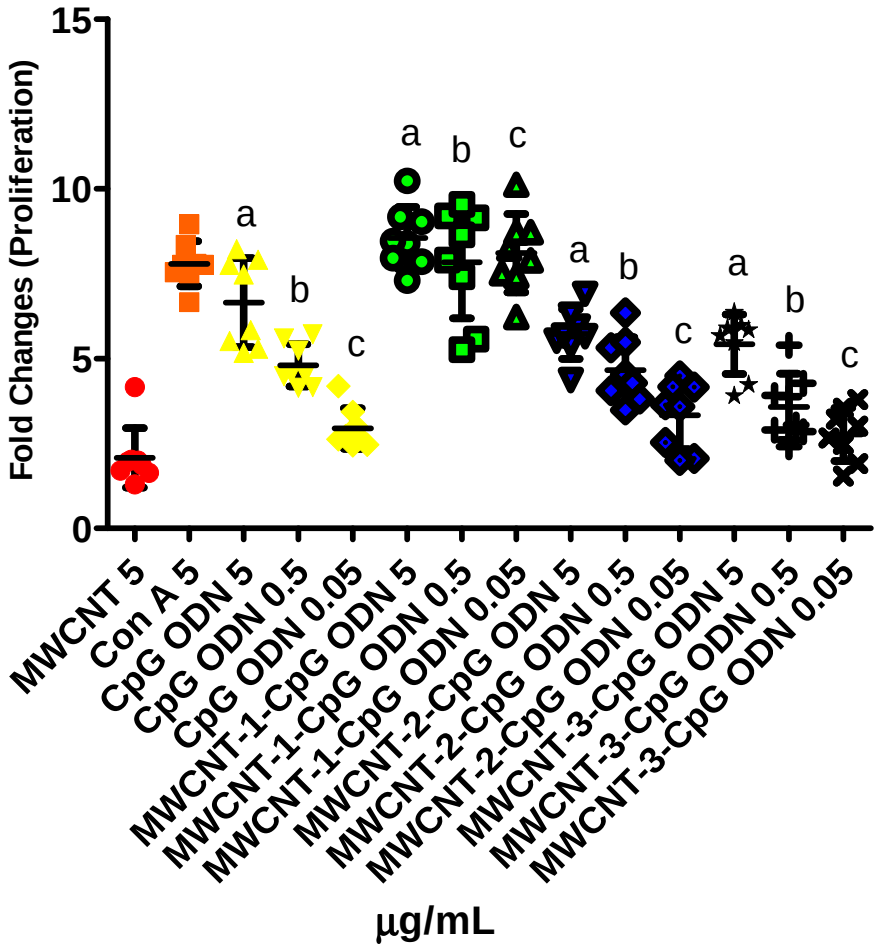
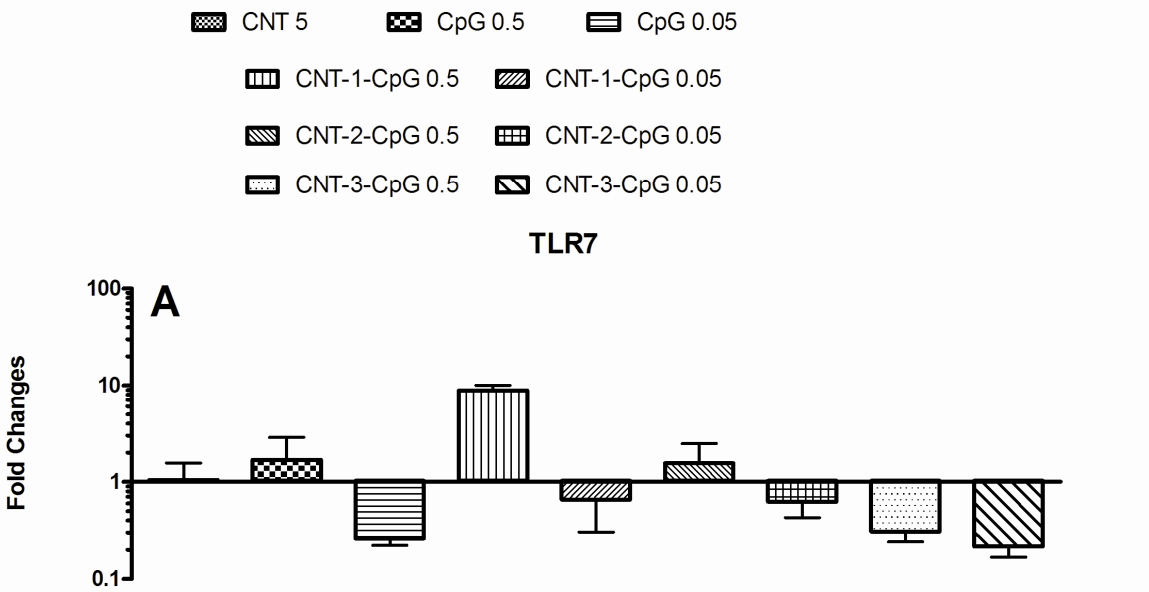
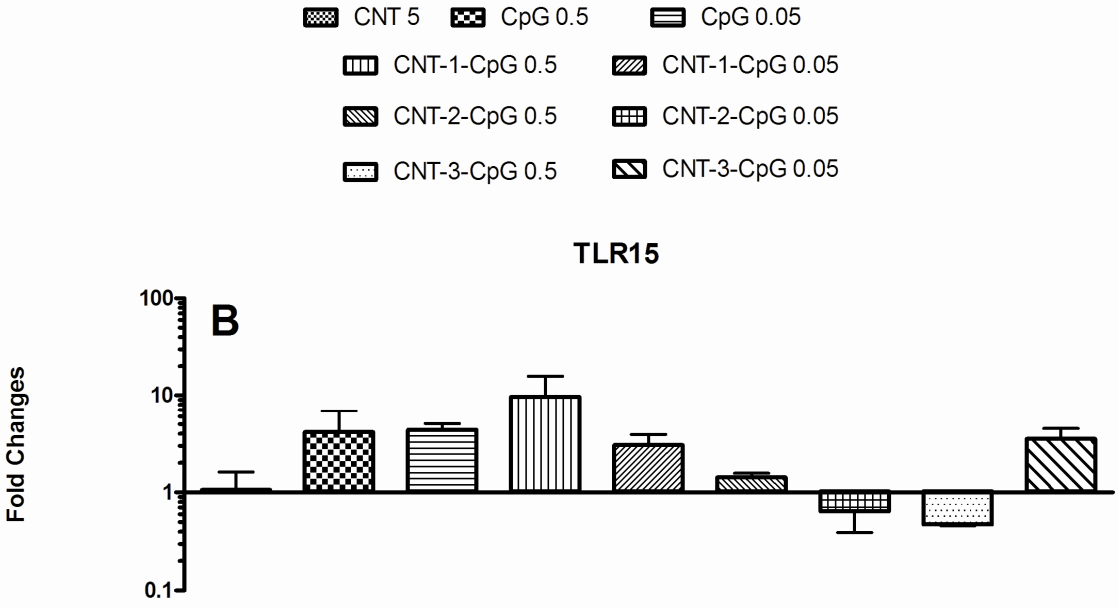


Figure 6.1: Cellular proliferation following stimulation with MWCNT-CpG ODN. Splenocytes isolated from chickens (n=8) were treated with various agents at a dose of 5 µg/mL, 0.5 µg/mL or 0.05 µg/mL. Control cells weretreated with 5 µg/mL of Con A (positive control). Cells were treated for 48 hours. Proliferative response is graphed as the fold change in cell number compared to the cell number of media treated cells. The median of the mean of three replicates for each bird is shown in the graph. MWCNT-1-CpG ODN at each dose generates a significantly higher proliferative response compared to the other treatments at the same dose. $p < 0.01$.


CNT 5
CpG 0.5
CpG 0.05
CNT-1-CpG 0.5
CNT-1-CpG 0.05
CNT-2-CpG 0.5
CNT-2-CpG 0.05
CNT-3-CpG 0.5
CNT-3-CpG 0.05
TLR7
A
Fold Changes
100
10
1
0.1


CNT 5
CpG 0.5
CpG 0.05
CNT-1-CpG 0.5
CNT-1-CpG 0.05
CNT-2-CpG 0.5
CNT-2-CpG 0.05
CNT-3-CpG 0.5
CNT-3-CpG 0.05
TLR15
B
Fold Changes
100
10
1
0.1

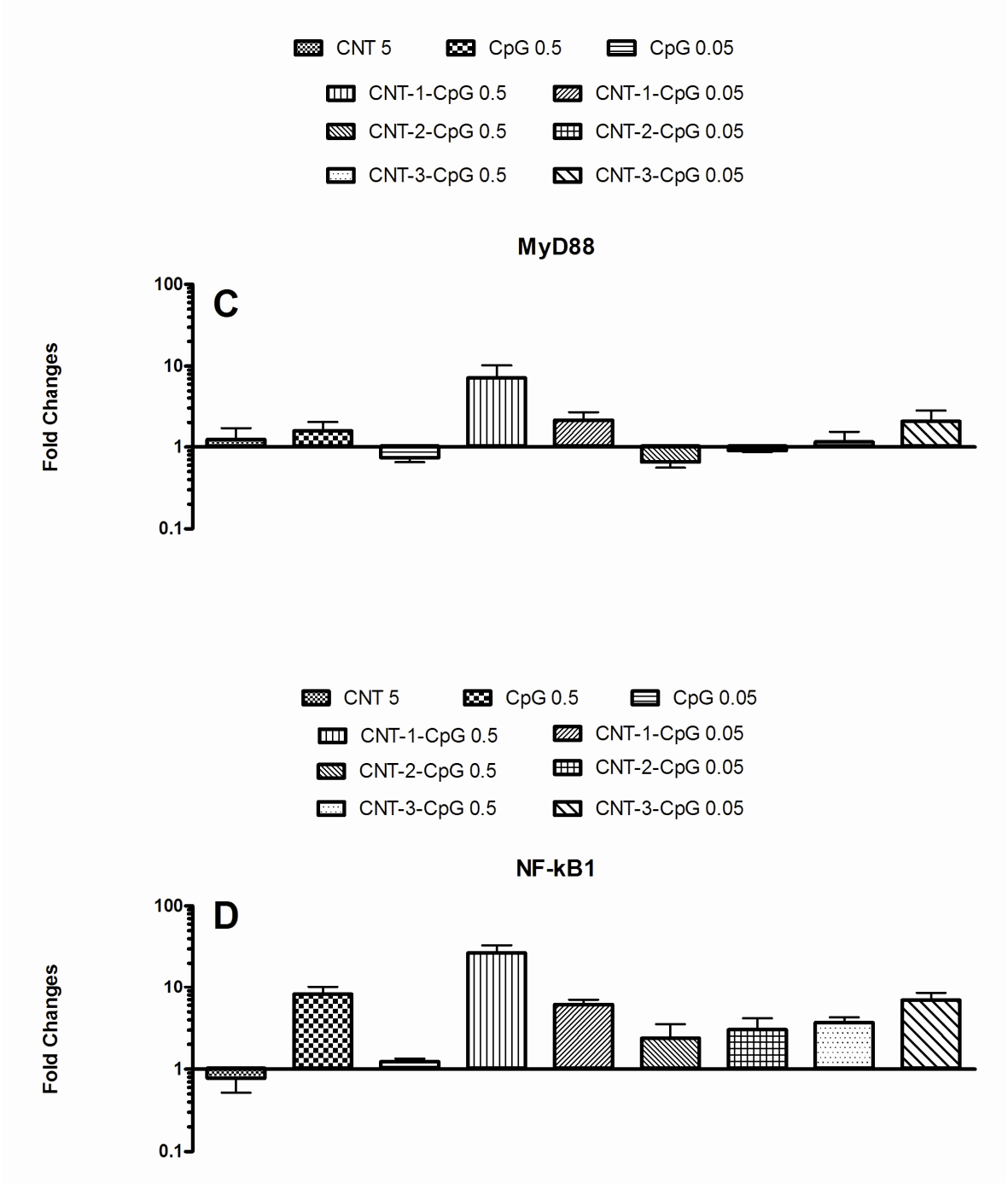


Figure 6.2 Relative fold changes in select innate immune gene expression. Fold changes of select innate immune genes in HD 11 cell with respect to the untreated media control following treatment for 4 hours with various agents at doses of 0.5 µg/mL or 0.05 µg/mL. A) TLR7 B) TLR15 C) MyD88 D) NFκ-β1. Presented as a bar diagram of the median of triplicates with ±1SD as error bars.

showed the same level of up-regulation following treatment with all three conjugates. However, certain innate immune genes, such as TLR7, TLR15, MyD88 and NF-κβ1, showed higher gene expression after treatment with 0.5 µg/mL MWCNT-1-CpG ODN compared to cells treated with the same dose of CpG ODN, MWCNT-2-CpG ODN or MWCNT-3-CpG ODN (Figure 6.2). These results led to the conclusion that MWCNT-1-CpG ODN is the best chemistry to link CpG ODN to MWCNT to get an optimal immune stimulating response from the linked CpG ODN, therefore this linkage using PySE was used for all further studies.

6.2 Biophysical characterization of MWCNT-CpG ODN

6.2.1 Raman Spectroscopy

To characterize the MWCNT-CpG ODN molecule further studies were conducted to see what the effect of CpG ODN has on the MWCNT and ensure CpG ODN is bound to MWCNT. First Raman Spectroscopy was conducted on MWCNT-COOH and then CpG ODN linked MWCNT. In figure 6.3 it is observed that the Raman profile of MWCNT-COOH is altered with the linkage of CpG ODN to the nanotube as the Raman intensity peaks differ significantly from free MWCNT to CpG ODN linked MWCNT. This indicates that CpG ODN is present with MWCNT but does not clearly indicate if it is bound.

6.2.2 Fluorescence Measurements

The next step in characterizing the MWCNT-CpG ODN molecule was to look at the fluorescence spectra of PySE, PySE-CpG ODN, MWCNT-PySE and MWCNT-PySE-CpG ODN. PySE fluoresces when excited at a wavelength of 340 nm. The two peaks seen in figure 6.4 are characteristic of PySE. The fluorescent spectra of PySE alone gives to peaks at approximately 380 nm and 400 nm at fluorescence of around 5 to 4.5 respectively using the scale on the left side of figure 6.4. When PySE is bound to MWCNT the fluorescence is quenched. When PySE is bound to CpG ODN there is increased fluorescence and when CpG ODN is bound to MWCNT through a PySE linkage the fluorescence increases to about 125. The increased fluorescence of MWCNT-CpG ODN compared to CpG-PySE indicates that CpG ODN is successfully bound to MWCNT.

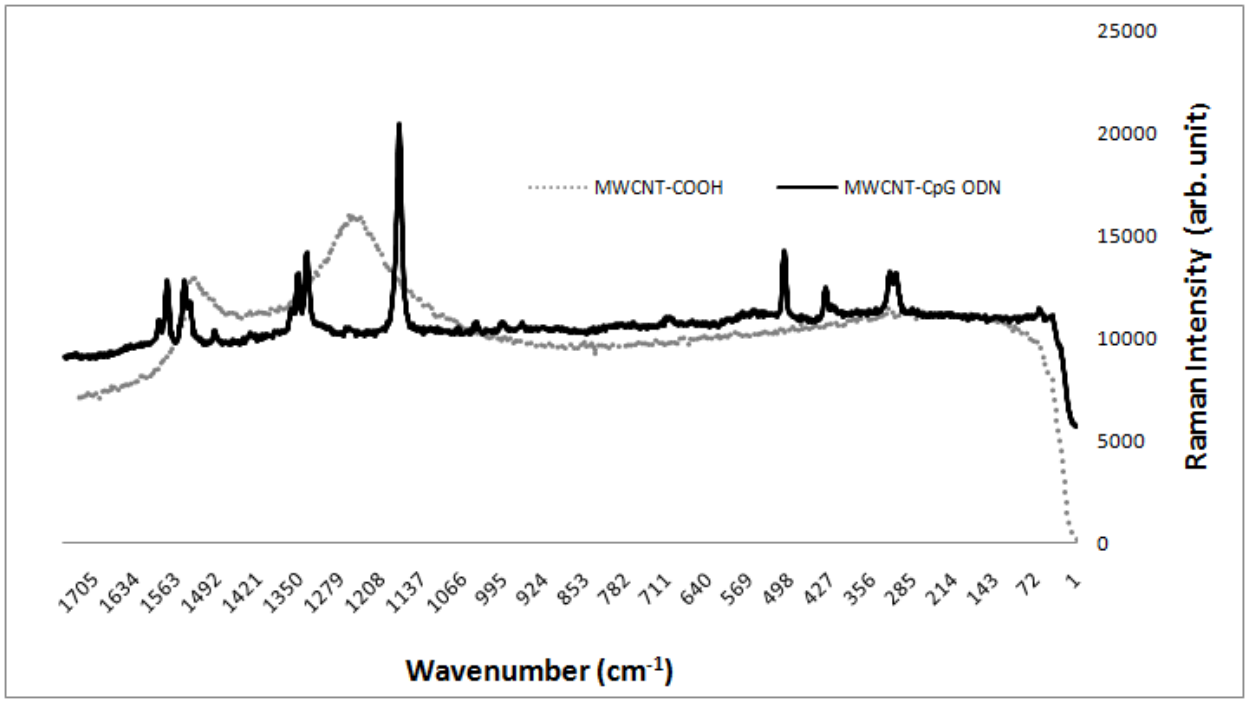


Figure 6.3 Raman Spectroscopy. The Raman spectra of MWCNT-COOH and MWCNT-CpG ODN.

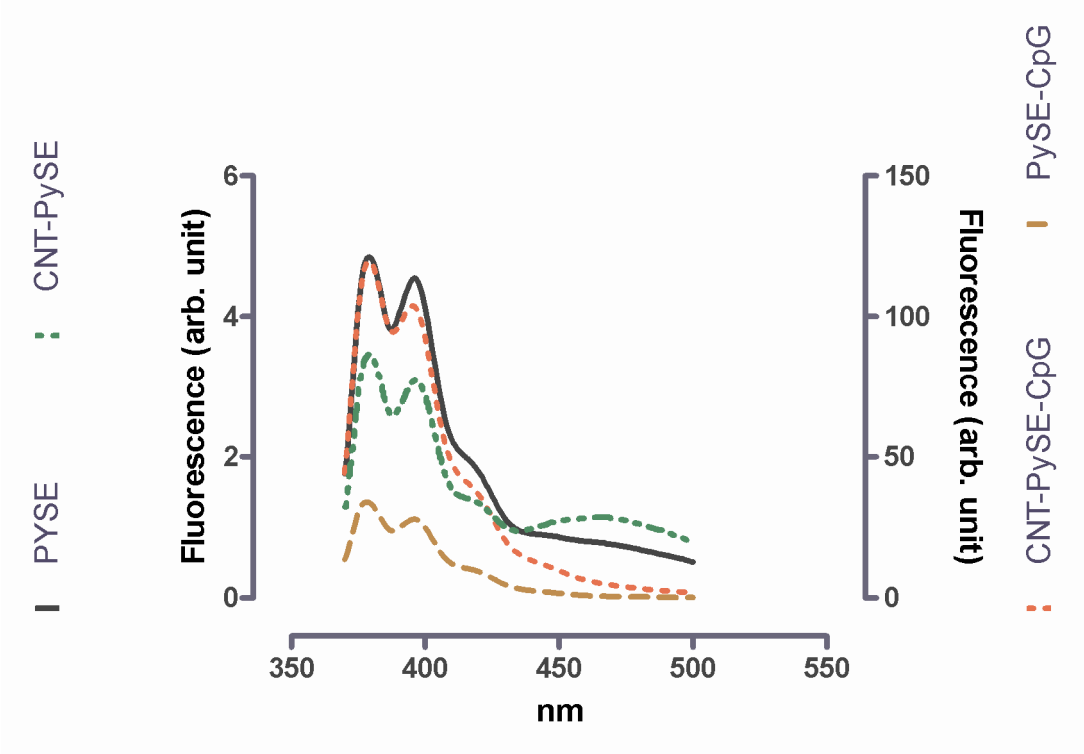


Figure 6.4. Fluorescence spectra. Normalized Fluorescence spectra of MWCNT, MWCNT-PySE, MWCNT-PySE-CpG and PySE-CpG at 2 µg/mL of PySE and 200 µg/mL CNT or CpG. Due to difference in fluorescence intensity spectra were either plotted on right or left Y-axis as shown by the axes with the symbols and the legend

6.3 Cellular Uptake

A comparative cellular uptake was performed with the chicken macrophage cell line HD11 to observe the uptake of both CpG ODN and MWCNT-CpG ODN by innate immune cells. To observe a difference in uptake a time course study was conducted to see if MWCNT-CpG ODN can enter the cell more rapidly. To measure uptake random sections of cells were observed so that at least one hundred cells were observed with each treatment and time point. Cells showing any sign of uptake were counted as cells taking up the molecules. After 30 minutes there is the beginning of uptake of both CpG ODN and MWCNT-CpG ODN (Figure 6.5 B,D). The rate of uptake appears to be similar as upon closer observation of the cells both CpG ODN and MWCNT-CpG ODN are localized at the cell membrane after 30 minutes post treatment (Figure 6.5 A, C). After 60 minutes post treatment both molecules begin to localize within the macrophage cell's cytoplasm (Figure 6.5 E, G). After 120 minutes post treatment the localization of both CpG ODN and MWCNT-CpG ODN is focused on certain areas within the cytoplasm and the intensity of the fluorescence is greater (Figure 6.5 I, K). After 240 minutes post treatment the localization still remains in the cytoplasm for both molecules but the intensity of fluorescence has increased indicating there is more uptake of the molecules with time (Figure 6.5 O, Q). It also appears that the cells receiving the MWCNT-CpG ODN show more fluorescence then the cells receiving CpG ODN treatment which could indicate more MWCNT-CpG ODN is being taken up by the cells or remaining in the cell longer. However, the shape of the nucleus in some cells with high levels of uptake of MWCNT-CpG ODN looks slightly altered indicating that cell death may be occurring at high doses allowing more uptake as the membrane becomes more permeable. However, not all cells receiving MWCNT-CpG ODN treatment had a disrupted nucleus after 240 minutes post treatment (Figure 6-5S). These cells also appear to have more uptake of the molecule compared to free CpG ODN. Cells treated with the Cy3 dye alone did not show the localization within the cell as the dye remained localized at the membrane even four hours post treatment (Figure 6.5 M). The conclusive observation is that attachment of CpG ODN to MWCNT does not alter the ability of the molecule to be taken up by viable cells.

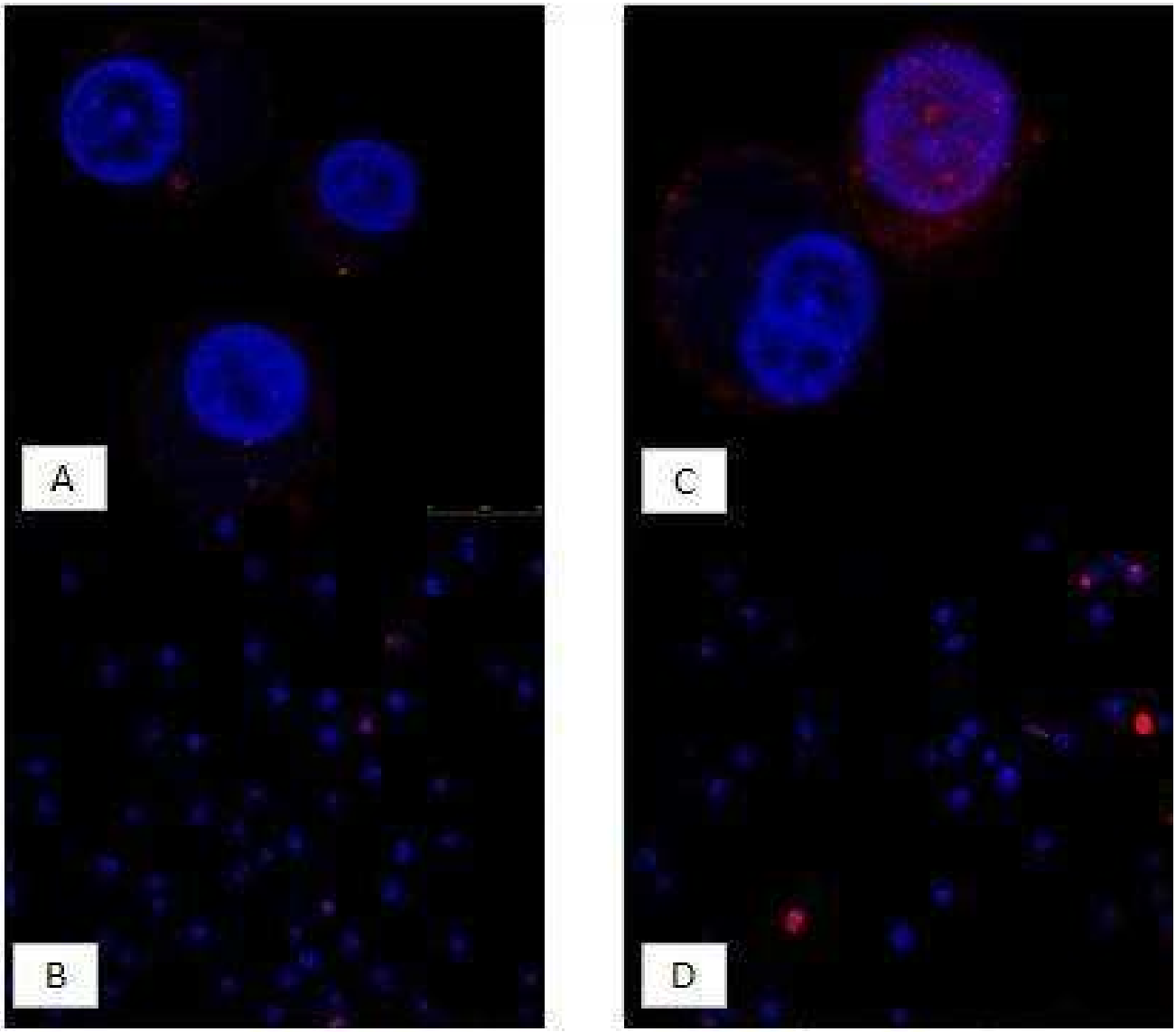


Figure 6.5 (A-D) Confocal microscopy of uptake by HD11 cells. Uptake of free and MWCNT-linked CpG ODN after 30 minutes of treatments. A and C are enlargements of representative cells in B and D respectively. A-B) CpG ODN treated cells; C-D) MWCNT-CpG ODN treated cells. Blue regions in the spectra indicate Hoechst 33342 stained nucleus and Red region indicates Cy3 labeled CpG ODN.

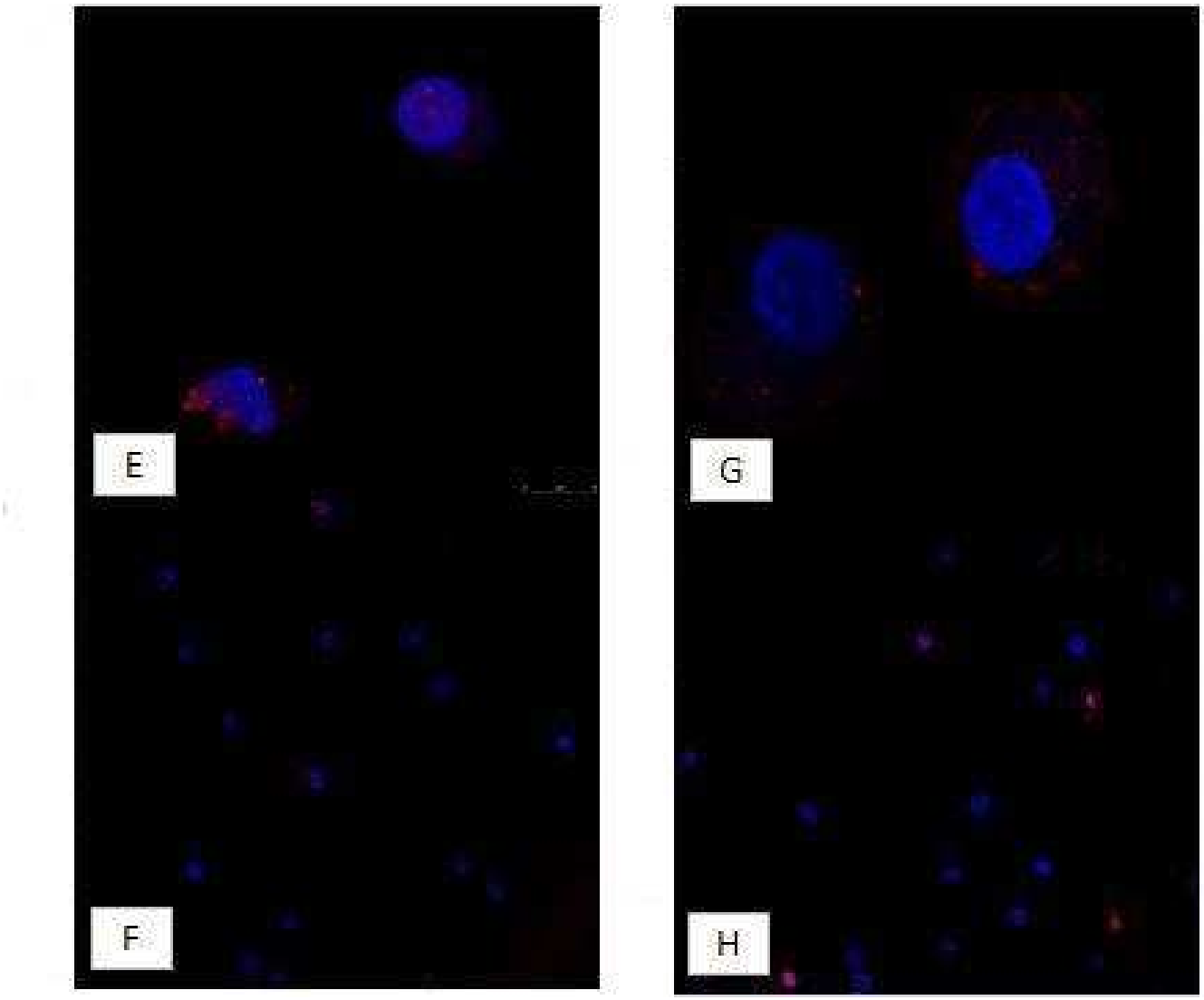


Figure 6.5 (E-H) Confocal microscopy of uptake by HD11 cells. Uptake of free and MWCNT-linked CpG ODN after 60 minutes of treatments. E and G are enlargements of representative cells in F and H respectively. E-F) CpG ODN treated cells; G-H) MWCNT-CpG ODN treated cells. Blue regions in the spectra indicate Hoechst 33342 stained nucleus and Red region indicates Cy3 labeled CpG ODN.

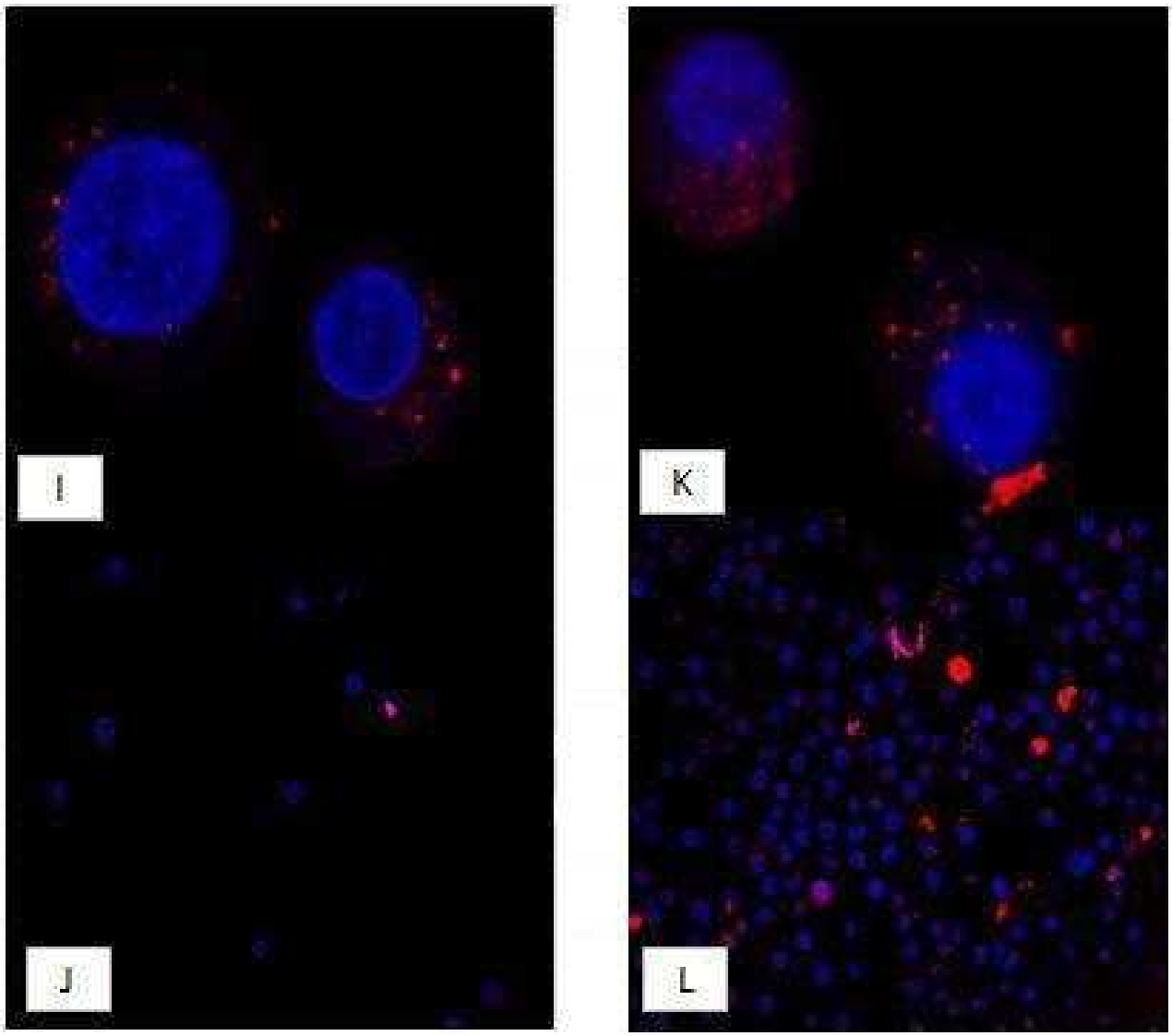


Figure 6.5 (I-L) Confocal microscopy of uptake by HD11 cells. Uptake of free and MWCNT-linked CpG ODN after 120 minutes of treatments. I and K are enlargements of representative cells in J and L respectively. I-J) CpG ODN treated cells; K-L) MWCNT-CpG ODN treated cells. Blue regions in the spectra indicate Hoechst 33342 stained nucleus and Red region indicates Cy3 labeled CpG ODN.

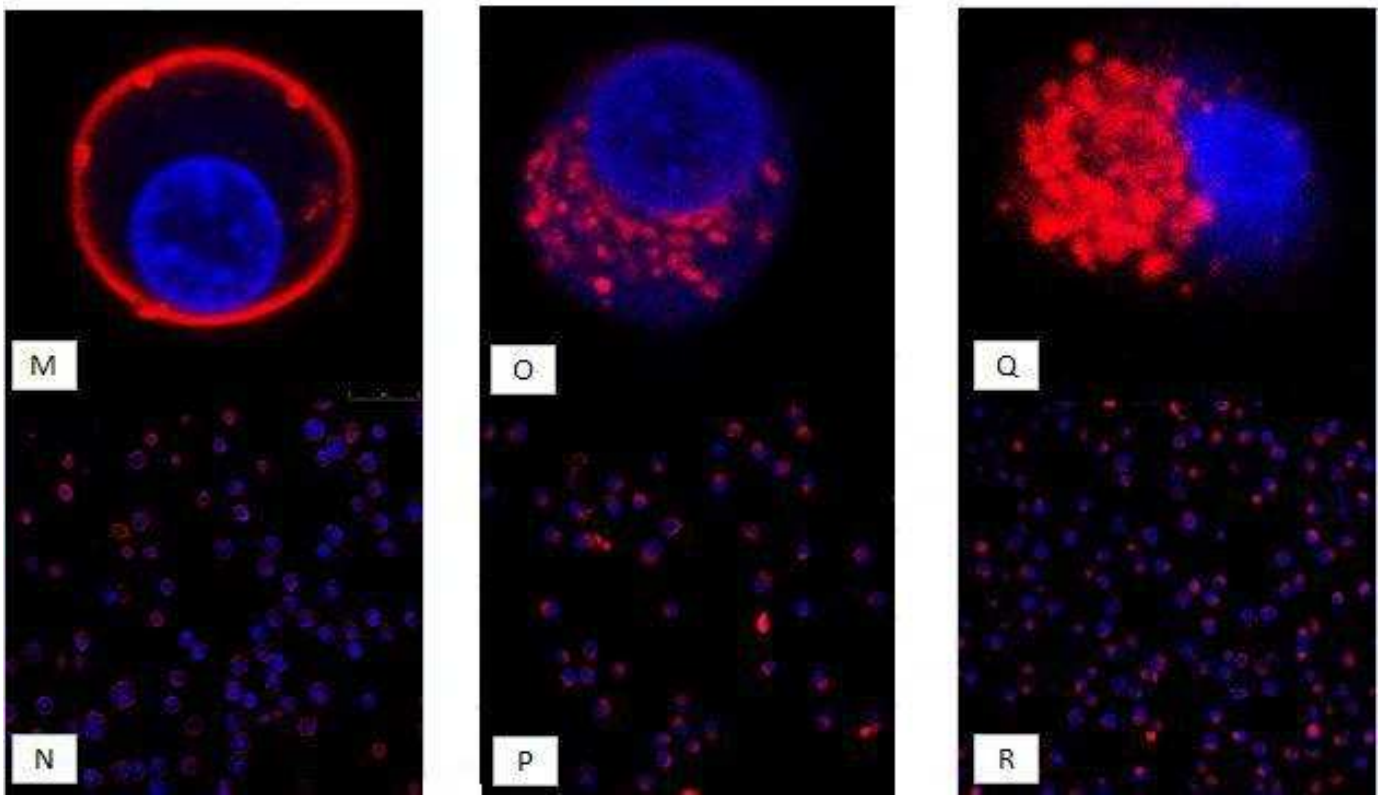


Figure 6.5 (M-R) Confocal microscopy of uptake by HD11 cells. Uptake of free and MWCNT-linked CpG ODN and Cy3 after 240 minutes of treatments. M,O and Q are enlargements of representative cells in N,P and R respectively. M-N) Cy3 treated cells O-P) CpG ODN treated cells; Q-R) MWCNT-CpG ODN treated cells. Blue regions in the spectra indicate Hoechst 33342 stained nucleus and Red region indicates Cy3 labeled CpG ODN.

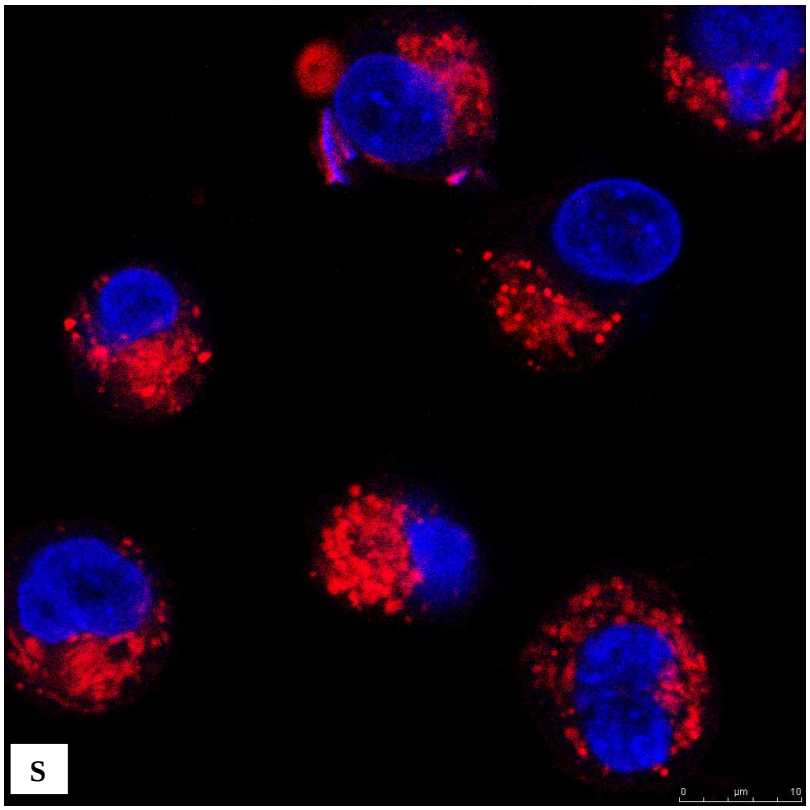


Figure 6.5 (S) Confocal microscopy of uptake by HD11 cells. Uptake of free and MWCNT-linked CpG ODN after 240 minutes of treatments. Blue regions in the spectra indicate Hoechst 33342 stained nucleus and Red region indicates Cy3 labeled CpG ODN.

6.4 *In vitro* Toxicity

6.4.1 Cell Viability

To test the toxicity of free MWCNT and CpG ODN linked MWCNT *in vitro* studies were conducted using the chicken macrophage cell line HD11 that were treated for various time points and various doses of free and linked CpG ODN as well as free MWCNT. Saponin treated cells were used as a positive control as saponin destroys cells by rapidly disrupting the cellular membrane. Media treated cells acted a negative control (Figure 6.6). The cells were treated with a 10-fold titration of treatments ranging from 100 µg/mL to 0.001 µg/mL. After one day post treatment cells receiving all doses of CpG ODN and MWCNT-CpG ODN showed no significant difference in cell viability compared to cells receiving no treatment, while cells receiving 100 µg/mL of MWCNT show a significant decrease in the percentage of viable cells (Figure 6.6 A). After three days post treatment the percent of viable cells receiving doses of CpG ODN remain near the media control level while there is a significant decrease in the percent of viable cells receiving 100 µg/mL of MWCNT-CpG ODN treatment (Figure 6.6 B). There is also a continued decrease in the percent of viable cells receiving 100 µg/mL of free MWCNT after three days post treatment as well as there is a significant decrease in the percentage of viable cells receiving 10 µg/mL of MWCNT after this time (Figure 6.6 B). After seven days post treatment the cells receiving doses of CpG ODN still remain close to the media control level while cells receiving 100 µg/mL or 10 µg/mL of either free MWCNT or CpG ODN linked MWCNT show a decrease in the percentage of viable cells to levels found with treatment of Saponin (Figure 6.6 C).

6.4.2 Free Radical Production

To examine if the treatments were causing death of the HD11 cells due to stimulation of free radicals or other means the levels of peroxide were measured following 24 hours post treatment with a 10-fold dose titration of MWCNT, CpG ODN or MWCNT-CpG ODN ranging from 100 µg/mL to 10 µg/mL. It was observed that the levels of peroxide being produced by cells treated with MWCNT-CpG ODN and CpG ODN were at the similar levels and even slightly below levels produced by the control cells treated with media alone (Figure 6.7 B-C). However, chicken macrophage cells receiving treatment of a high dose of MWCNT at

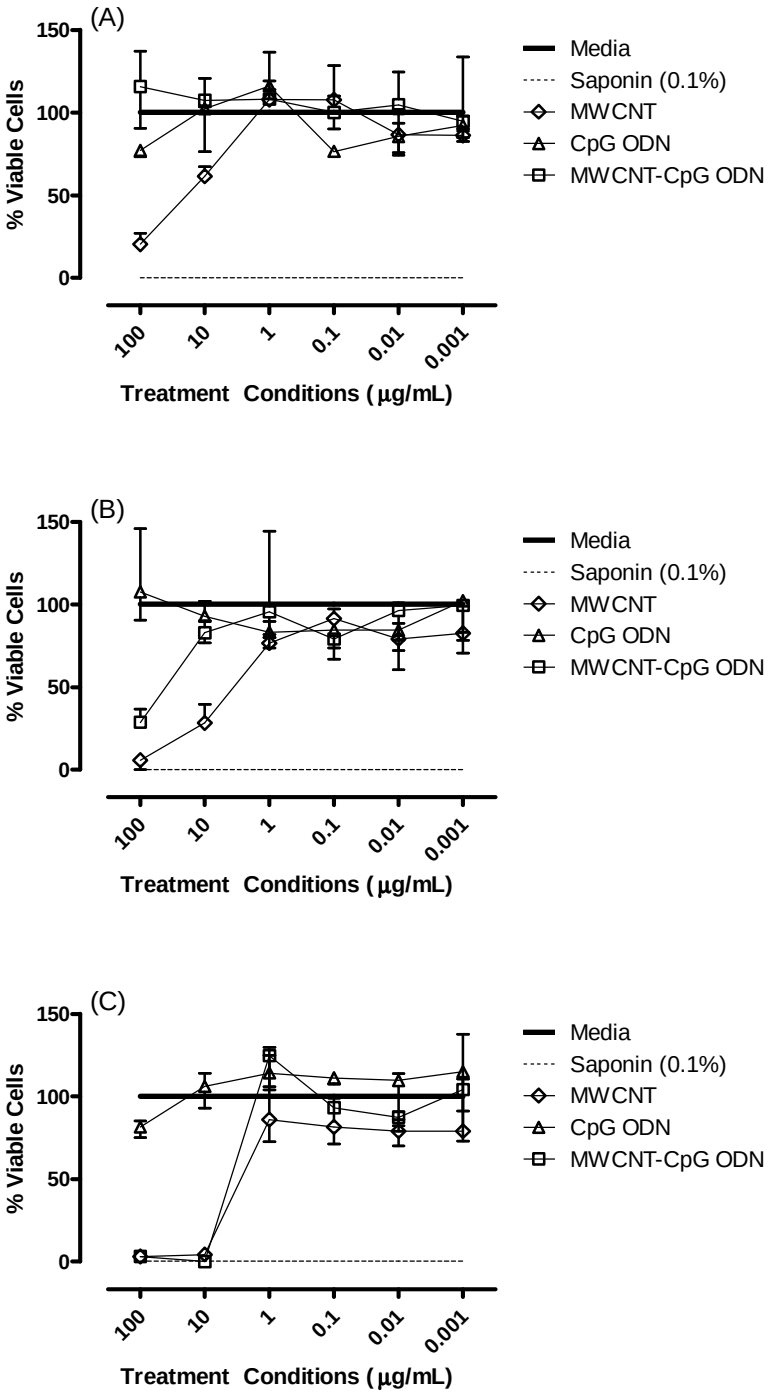


Figure 6.6 ***In vitro*** **viability studies with Chicken macrophage cells (HD11).** Percent (%) of viable cells on days A) 1 B) 3 and C) 7 following various treatments as shown on X-axis are plotted. Mean values of three replicates for each treatment with 1-SD are plotted. Symbols for various conditions are shown on the side.

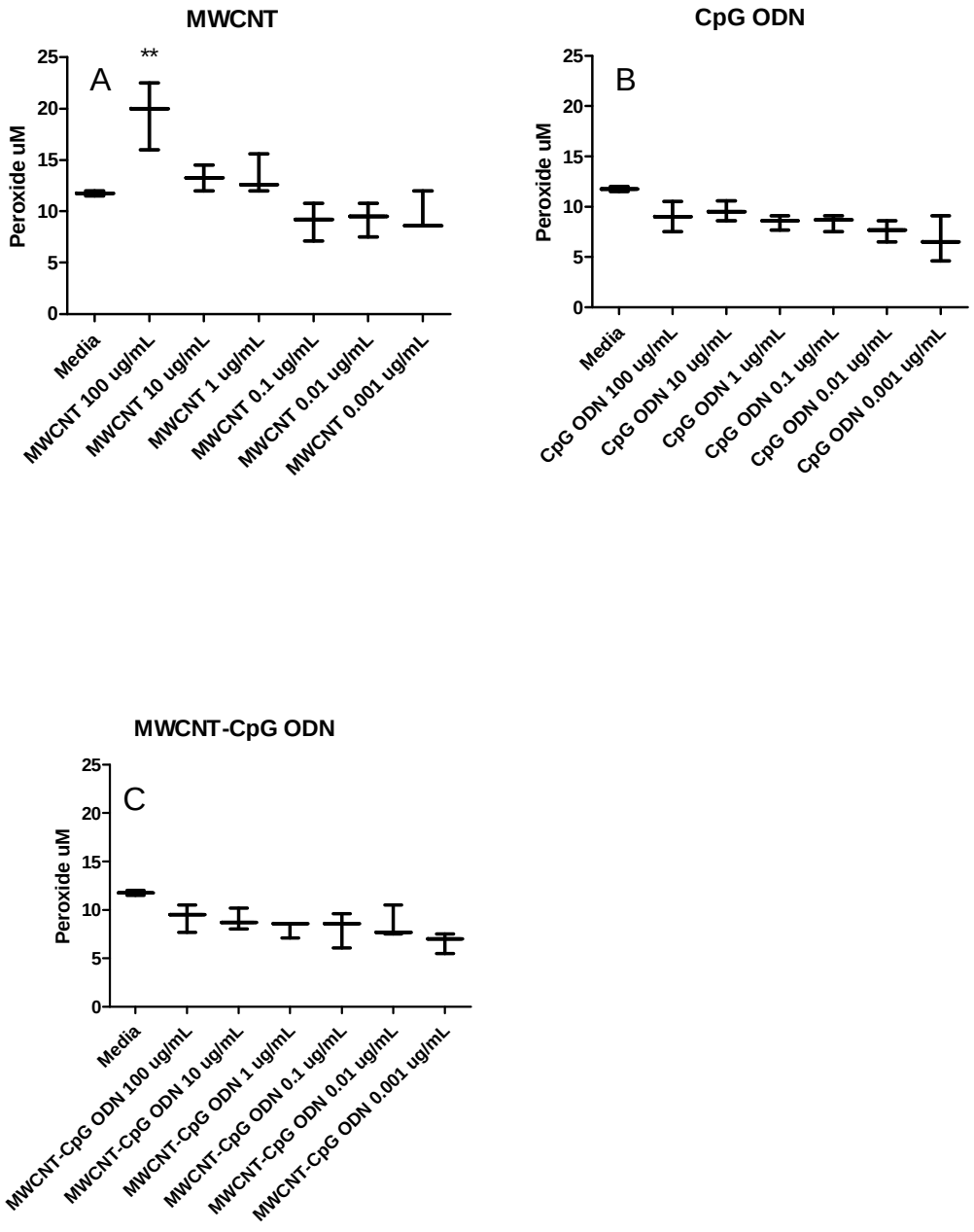


Figure 6.7 Peroxide production of treated HD11 cells. The levels of peroxide were measured in HD11 cells treated with various doses of A) MWCNT B) CpG ODN or C) MWCNT-CpG ODN. Cells were treated for 24 hours. The median of three replicates is graphed. ** significant difference compared to media treated control cells $p < 0.001$.

100 µg/mL produced significantly higher levels of peroxide after 24 hours post treatment (Figure 6.7 A). The results of increased peroxide production with the high treatment of MWCNT correlates with the decreased cell viability at this time point.

6.4.3 Production of Nitric Oxide

The production of nitric oxide in certain cells can act as a free radical. In macrophages it also has an important immune stimulating ability. To examine MWCNT, CpG ODN and MWCNT-CpG ODN's ability to stimulate nitric oxide production in macrophages the chicken macrophage cell line HD11 was again treated with a 10-fold dose titration of the three molecules over a 168 hours (seven day) time course. LPS (1 µg/mL) was used as a positive control as it stimulates nitric oxide production in macrophages while media treated cells acted as the negative control. To measure nitric oxide the more stable product of nitric oxide, nitrite was measured. It was seen that MWCNT by itself, at all doses, did not stimulate nitrite production as the levels remained the same as the media treated cells for 168 hours (Figure 6.8). At high doses of MWCNT-CpG ODN there was an initial increase in nitrite production comparable to CpG ODN and LPS treated cells but by 72 hours the levels of nitrite were reduced to that of the negative control cells (Figure 6.8 A,B). At the concentrations of 1 µg/mL and 0.1 µg/mL the macrophages responded to stimulation with LPS, CpG ODN and MWCNT-CpG ODN treatment similarly. There was an initial increase in nitrite after 24 hours and peak nitrite production was reached at 72 hours post treatment and maintained to 168 hours (Figure 6.8 C,D). When the concentration was lowered to 0.01 µg/mL for both MWCNT-CpG ODN and free CpG ODN the stimulation of nitrite production in the HD11 cells changed. After 24 hours there was nitrite production comparable to the negative control cells treated with media but by 72 hours there was an increase in nitrite production in cells treated with MWCNT-CpG ODN to the same levels being produced by the positive control cells treated with LPS (Figure 6.8 E). At 72 hours the cells treated with the same dose of CpG ODN also showed an increase in nitrite production but it was significantly less than the nitrite being produced by the MWCNT-CpG ODN treated cells (Figure 6.8 E). At 168 hours the levels being produced by cells treated with 0.01 µg/mL of either CpG ODN or MWCNT-CpG ODN was reduced to levels similar to that of the negative controls. Cells treated with 0.001 µg/mL did not show an

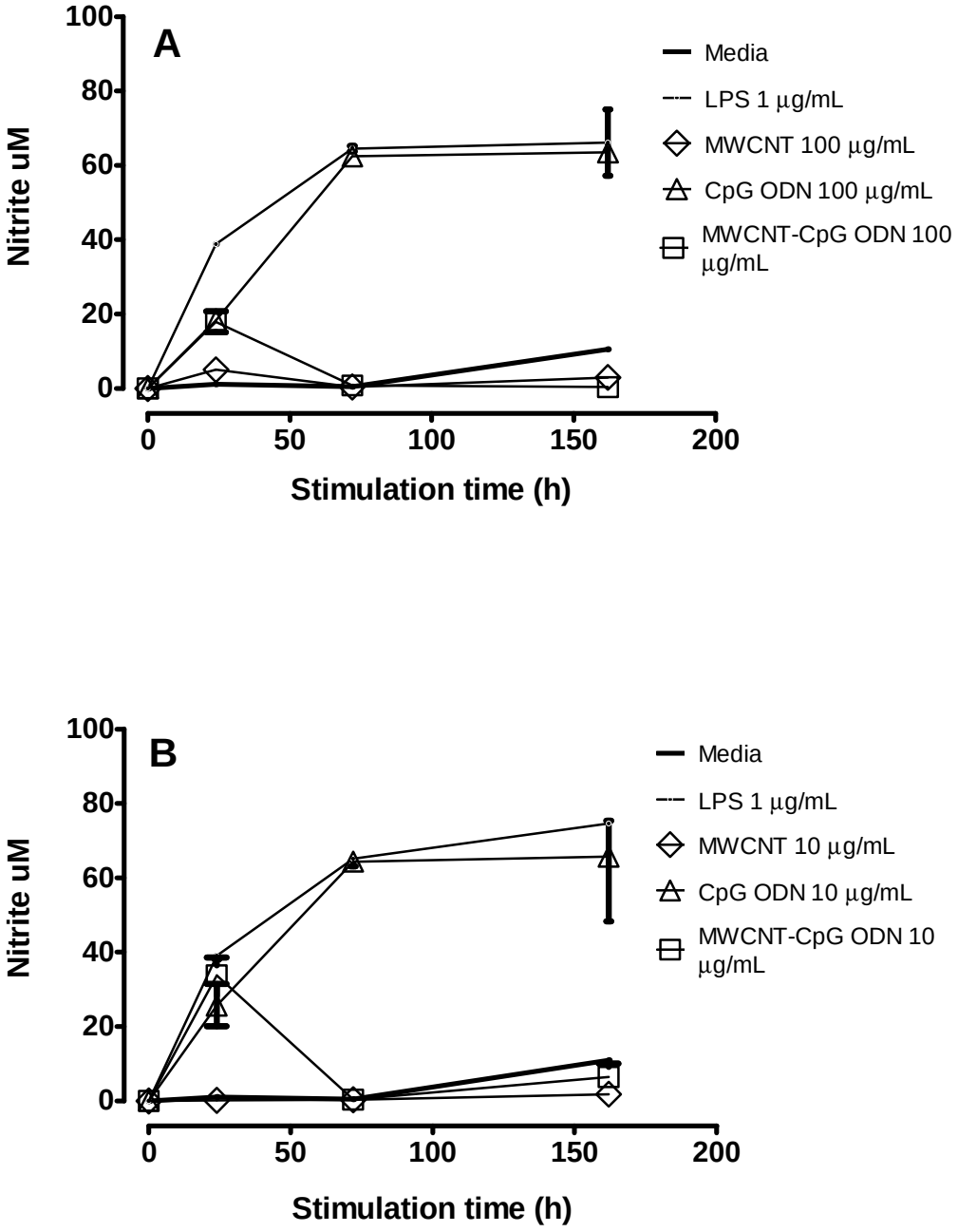


Figure 6.8. Nitrite production by chicken macrophage cells (HD11). Kinetics of production of nitrites in HD11 cells following treatments with MWCNT, CpG ODN and MWCNT-CpG ODN at A) 100 µg/mL B) 10 µg/mL are shown. Kinetics of production of nitrite following treatment of the cells with LPS (1 µg/mL) is shown as a positive control. Kinetics of nitrite production without any treatment in media is shown as a negative control. Median values of three replicates with 1-SD for each condition are plotted.

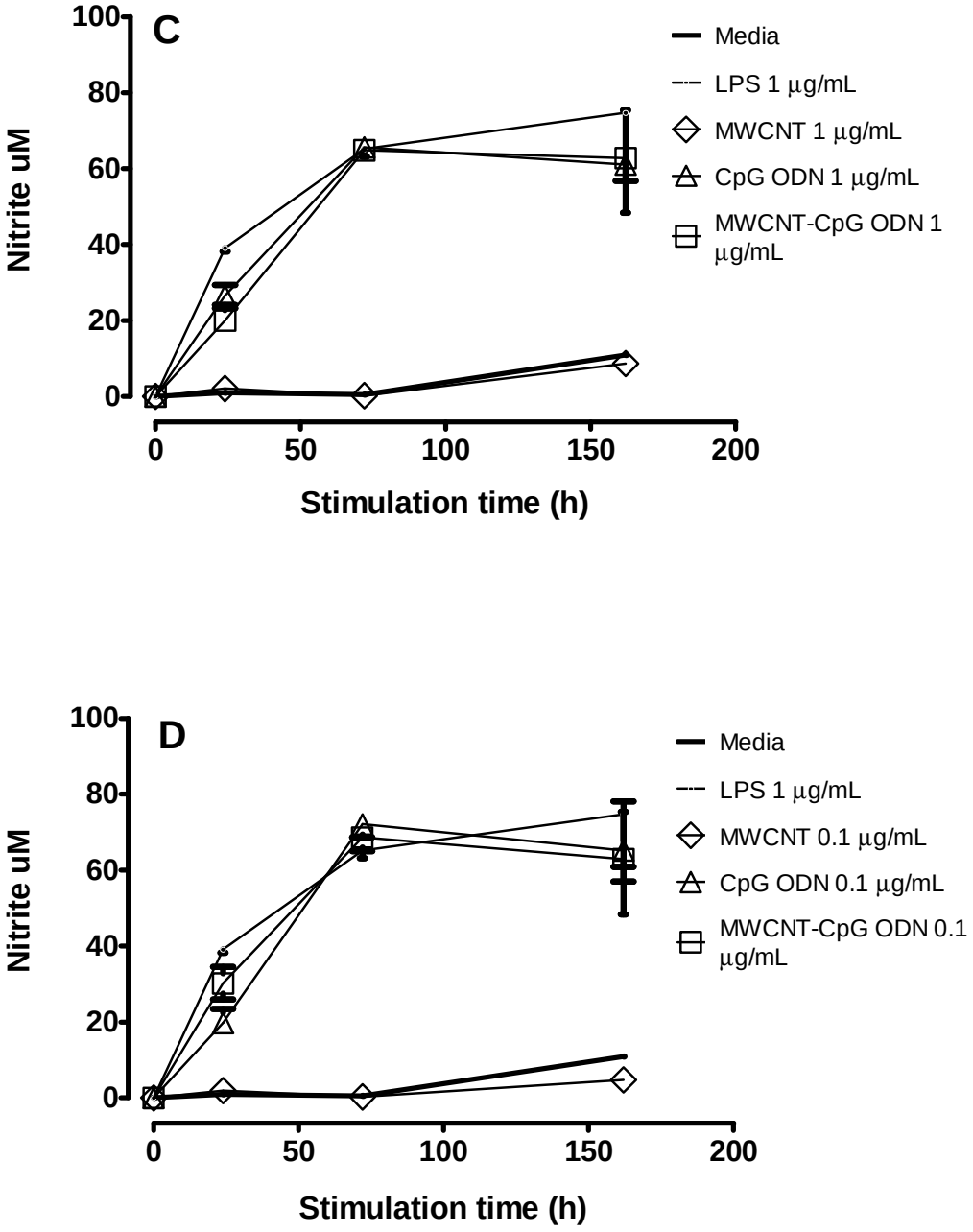


Figure 6.8. Nitrite production by chicken macrophage cells (HD11). Kinetics of production of nitrites in HD11 cells following treatments with MWCNT, CpG ODN and MWCNT-CpG ODN at C) 1 µg/mL D) 0.1 µg/mL are shown. Kinetics of production of nitrite following treatment of the cells with LPS (1 µg/mL) is shown as a positive control. Kinetics of nitrite production without any treatment in media is shown as a negative control. Median values of three replicates with 1-SD for each condition are plotted.

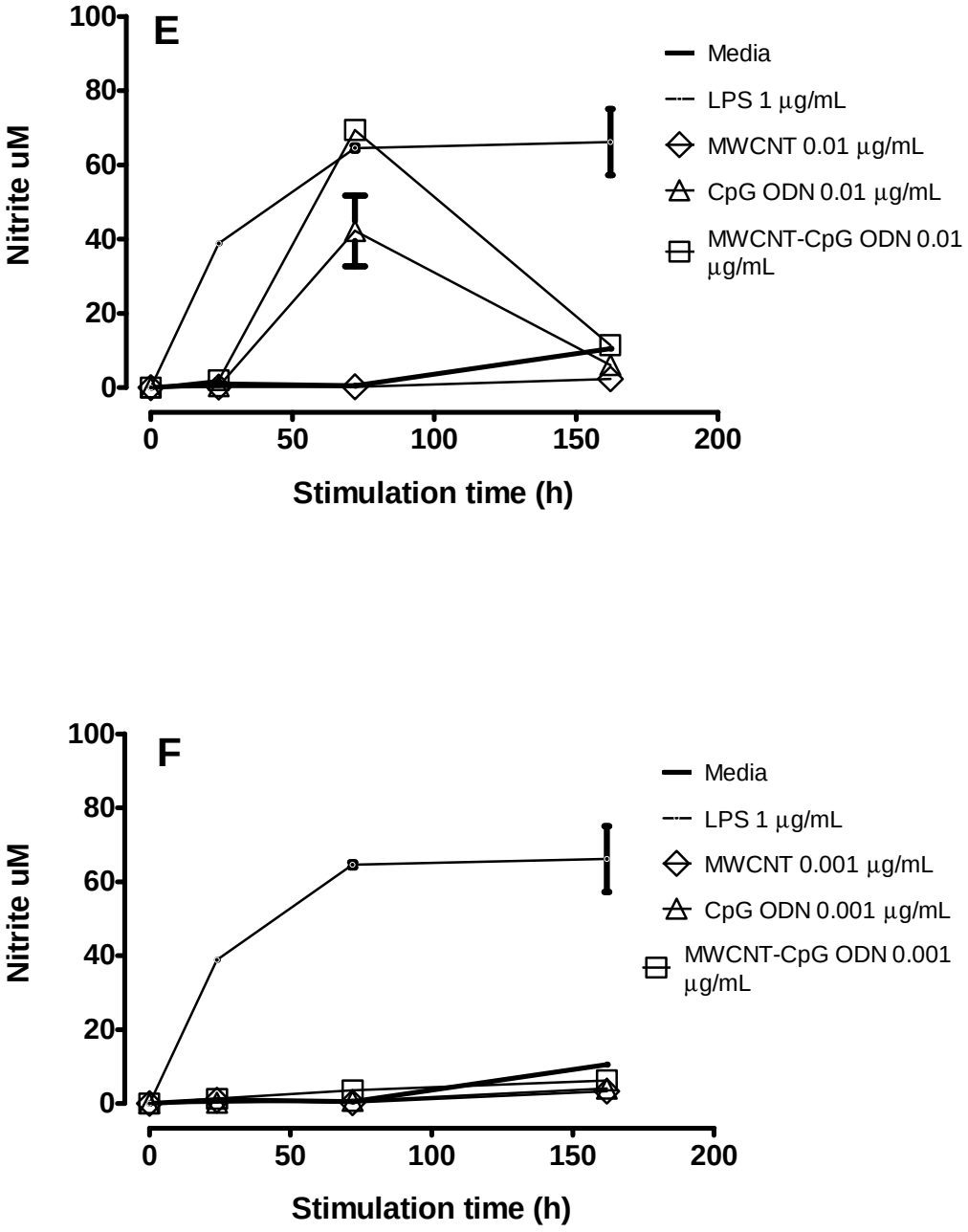


Figure 6.8. Nitrite production by chicken macrophage cells (HD11). Kinetics of production of nitrites in HD11 cells following treatments with MWCNT, CpG ODN and MWCNT-CpG ODN at E) 0.01 µg/mL and F) 0.001 µg/mL are shown. Kinetics of production of nitrite following treatment of the cells with LPS (1 µg/mL) is shown as a positive control. Kinetics of nitrite production without any treatment in media is shown as a negative control. Median values of three replicates with 1-SD for each condition are plotted.

increase in nitrite production by the chicken macrophage cell line at any time point (Figure 6.8F).

6.5 Inhibition of Induced Nitric Oxide Production in Macrophages

6.5.1 Endocytosis and Endosomal Maturation

The next step in ensuring that MWCNT linkage to CpG ODN did not disrupt the biological function of CpG ODN was to examine the pathway of nitric oxide stimulation by CpG ODN and MWCNT-CpG ODN in the chicken macrophage cell line HD11. First the role of uptake of both molecules was examined by using the clathrin inhibitor MDS which disrupts clathrin-mediated endocytosis so vesicle mediated uptake cannot occur. HD11 cells were pre-treated with this inhibitor for one hour then treated with CpG ODN, MWCNT-CpG ODN or the control LPS. After 24 hours the levels of nitrite were measured to examine cellular immune activation in the absence of endocytosis. It was observed that with inhibition of clathrin-dependent endocytosis there was a significant reduction in nitrite production by cells treated with both CpG ODN and MWCNT-CpG ODN (Figure 6.9 A). At certain concentrations of MDS, endocytosis was shut down to a point that there was no nitrite production by CpG ODN and MWCNT-CpG ODN treated cells. Cells treated with LPS were still able to produce high levels of nitrite.

Another key step in CpG ODN initiating nitric oxide production in macrophages is the binding to the target receptor which is located in the endosome. To see if both CpG ODN and MWCNT-CpG ODN use the same intracellular receptor the endosomal maturation inhibitor chloroquine was used to pre-treat the HD11 cells for one hour. After 24 hours post treatment the nitrite levels were measured. It was seen that nitrite production for cells treated with either CpG ODN or MWCNT-CpG ODN was significantly reduced in the absence of endosome maturation (Figure 6.9 B). This indicates that linking CpG ODN to MWCNT likely does not alter the molecules ability to recognize and bind to the endosomally located target receptor.

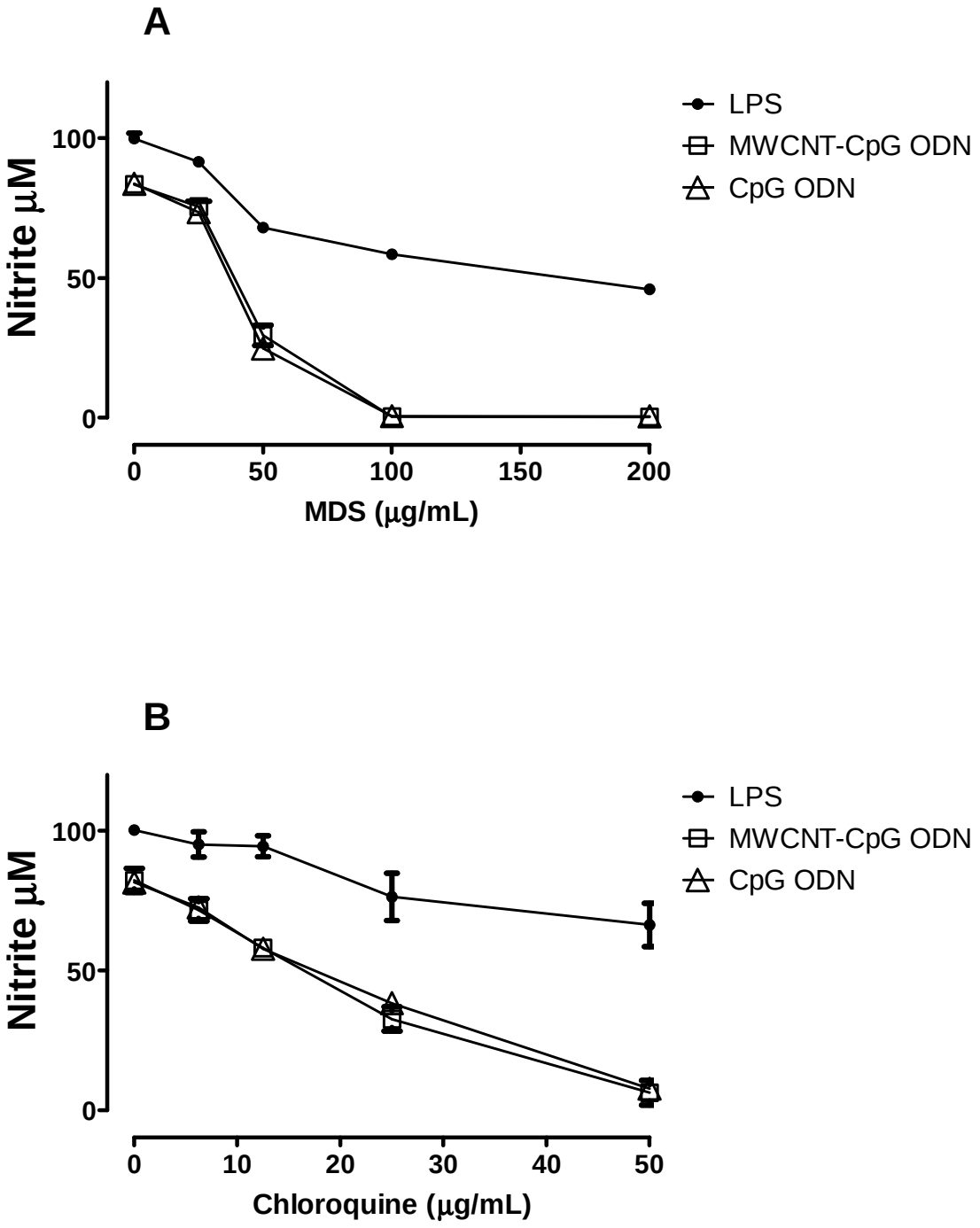


Figure 6.9 Inhibition of nitric oxide production – endocytosis and endosmal maturation. Inhibition of nitrite production in HD11 cells pretreated with different concentrations of inhibitors A) MDS and B) Chloroquine following 24h treatment with either 1 µg/mL of CpG ODN, or MWCNT-CpG or LPS. Mean values of three biological replicates with 1-SD are plotted.

6.5.2 Nitric Oxide Signal Pathway

To ensure that the stimulation of the macrophage cells to produce nitric oxide with MWCNT-CpG ODN treatment uses the same signaling pathway as that of CpG ODN treated cells, inhibitors of key enzymes were used to see if there was differential activation between the two molecules. The immune stimulating form of nitric oxide is produced by the enzyme, inducible nitric oxide synthase or iNOS. To make sure that both CpG ODN and MWCNT-CpG ODN induced nitric oxide production through the activation of this enzyme and not different isoforms of NOS, the iNOS inhibitor 1400W was used to pre-treat the cells before 24 hour treatment with CpG ODN or MWCNT-CpG ODN. Inhibition of iNOS resulted in the reduction of nitrite being produced by both CpG ODN and MWCNT-CpG ODN treated cells as well as with treatment of the positive control, LPS (Figure 6.10 A). This indicates that this enzyme plays a key role in free and MWCNT linked CpG ODN stimulation of macrophage nitric oxide production. The pathway to iNOS activation was then examined using inhibitors of key enzymes in the signaling pathway.

PKC is a protein that plays a central role in the entry into the pathway leading to nitric oxide production with CpG ODN treatment. To ensure MWCNT-CpG ODN also requires this protein's activity to stimulate nitric oxide production the PKCα inhibitor Ro 32-0432 was used to pre-treat the HD11 cells. Inhibition of this protein completely shut down nitrite production in cells treated with both CpG ODN and MWCNT-CpG ODN as well as LPS treated cells (Figure 6.10B). This indicates this protein plays a central role in nitric oxide production in both CpG ODN and MWCNT-CpG ODN treated macrophages.

The pathway of nitric oxide production after stimulation with CpG ODN and MWCNT-CpG ODN was examined by using select inhibitors of the MAPK signaling cascade. SB-203590 inhibited p38 MAPK (Figure 6.11B) and U-0126 inhibits MEK1/2 activation (Figure 6.11A) which are both required for nitric oxide production via free and MWCNT-linked CpG ODN treatment. The MAPK cascade is required to activate NF-κB which plays a central role in inflammation by regulating inflammatory cytokines and iNOS. In figure 6.11C pre-treatment with Bay 11-7082 which can block the activation of NF-κB also inhibits nitrite production for

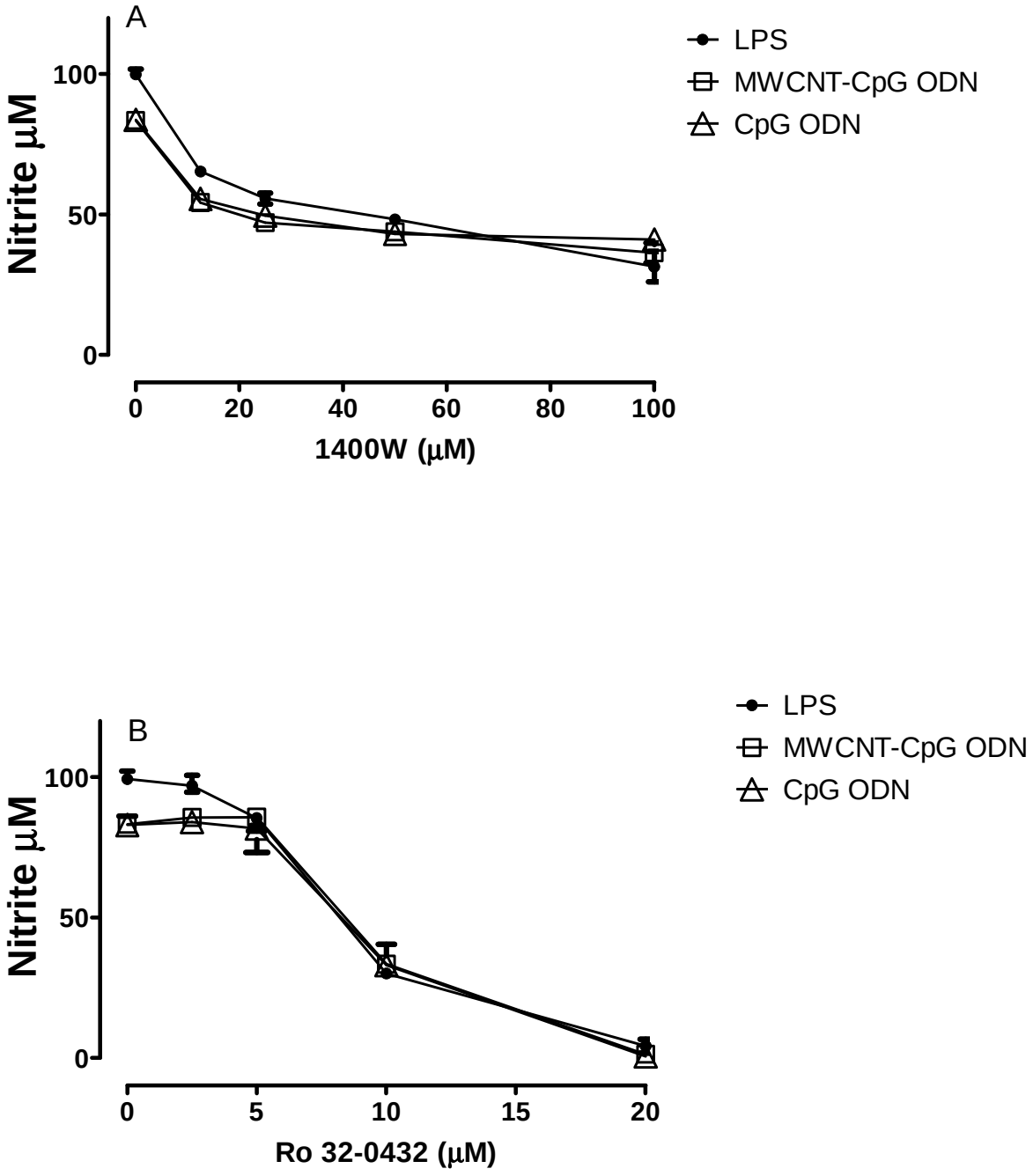


Figure 6.10 Inhibition of nitric oxide production – iNOS and PKC. Inhibition of nitrite production in HD 11 cells pretreated with different concentrations of inhibitors (A) 1400W and (B) Ro 32-0432 following 24h treatment with either 1 µg/mL of CpG ODN, or MWCNT-CpG or LPS. Mean values of three biological replicates with 1-SD are plotted

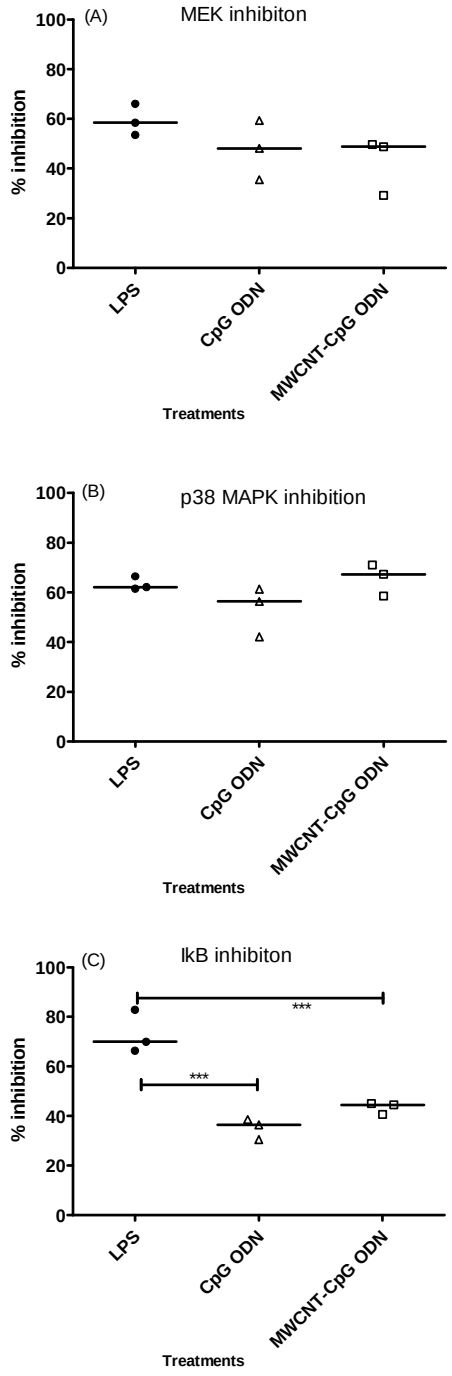


Figure 6.11 Inhibition of nitric oxide production – p38, MEK ½, and NF-κβ. Cells were pretreated with 10 µM of inhibitors A) U1026 B) SB 203580 and C) Bay 11-7082 followed by 24h treatment with either CpG ODN or MWCNT-CpG or LPS. The median of three biological replicates with 1-SD are plotted. Asterisks in panel (C) indicate significant difference between treatments. $p < 0.05$.

both free and MWCNT-linked CpG ODN. The similarity of these specific inhibitory effects on the production on nitric oxide indicated that the conjugation of MWCNT to CpG ODN did not alter the way in which conjugated CpG ODN can stimulate immune responses at the signalling level.

6.5 *In vivo* studies

Now that it has been established that MWCNT-CpG ODN can stimulate an immune response *in vitro,* the study moved *in vivo* to see if MWCNT-CpG ODN can provide a better protective immune response then free CpG ODN in chickens.

6.5.1 Protection study

An essential part of developing an effective immunotherapeutic vaccine is the ability to protect the host from infection. A protection study was performed to see the *in vivo* effect of MWCNT-CpG ODN treatment on the immune system using a chicken model. Day old chicks received a dose of MWCNT-CpG ODN or CpG ODN ranging from 10 µg to 0.01 µg per injection. For a negative control birds were injected with PBS or 10 µg of free MWCNT. Three days post treatment the birds were challenged with a lethal dose of *S. typhimurium*. MWCNT alone was ineffective in protecting the chickens against a lethal dose of bacteria as the survival percentage of these birds matched the survival percentage of PBS treated birds (Figures 6.12, 6.13). Therefore, any protection provided by MWCNT-CpG ODN was due to the CpG ODN linked to the MWCNT and not an immune response to the MWCNT. The MWCNT linked CpG ODN did not lose any ability to protect the chickens as high concentrations of MWCNT-CpG ODN and CpG ODN are just as effective at protecting the chickens (Figure 6.12). However, at the lower concentration of 0.1 µg per injection MWCNT-CpG ODN was significantly more effective at protecting chickens against a lethal bacterial dose compared to the same treatment dose of CpG ODN alone (Figure 6.13). From this study it was demonstrated that MWCNT-CpG ODN can boost the immune system to provide protection against infection at a concentration 10 fold less than that of free CpG ODN. This is a key observation in the development of an effective preventative treatment using MWCNT to enhance the biological activity of low doses of CpG ODN.

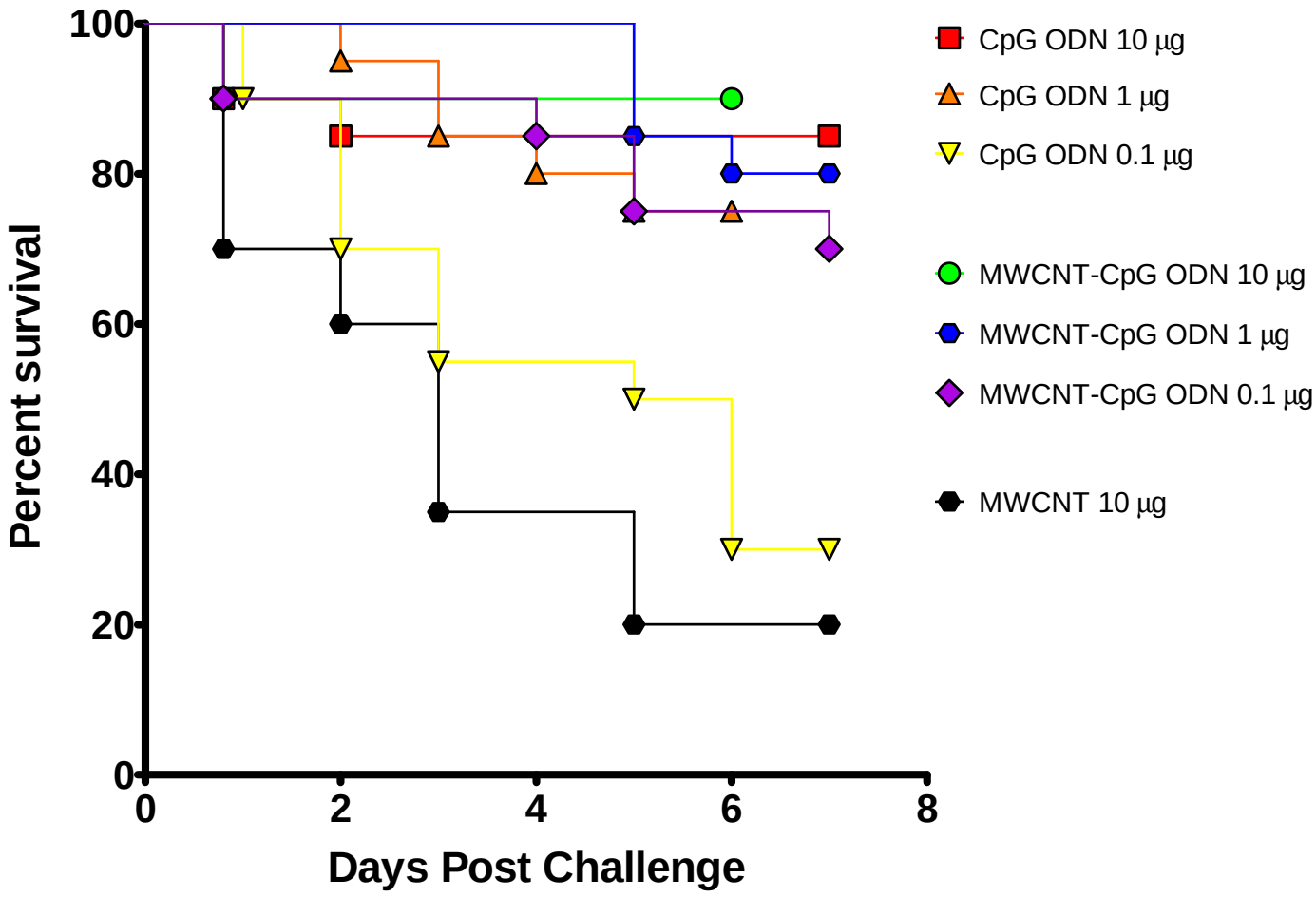


Figure 6.12 Survival graph of *S. typhimurium* challenged chickens. Day old chickens were placed in groups (n=25) and treated with either MWCNT (10 µg), CpG (10 – 0.1 µg), or MWCNT-1-CpG (10 – 0.1 µg). Three days after treatment the chickens were challenged with 10^8 cfu/chicken of *S.typhimurium*. Percent survival of challenged chickens 7 days post challenge is graphed.

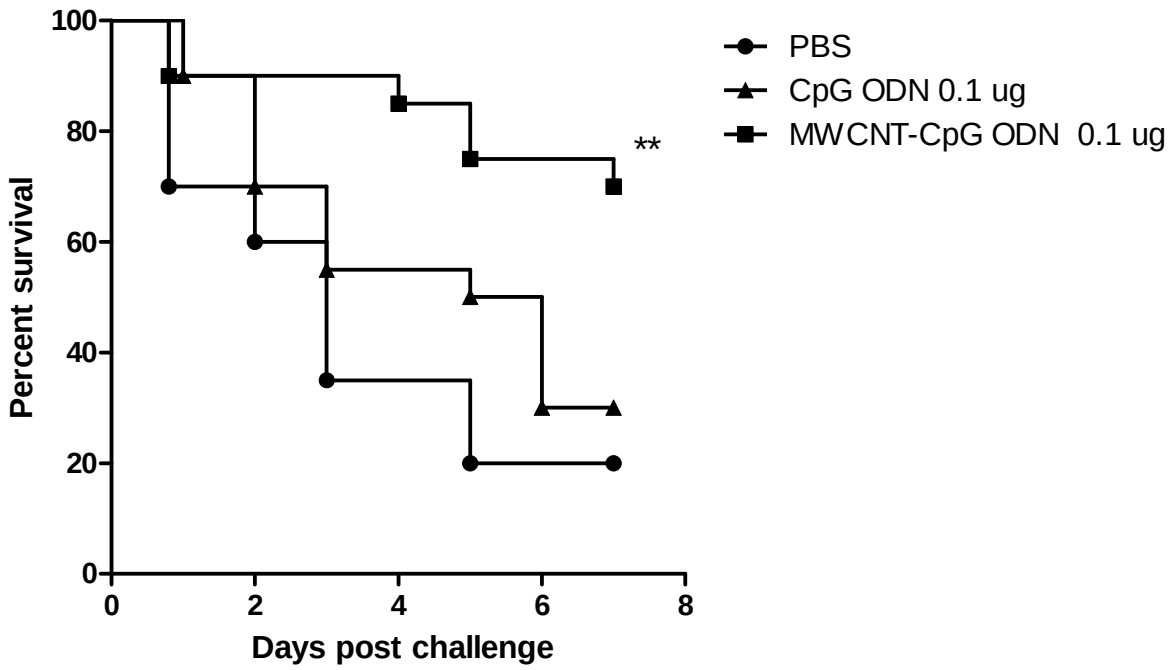


Figure 6.13. Survival of chickens at lower doses. Percent survival curve of one day old chickens treated with PBS, CpG ODN (0.1 µg) or CNT linked CpG ODN (0.1 µg) followed by challenge with 1 x 10^8 cfu/bird and of *Salmonella typhimurium* ST5. ** indicates significant difference with MWCNT-CpG ODN treatment compared to PBS or CpG ODN treatment. $p < 0.001$

6.5.2 Cell Recruitment

An *in vivo* study was done in chickens to observe the amount of cellular recruitment that occurs to the site of injection with various doses of CpG ODN and MWCNT-CpG ODN as well as negative controls PBS and MWCNT. The severity of the cell infiltration was scored on a scale from 0 to 4, with 4 being the most severe amount of cell recruitment to the injection site. A representative picture of severity is given in table 6.1. The amount of cell recruitment was measured with a time course of 1,2,3 and 7 days post treatment of CpG ODN and MWCNT-CpG ODN at a dose of 1 µg and 0.1 µg per injection. Higher doses of CpG ODN (10 µg) showed severe cellular recruitment and inflammation after 1 day which remained until day 7 (Table 6.2). At day 2 the higher CpG ODN doses (10 µg and 1 µg) showed extreme inflammation with tissue damaging myositis and cellulitis. This was not observed with the MWCNT-CpG ODN conjugates. At a dose of 1 µg both CpG ODN and MWCNT-CpG ODN were able to generate similar levels of cell recruitment which did not peak until day 2 (Table 6.2; Figure 6.14A). The key dose was the 0.1 µg per injection which for MWCNT-CpG ODN was shown to be significantly more effective then the same dose of CpG ODN in protecting against a lethal challenge of bacteria. At day 1 and day 2 there is more cellular recruitment to the injection site for the 0.1 µg MWCNT-CpG ODN treated chickens than the 0.1 µg CpG ODN treated chickens. By day 3 MWCNT-CpG ODN treated chicken cellular recruitment to the injection site drops to the same level as the CpG ODN treated chicken then further drops by day 7 (Table 6.2; Figure 6.14B).

Table 6.1 – Cell Recruitment Severity

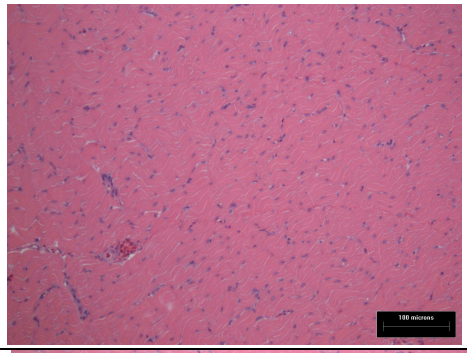
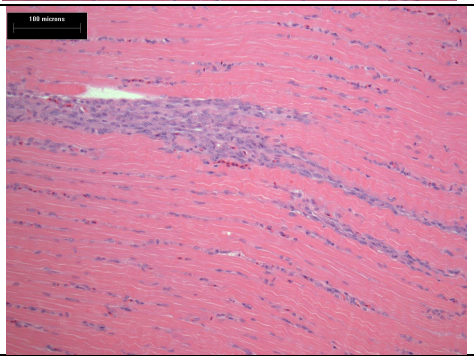
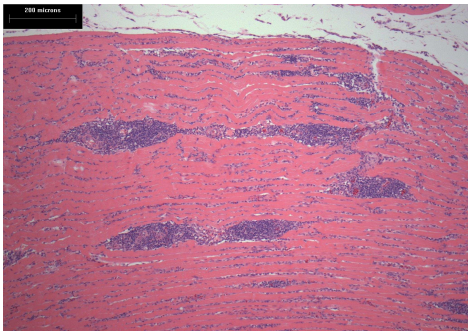
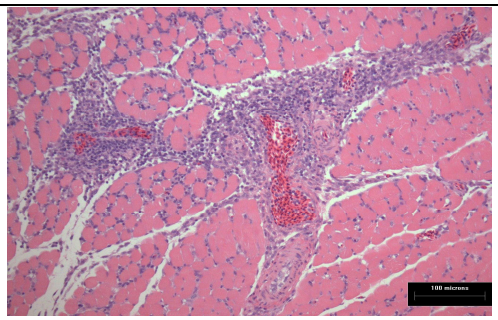
Severity of Infiltration	Infiltration Score	
Normal	**0**	
Mild	**1**	
Moderate to Moderate-severe	**2.5**	
Severe	**4**	

Table 6.1 Representative pictures of the severity of cell recruitment to injection site. Scoring: 1=mild; 2= moderate; 3= moderate-severe; 4= severe

Table 6.2 Overview of infiltration scores for subgroups

TREATMENT	Day 1	Day 2	Day 3	Day 7
PBS 20mM	none	none	none	none
MWCNT- 10ug	none	none	none	none
CpG ODN - 10ug	Severe	Severe	Severe	Severe
CpG ODN - 1ug	Mod	Severe	Severe	Severe
CpG ODN - 0.1ug	none	Mod	Mild	Mod
MWCNT-CpG ODN - 1ug	Mild	Severe	Mod	Severe
MWCNT-CpG ODN - 0.1ug	Mild	Severe	Mild	None

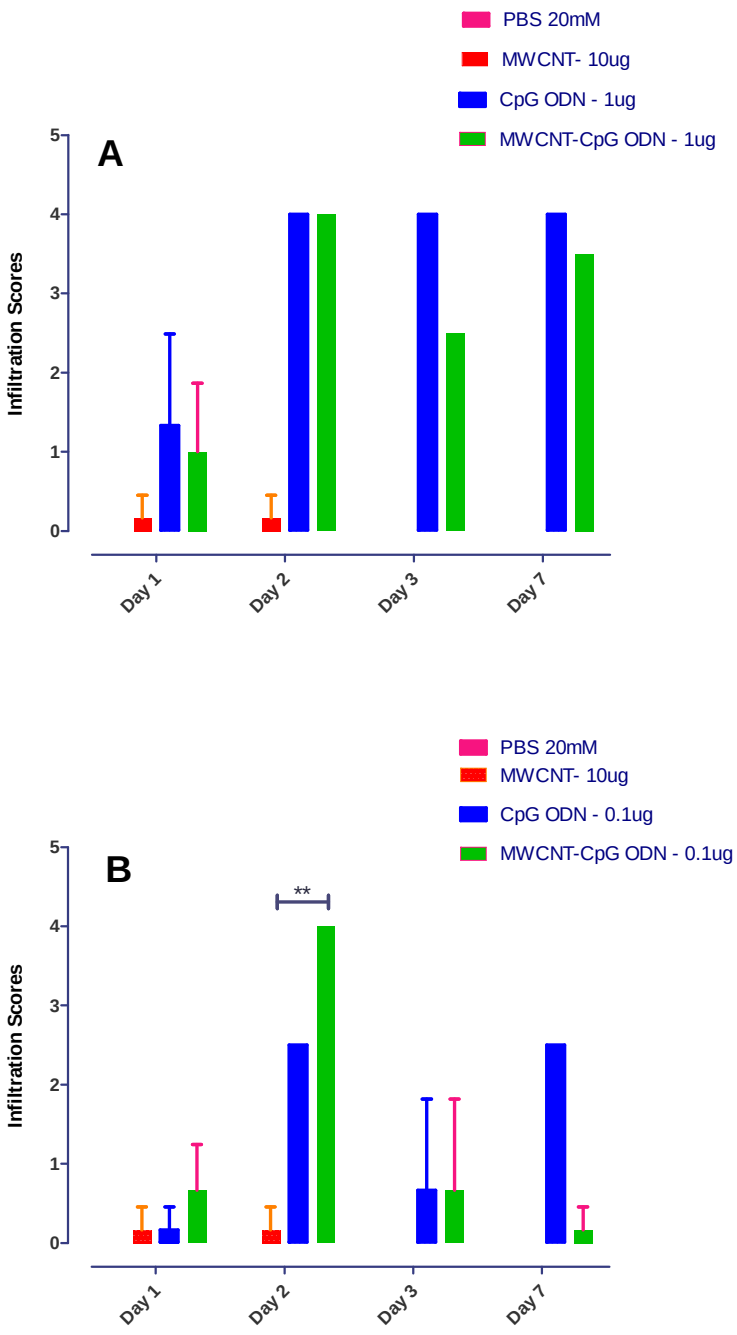


Figure 6.14 Cell recruitment scores. Two week old chicks were injected with various doses of either CpG ODN, MWCNT-CpG ODN or controls PBS and MWCNT. Subgroups were humanly euthanized and biopsies were taken of the injection site and examined for cell recruitment. Subgroups = 1,2,3 and 7 days post treatment. Scoring: 1=mild; 2= moderate; 3= moderate-severe; 4= severe. A) 1 µg of MWCNT-CpG ODN or CpG ODN B) 0.1 µg MWCNT-CpG ODN or CpG ODN. ** indicates significant difference $p < 0.01$.

6.7 Surface Antigens

To try to better understand the mechanism of immune activation by CpG ODN and MWCNT-CpG ODN, antigen expression of CpG ODN or MWCNT-CpG ODN treated HD11 cells was examined. MHC class I and II are important antigens expressed on certain immune cells. MHC I is expressed on all nucleated cells and upon viral infection cells present cytoplasmic produced proteins via their MHC I antigen to T cells resulting in apoptosis of the cell. MHC II antigens can only be expressed by certain antigen expressing cells including macrophages (He et al., 2003; van Duin et al., 2006). Using FACS analysis it was observed that there was no change in MHC I antigen expression between the media treated control and the CpG ODN and MWCNT-CpG ODN treated HD11 cells (Figure 6.15A). The expression of MHC II increased with CpG ODN and MWCNT-CpG ODN treatment compared to the media treated cells but there was no significant difference between the two treatments even at lower doses (Figure 6.15B). Therefore the improved immune stimulation by lower doses of MWCNT-CpG ODN is not due to increased antigen presentation.

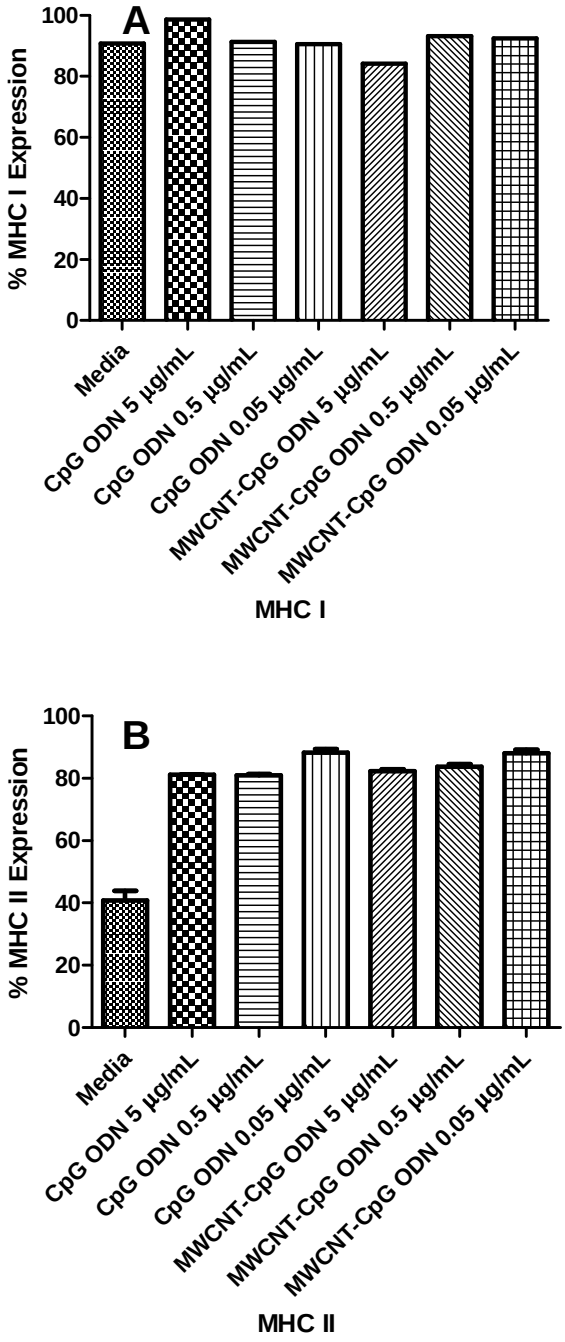


Figure 6.15. MHC I and MHC II antigen expression on HD11 cells. HD11 cells were treated for 24 hours with CpG ODN, MWCNT-CpG ODN or media alone. A) MHC I and B) MHC II antigen expression was measured using FACS analysis. Graphed as percent of cells expressing MHC I or MHC II. All treatments were done in triplicate.

6.8 Innate Immune Gene Expression

Since immune cells produce important cytokines in response to activation by molecules such as CpG ODN it may be possible that the immune stimulation of effective lower doses of MWCNT-CpG ODN is due to the ability to more effectively activate cells to produce immune stimulating cytokines. Select innate immune genes that are expressed by activated macrophages including IL-6, IL-12, IL-8 and IL-1β. IL-6 and IL-12 are produced by activated macrophages and act as inflammatory cytokines that also stimulate T cells. IL-6, IL-12, IL-8 and IL-1β are macrophage produced cytokines important to inflammatory responses (Kumar et al., 2009). To observe the effect of CpG ODN and MWCNT-CpG ODN on HD11 cells these cytokines were observed at the gene level using qRT-PCR. Cells were treated with various doses of either CpG ODN or MWCNT-CpG ODN for 24 hours. All four genes were increased at 0.05 µg/mL MWCNT-CpG ODN treatment compared to the same concentration of CpG ODN treatment (Figure 6.16). There was a significant increase in IL-6, IL-12, IL-8 and IL-1β production by CpG ODN and MWCNT-CpG ODN treated cells. There was also a significant difference between CpG ODN and MWCNT-CpG ODN treated cells with cells treated with a lower dose of MWCNT-CpG ODN compared to cells treated with the same dose of CpG ODN. These four genes are important cytokines in inflammatory responses to pathogens and play an important role in macrophage nitric oxide production.

It was observed that there was a significant increase in the level of the four cytokine genes expressed in cells treated with CpG ODN and MWCNT-CpG ODN. There was also a significant difference between the expressions of these genes in cells treated with the two molecules. The expression of IL-6 and IL-12 were not as high as the other two cytokines but there is an increased expression with CpG ODN and MWCNT-CpG ODN treatment. There was also a significant different in the fold change of these cytokines with treatment of 0.05 µg/mL of MWCNT-CpG ODN compared to cells treated with the same dose of free CpG ODN (Figure 6.16A-B). The expression of IL-1β in cells treated with free CpG ODN remains similar for all doses; however, cells treated with MWCNT-CpG ODN there was an increase in IL-1β expression as the dose lowers. There is also significantly higher expression in cells treated with a lower dose of MWCNT-CpG ODN compared to cells treated with CpG ODN with a

concentration of 0.05 µg/mL of MWCNT-CpG ODN stimulating the most gene expression (Figure 6.16C). The expression of IL-8 was also significantly higher for cells treated with MWCNT-CpG ODN compared to cells treated with CpG ODN with the highest response being generated with 0.5 µg/mL treatment with MWCNT-CpG ODN (Figure 6.16D).

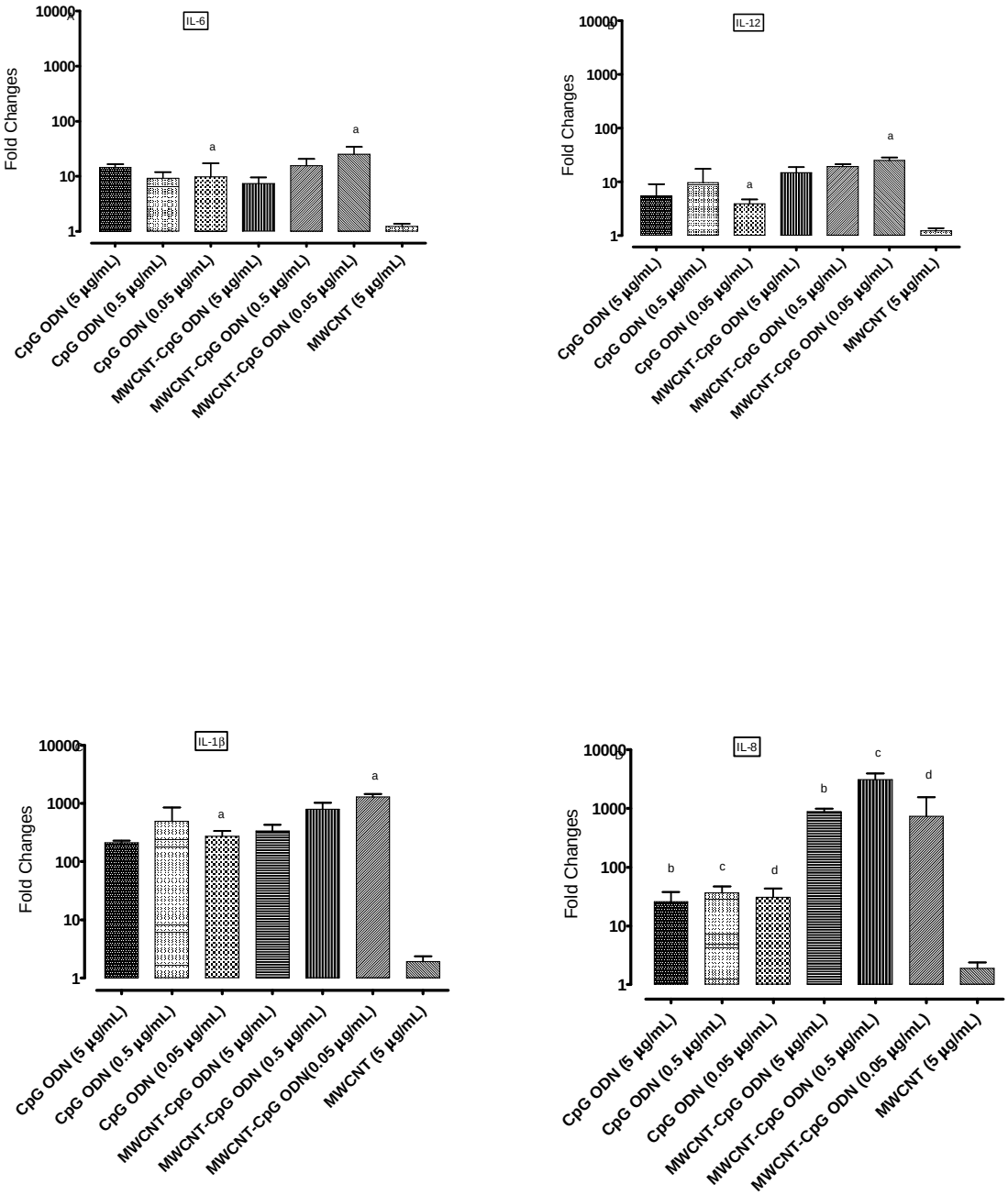


Figure 6.16. Fold Changes of select innate immune genes. Fold change values for transcriptional expression of (A) IL-6 (B) IL-12 (C) IL-1β and D) IL-8 in HD-11 cells following 24h treatment with various doses of free and MWCNT-linked CpG ODN with respect to media treated cells are shown. Fold changes for CNT treated cells are also shown as negative control. Mean values from three biological replicates are plotted with 1-SD. Bars with similar letters indicate statistically significant difference. $p < 0.05$

7.0 Discussion and Conclusion

7.1 Selection of best conjugate

The first step in using MWCNTs to aid in the delivery of CpG ODN is selecting the most effective means to link the two molecules. There is a wide variety of techniques available to link carbon nanotubes to nucleic acids such as CpG ODN involving direct adsorption with or without a linker, electrostatic linkers and other chemical reactions (Chicharro et al., 2007; Lei et al., 2008; Shim et al., 2002; Yang et al., 2008). It was also important that non-covalant interactions were involved in order not to disrupt the structure of CpG ODN which could disrupt the biological activity and function of the molecule. We wanted to select the best method that provided the strongest immune response at lower doses. To test which linkage was the best we first looked at the lymphocyte proliferative response when treated with each of the three methods of linkages. Proliferation of B cells in response to proper stimuli is a sign of the generation of an immune response (Miyamoto et al., 2002). It has been shown in other species that CpG ODN is able to stimulate a proliferative B cell response (Booth et al., 2007). Here we isolated lymphocytes from chicken's spleens as this is an important site of lymphocyte conjugation and there is a mix of B lymphocytes, T lymphocytes and other mononuclear cells (Neelima et al., 2003). After 48 hours the proliferative responses were measured and it was found that the cells treated with CpG ODN, MWCNT-2-CpG ODN, and MWCNT-3-CpG ODN showed significant proliferation at treatments of 5 µg/mL but when the dose was lowered ten-fold the proliferative response dropped dramatically to levels comparable to the negative control cells treated with MWCNT or media. It has been shown that at lower doses free CpG ODN is not able to give strong immune responses, likely due to instability of the molecule (Dar et al., 2009). However, it was observed that treatment with MWCNT-1-CpG ODN gave a high proliferative result at 5 µg/mL which remained constant even to 0.05 µg/mL (Figure 6.1). This indicates that this linkage would appear to be the best for improving the biological activity of CpG ODN. The use of PySE as a linker may be better due to the strong Π-Π stacking interactions between the basal plane of the graphite of the side wall of the carbon and the pyrene of the PySE which leads to a stable and strong interaction (Chen et al., 2001). This interaction makes CNTs more soluble as well as it allows a strong non-covalent interaction with

biologically active molecules like CpG ODN without disrupting their structure (Chen et al., 2001; Yang et al., 2008). The ester of PySE can bind to the amine groups of the nucleotides to form a strong interaction with CpG ODN. The use of direct adsorption of CpG ODN unto the carbon nanotubes does not have as strong as a linkage provided by PySE. The same may be true for using the electro-static linker PEI.

We also looked at the up-regulation of some important innate immune genes in HD11 chicken macrophage cells treated with a dose titration of the three MWCNT-CpG ODN linkages. It was observed that certain genes were expressed at higher levels with treatment with MWCNT-1-CpG ODN compared to cells treated with MWCNT-2-CpG ODN, MWCNT-3-CpG ODN and free CpG ODN (Figure 6.2). The genes expressed are vital to immune responses generated by CpG ODN. Namely TLR7, TLR15, MyD88 and NF-kB. MyD88 is an important adapter protein that is recruited to TLRs when they come in contact with their ligands. MyD88 interacts with other proteins such as IRAK and TRAF6 which forms a complex that results in the up-regulation of innate immune cytokines (Krieg, 2006). NF-κB is an important transcription factor which can be stimulated by mitogens such as LPS and CpG ODN (He and Kogut, 2003). Once active, NF-κβ translocates into the nucleus where it is involved in the transcription of innate immune cytokines genes. The up-regulation of TLR7 and TLR15 may indicate how CpG ODN is recognized in chicken cells as avian species lack TLR9 which mammals use to recognize CpG ODN. TLR7 is also located within the endosome like TLR9 is and may be possible that it functions like TLR9 in avian species. It has also recently been discovered that chickens infected with *Salmonella* have an up-regulation of TLR15 (Higgs et al., 2006). *Salmonella* contains CpG ODN motifs that can trigger an immune response, so the up-regulation of TLR15 in this study and in others may indicate that TLR15 is involved in CpG ODN recognition in the avian species or is working in tandem with TLR7. However, a recent study indicates that TLR21 is the possible avian homologue to mammalian TLR9 (Brownlie et al., 2009). This receptor is also located in the endosome and reacts to microbial DNA as a danger signal. Therefore, the up-regulation of TLR7 and TLR15 may not have as significant impact. The observations here do show that CpG ODN bound to MWCNT with the use of PySE as a non-covalent linker is able to stimulate these innate immune genes better then free CpG ODN or any other linking chemistry attempted in this study. This may be to due to a stronger

interaction between MWCNT and CpG ODN due to the PySE lending more stability to CpG ODN or resulting in more CpG ODN being delivered per bird at one time. Also the percent of CpG ODN bound to MWCNT during the conjugation process indicates that adsorption of CpG ODN onto MWCNT is better than use of an electrostatic linker as around 50-60% of the CpG ODN is being bound compared to the 19% bound by the electrostatic linker PEI (Table 7.1).

After selecting which chemistry to use it was vital to ensure that CpG ODN is indeed linked to the MWCNT. First Raman spectometery was conducted on free MWCNT and MWCNT-CpG ODN to see if there was a difference in the Raman spectra profile. The peaks of the Raman spectra appear when the molecule scatters light at a specific wavelength. The spectra profile of carbon nanotubes is different depending on the type of CNT and the manufacturing process (Costa et al., 2008). The MWCNT-COOH used in this study had a Raman spectra with peaks at certain wavelengths with a large peak around 1200 nm (Figure 6.3). This is a typical spectra found for most MWCNT (Costa et al., 2008). The spectra of the MWCNT-CpG ODN gave a different spectra than MWCNT-COOH where the peaks generated at wavelengths over 1300 nm were wider. The difference in Raman spectra may indicate that CpG ODN is bound to MWCNT as the Raman spectra profile is similar but different than the free MWCNT spectra. To further examine and confirm the linkage between CpG ODN and MWCNT using the PySE linker a fluorescence study was also conducted. PySE fluoresces naturally due to the pyrene moiety, therefore alterations in the fluorescence spectra when bound to MWCNT, CpG ODN and MWCNT-CpG ODN would indicate different interactions. As seen in figure 6.4 the fluorescence spectra of free PySE has a profile with two peaks with fluorescence value around 5. When linked to MWCNT there is quenching of PySE as the fluorescence is reduced. When PySE is bound to CpG ODN there is an increase in fluorescence. However, when PySE is linking CpG ODN to MWCNT there is a further increase in fluorescence. This difference between MWCNT, CpG ODN and MWCNT-CpG ODN indicates different interactions and the linkage of MWCNT to CpG ODN by PySE. To ensure this observation is due to the linkage MWCNT-PySE was spiked with CpG ODN before fluorescence measurements and it was observed the emission spectra of MWCNT-PySE was not changed. These studies indicate that CpG ODN is indeed being linked to MWCNT and that adsorption with the non-covalent linker,

PySE, is the best method attempted to get optimal conjugation between CpG ODN and MWCNT which results in an improved immune response at lower doses.

Table 7.1 Percent of CpG ODN conjugated to MWCNT

Chemistry	Denotation	CpG ODN added (µg/mL)	CpG ODN bound (µg/mL)	CpG ODN bound (%)
Adsorption with a linker (PySE)	MWCNT-1-CpG ODN	500	261	52.2
Direct Adsorption	MWCNT-2-CpG ODN	500	297	59.4
Electostatic linker (PEI)	MWCNT-3-CpG ODN	500	97	19.4

7.2 Cellular Uptake

It is critical that target cells are able to internalize MWCNT linked CpG ODN in order for the molecule to stimulate an immune response. Internalization of CpG ODN is essential because in most species the receptor that recognizes CpG ODN leading to immune activation is TLR9 which compartmentalized inside the cell (Hacker et al., 1998; Hemmi et al., 2000). Until very recently the receptor for recognizing CpG ODN was unknown as avian species lack TLR9. However, it is now believed that TLR21 is able to recognize and respond to CpG ODN in chickens (Brownlie et al., 2009). This receptor is also located inside the cell in the same compartment, the endosome, where TLR9 is located in mammals. It has been observed in this study and others that inhibition of endosomal maturation prevents immune responses in chicken cells to CpG ODN treatment (He and Kogut, 2003). Therefore, CpG ODN linked to MWCNT must also be able to enter the cell to reach the target receptor.

It has been observed that the cellular uptake of free CpG ODN requires receptor-mediated endocytosis in order for the nucleic acid to cross the cellular membrane (Chelobanov et al., 2006). The cellular uptake of free functionalized MWCNTs involves an energy-independent non-endocytotic pathway as these molecules can enter the cell in the presence of endoctyosis inhibitors (Pantarotto et al., 2004a). The size and chemical structure allows functionalized carbon nanotubes to almost freely pass across the cellular membrane without the need for receptor mediated endocytosis making them ideal for therapeutic delivery (Lacerda et al., 2006; Pantarotto et al., 2004b). This characteristic of carbon nanotubes led to the hypothesis that the improved immune stimulation by MWCNT-CpG ODN compared to free CpG ODN may be due to more rapid uptake of the MWCNT-CpG ODN molecules resulting in more immune stimulation before cellular degradation of CpG ODN. To test this we did a time course study to observe any difference in uptake between free and MWCNT linked CpG ODN. Chicken macrophage cells were used as the target cell as they are important innate immune cells that have protective immune function themselves and also can act as antigen presenting cells for adaptive immune cells. They have also been shown to respond strongly to CpG ODN treatment (He et al., 2003).

It was observed that MWCNT-CpG ODN and free CpG ODN both start the process of cellular uptake rapidly. To assess this I counted a hundred cells at every time point and found that after 30 minutes the majority of both molecules are localized around the cellular membrane. Nearly all of the macrophages are in some stage of taking up the molecules (Figure 6.5 A-D). The uptake process continues and by two hours there is the beginning of localization in specific areas in the cytoplasm of both molecules as the intensity of fluorescence is greater for both molecules and concentrated in small areas inside the cell (Figure 6.5 I-L). Previous studies have indicated that it takes at least four hours for CpG ODN to stimulate an immune response (Utaisincharoen et al., 2002; Xu et al., 2003). After 4 hours post initial treatment the localization in the cytoplasm of both molecules is even more intense but there appears to be more intensity of fluorescence in cells treated with MWCNT-CpG ODN compared to cells treated with CpG ODN (Figure 6.5 O-R). Since the control cells treated with Cy3 dye alone did not have this localization even 4 hours post treatment, the localization seen is due to the labelled CpG ODN entering the cell. The localization in the cytoplasm is likely at the endosome where the intracellular target of CpG ODN is located. From these observations it indicates that speed of uptake and intracellular localization is not altered by linking CpG ODN to MWCNT which dismisses the possibility that MWCNT-CpG ODN is able to freely pass through the cell membrane and enter the cells more rapidly. It was also observed that the use of a clathrin inhibitor which prevents endocytosis inhibits the ability of MWCNT-CpG ODN to stimulate HD11 cells to produce nitric oxide (Figure 6.9 A). This indicates that MWCNT-CpG ODN still requires receptor mediated endocytosis to enter the cell to stimulate an immune response. It may be the bulk of the CpG ODN molecule or its charge that prevents passage across the membrane without the aid of endocytosis.

It was also observed that with time it appears more MWCNT-CpG ODN is localized within the cell compared to the localization of free CpG ODN. However, the nucleuses of some of the cells showing extremely high uptake do not appear as healthy. This may be due to the large dose required to get a visualization of uptake. As was indicated in this study, high doses of MWCNT can be toxic towards the HD11 cells. The observation that there appears to be more MWCNT-CpG ODN in the cells with time may be due to more MWCNT-CpG ODN entering the cell or MWCNT providing protection from intracellular degradation both of which may be

occurring which would lead to more CpG ODN present to stimulate a stronger immune response. Free CpG ODN is typically degraded by 3' exonucleases fairly rapidly (Noll et al., 2005). The increased protection of MWCNT could lead to a smaller dose of CpG ODN which would typically be degraded rapidly before it can generate a response to be protected and stimulate an effective immune response. The linkage of CpG ODN to MWCNT may also provide stability to the CpG ODN molecule *in vivo* which would allow more of the molecule to reach target cells before the molecule is destroyed in the system. The uptake study indicates that there is no improved uptake between the molecules regarding time but there may be an increased amount of MWCNT-CpG ODN remaining or entering the cell. This could explain why a smaller dose of MWCNT-CpG ODN is more effective at stimulating a protective immune response then the same dose of free CpG ODN. It is clear that linking CpG ODN to MWCNT does not adversely affect the uptake of the CpG ODN molecule. It is for certain that the attachment of CpG ODN to MWCNT does not inhibit the CpG ODN from entering the cell.

7.3 Toxicity

Now that it has been established that MWCNT-CpG ODN are able to enter the cell and stimulate an improved immune response at lower doses, it is vital that the molecule is not toxic towards immune cells at the target dose. With the rise in manufacturing and use of carbon nanotubes several studies have been attempted to determine what kind of effect they have on human and animal health. The use of nanoparticles in the form of carbon nanotubes is fairly new so there is a lag in information regarding the long term exposure to these molecules but several short term *in vivo* and *in vitro* studies have been conducted. There are many contradictory reports regarding the cytotoxicity of carbon naotubes. Some reports indicate that carbon nanotubes show very high toxicity (Jia et al., 2005; Manna et al., 2005; Murr et al., 2005). However, several reports also indicate that carbon nanotubes show very low toxicity or no toxicity at all (Kam et al., 2005; Murakami et al., 2004; Pantarotto et al., 2004a). It seems that several factors are involved with the toxicity generated from carbon nanotubes including its length and size distribution (Poland et al., 2008; Sato et al., 2005), surface area chemistry (Saxena et al., 2007) and aggregation status (Wick et al., 2007). The manufacturing process also

plays a role in the toxicity of the carbon nanotubes. Unpurified CNTs may contain metal traces such as iron and nickel associated with the manufacturing process (Pulskamp et al., 2007). These various factors make it essential to test the cytotoxicity of the carbon nanotube used as CNTs purchased elsewhere may have different toxicity profiles. In general CpG ODN shows no severe toxicity at lower doses as has been seen in other studies including this one (Dar et al., 2009). Therefore, if toxicity is seen with the MWCNT-CpG ODN molecule it likely due to the MWCNT rather than the linkage between CpG ODN and MWCNT.

We examined the toxicity of free MWCNT and CpG ODN bound MWCNT as well as the toxicity of CpG ODN *in vitro* using the chicken macrophage cell line HD11 as macrophages are important in immune responses as they come in first contact with a molecule. To examine the cytotoxicity of the molecules a cell viability study was done looking at certain time points during a 7 day study post initial treatment. It was observed that CpG ODN alone did not show any significant reduction in the number of viable cells at any time point or at any concentration as the percent of viable cells was comparable to the control cells treated with media alone (Figure 6.6). Cells that were treated with free MWCNT began to show a significant decrease in the percent of viable cells after only 24 hours when treated with the high dose of 100 µg/mL and the beginning of toxicity with treatment of 10 µg/mL (Figure 6.6A). This toxicity of MWCNT at these higher doses continues to increase with time, as by day three post treatment the percent of viable cells treated with 100 µg/mL of MWCNT is at the same level as the negative control cells treated with Saponin which rapidly kills cells by lysing the cell membrane (Figure 6.6B). By day seven post treatment both 100 and 10 µg/mL of MWCNT treatment are at the same level of percent viable cells as the negative control cells. However, the lower doses of MWCNT do not show any toxicity towards the cells. This observation indicates that free MWCNT shows cytotoxity towards macrophages at higher doses > 1 µg/mL, at least *in vitro*. This toxicity is rapid at very high doses and increases with time. Cells treated with MWCNT-CpG ODN do not show this toxicity towards the cells after 24 hours of treatment even at the very high doses (Figure 6.6A). However, by three days post treatment cells receiving 100 µg/mL of MWCNT-CpG ODN begin to show a significant decrease in the percentage of viable cells and by seven days post treatment cells that received 100 and 10 µg/mL of MWCNT-CpG ODN show percentage of viable cells comparable to the negative

control and cells receiving the same doses of free MWCNT (Figure 6.6B-C). This indicates that when linked to CpG ODN MWCNT at high doses can be toxic to the macrophages *in vitro* just as free MWCNT is. This toxicity for the CpG ODN linked MWCNT is delayed compared to the free MWCNT toxicity but by day seven post treatment the results are similar.

This study did indicate *in vitro* toxicity with very high doses of free MWCNT and even with high doses of CpG ODN linked MWCNT. However, the effective target dose of a MWCNT-CpG ODN treatment is 1000-fold less than these toxic MWCNT concentrations. The lower doses of free and linked MWCNT did not show any toxicity towards the cells. The use of MWCNTs in the biological system is a relatively new concept and there is not much information regarding the long term exposure to the molecules. It may be possible that MWCNT can build up in the system and reach toxic levels. Therefore, further studies need to be conducted to see the long term effect of even small doses of MWCNT. In practice, the dose of MWCNT-linked CpG ODN would be considerably lower than the dose shown to be toxic. The animals would likely only require a single dose and there would not be a build up to a toxic level before they pass their life expectancy. It may be possible that eating MWCNT-linked CpG ODN treated animals would result in the build up of CNTs in the human body but studies into the clearance of MWCNT-linked CpG ODN from the body need to be conducted to know for certain. It has also been observed in other studies that functionalized CNTs are cleared from the blood system after 3 to 4 hours by the liver and are excreted from the host as intact carbon tubes in the urine (Singh et al., 2006). The clearance of the carbon nanotubes from the host would prevent the possible build-up of MWCNTs to the level that was seen to show toxicity *in vitro*.

It has been observed in previous studies that treatment with high doses of CNTs can lead to cell death in macrophages. These cells show an increase in calcium within the cell which is linked to oxidative stress (Brown et al., 2007; Bussy et al., 2008; Donaldson et al., 2003). Macrophages can produce reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide to help fight intracellular infections but too much production can cause oxidative stress and damage to the cell, and surrounding cells and tissue (Dietert and Golemboski, 1998). To observe how MWCNT are toxic to the chicken macrophage cell line the production of peroxide was measured 24 hours post initial treatment. At this time point in the

viability assay, it was observed that all treatments did not reveal such toxicity with the exception of 100 µg/mL of MWCNT alone. When examining the production of peroxide of cells treated with 100 µg/mL of MWCNT showed a significant increase in peroxide levels compared to the media treated control cells (Figure 6.7A). This observation indicates that MWCNT at high doses are causing oxidative stress in the chicken macrophages which is resulting in their cell death.

7.4 Comparison of free and MWCNT linked CpG ODN's biological function

Another free radical that can be produced by macrophages that is beneficial to an innate immune response is nitric oxide (NO). Extensive studies have been done on CpG ODNs ability to stimulate HD11 cells to produce nitric oxide (Hemmi et al., 2000). It has been demonstrated in several studies that CpG ODN by itself is able to stimulate nitric oxide production in HD11 cells (He et al., 2003; Utaisincharoen et al., 2002). The stimulation to produce nitric oxide is essential to marcophage's immunological function, as nitric oxide helps destroy intracellular pathogens such as *Salmonella* (Eisenstein, 2001; He et al., 2003). Therefore it is vital that CpG ODN linked to MWCNT also stimulates these cells to produce nitric oxide that is beneficial to the macrophage's immune function. It was observed that both free and linked CpG ODN are able to stimulate the production of nitric oxide in the HD11 cells comparable to the positive control LPS and what has been found in other studies (Crippen et al., 2003; He et al., 2003). At the higher doses of MWCNT-linked CpG ODN (100 and 10 µg/mL) there was the initial increase in NO after 24 hours but there was a drop by day 3 due to the toxic effects of the high dose MWCNT (Figure 6.9A-B). Free MWCNT itself was unable to stimulate any NO production at any concentration. This indicates that the NO being stimulated by MWCNT-linked CpG ODN is the result of the CpG ODN activating the macrophage to produce nitric oxide that is beneficial to immune responses and is not acting as a free radical. Both MWCNT-CpG ODN and CpG ODN are able to stimulate NO production at comparable levels with each other and the positive control at concentrations ranging from 1 µg/mL to 0.1 µg/mL. However at a concentration of 0.01 µg/mL there is a delayed stimulation of NO production and it is not until day 3 post treatment that there is any NO production being stimulated. At this time point

the level of NO stimulation by MWCNT-CpG ODN is at the maximal level found with LPS treatment but the NO level stimulated by free CpG ODN is significantly less (Figure 6.8E). This indicates that MWCNT-CpG ODN is more effective at stimulating activation of macrophages at a lower dose then free CpG ODN even though the level of stimulation cannot be maintained to day 7 like the higher doses. Below this concentration both free and MWCNT linked CpG ODN are unable to stimulate NO production in the HD11 cells. To ensure that there is no alteration to the biological function of CpG ODN when linking it to MWCNT the pathway of NO stimulation of these two molecules was examined at an optimal concentration for stimulation of NO for both molecules.

An important first step in the stimulation of an immune cell by CpG ODN is the cellular uptake of the molecule. As seen with the uptake study both free and MWCNT-linked CpG ODN are taken up by the HD11 cell. The uptake of extra-cellular molecules by the cell requires endocytosis which is mediated by a small peripheral membrane protein called clathrin which forms a lattice coat around the inner membrane that is destined for vesicle formation to aid in vesicle budding (Royle, 2006). By using an inhibitor of clathrin-dependent endocytosis the ability of both free and MWCNT-linked CpG ODN to stimulate nitric oxide production were dramatically reduced to the same level (Figure 6.9A). LPS stimulation of NO production was not as disrupted as its target receptor is TLR4 which is located on the cell surface. Accordingly cellular uptake is not required for LPS stimulation. The need of receptor-mediated endocytosis for CpG ODN to stimulate HD11 cells had been observed in other studies (He and Kogut, 2003). This study also indicates that MWCNT-linked CpG ODN also requires clathrin-mediated uptake into the cell and is not free to pass through the cellular membrane. MWCNT have been shown to be able to pass through cellular membranes on their own. However, here it is clear that endocytosis is required to allow MWCNT-linked CpG ODN entry into the cell, likely due to the bulk or the charge of the CpG ODN (Bianco et al., 2005).

It is known that the target receptor for CpG ODN in mammals is TLR 9 located in the endosome (Krieg, 2006). Unfortunately so far there has been no TLR 9 orthologue found in avian species but they still respond to CpG ODN (Boyd et al., 2007; He et al., 2006). It could be that TLR9 still needs to be defined in chickens or a different receptor is involved. Previous studies have indicated that inhibition of endosomal maturation with an inhibitor like

chloroquine prevents CpG ODN from stimulating macrophages to produce NO (He and Kogut, 2003). This would indicate that the chicken receptor that recognizes CpG ODN is also located in the endosome like the mammalian TLR 9. By using chloroquine we observed that MWCNT-linked CpG ODN also requires an endosomally located receptor to stimulate NO production in HD11 cells as there was a dramatic reduction in NO production by inhibiting endosome maturation for both free and MWCNT-linked CpG ODN (Figure 6.9B). Again LPS stimulation is not as greatly effected as TLR 4 is located on the cell surface. Thus endosomal maturation is not required for LPS to stimulate NO production. This study indicates that linking CpG ODN to MWCNT does not structurally alter CpG ODN, as it is still recognized by the same endosomally located receptor.

It has also been demonstrated in other studies that both LPS and CpG ODN induce NO production in HD11 cells by stimulating the activation of the iNOS enzyme responsible for producing NO (Utaisincharoen et al., 2002). There are three isoforms of nitric oxide synthase (NOS) but it is the inducible form which is stimulated by extracellular molecules in macrophages to produce NO with immune functions (Dietert and Golemboski, 1998). In this study we also observed that MWCNT-linked CpG ODN treated HD11 cells have a significant reduction in NO production when pre-treated with an iNOS inhibitor (Figure 6.10A). This indicates that both free and MWCNT-linked CpG ODN activate iNOS in these cells resulting in the inducible form of NO. To ensure that the same signalling pathway is being activated by free and MWCNT-linked CpG ODN several inhibitors were used that inhibit key steps in the pathway of iNOS activation.

It has been demonstrated that PKC activity is essential for the stimulation of NO production in macrophages (Clemens et al., 1992). PKC isozymes have been shown to play critical roles in NF-κB activation and in the MAPK cascade which can lead to iNOS activation (Xu et al., 2003; Zhang et al., 2000). Studies have shown that inhibiting PKCα leads to an almost complete shutdown of NO production in LPS and CpG ODN treated macrophages (He and Kogut, 2003). This study also indicates the this enzyme plays a key role in NO production of MWCNT-linked CpG ODN treated HD11 cells, as there is almost complete inhibition of NO production in cells pre-treated with a PKC inhibitor (Figure 6.10B).

PKC plays a central role in the entry into the MAPK pathway which can lead to NF-κB activation (Liebmann, 2001). It has been demonstrated that the MAPK cascade and NF-κB activation are essential to CpG ODN stimulated NO production in HD11 cells (He and Kogut, 2003; Seger and Krebs, 1995). Here we used inhibitors of key factors in the MAPK cascade and NF-κB activation to observe if this pathway also plays an essential role in NO production in MWCNT-linked CpG ODN cells. SB-203580 and U-0126 were used to inhibit p38 MAPK and MEK 1/2 respectively. These serine/threonine protein kinases participate in the MAPK signalling cascade which can be a response to extracellular products such as LPS and CpG ODN. By inhibiting one or the other enzymes there is a significant reduction in the level of NO being produced by both CpG ODN and MWCNT-linked CpG ODN indicating that the MAPK pathway is essential for macrophages stimulation for both molecules. There is not complete inhibition of NO production, as it has been shown in other studies that there are other pathways involved in NO production in macrophages treated with CpG ODN (He and Kogut, 2003). Although MAPK signalling cascade can lead to the eventual activation of NF-κB, direct NF-κB activation can also lead to iNOS activation and NO production and plays a central role in inflammation and cytokine production (Scott and Owens, 2008). We also used a direct NF-κB inhibitor in the form of BAY 11-7082 which also significantly inhibits NO production for both free and MWCNT-linked CpG ODN treated cells (Figure 6.12C). The pathway studies revealed that linking CpG ODN to MWCNT did not alter its ability to be taken up the cell, interact with the target receptor and stimulate the MAPK signalling cascade and NF-κB activation, in turn resulting in the activation of iNOS and NO production by the HD11 cells. This demonstrates that the biological function of CpG ODN is not altered by linking it to MWCNT.

Another study conducted to observe any alterations to the biological function or action of CpG ODN when linked to MWCNT was to monitor MHC antigen levels with treatment. Since macrophages also act as antigen presenting cells to adaptive immune cells, the presentation of these antigens requires MHC complexes to be present on the macrophage cell surface in order to interact with the adaptive immune cells. Previous studies in mice have shown a decreased MHC II processing with treatment of CpG ODN (Chu et al., 1999) while other studies in chickens have shown little or no change in MHC II with treatment of CpG ODN (Xie et al., 2003). In this study the percent of HD11 cells expressing MHC I or MHC II

following treatment with a dose titration of CpG ODN or MWCNT CpG ODN indicated that there was no change in MHC I expression (Figure 6.15 A) while there was an increase on MHC II expression (Figure 6.15B). Although there is a definite increase in the percent of cells expressing MHC II treated with CpG ODN or MWCNT-CpG ODN compared to the media control, there is no significant difference between cells treated with CpG ODN and MWCNT-CpG ODN. CpG ODN bound to MWCNT does not improve MHC II expression but it does not decrease the MHC II expression compared to CpG ODN treatment. This indicates that the biological activity may not be improved at the antigen presenting level but the biological function of CpG ODN is not altered with linkage to MWCNT. MWCNT does not interfere with the presentation of CpG by antigen presenting cells. Also the presence of MHC II may increase on the macrophage cell surface but it does not indicate if the processing and presentation of the molecules is upregulated at the same level or at all.

7.5 *In vivo* studies: Protection of chickens against bacterial infection and cell recruitment

It is vital to the development of a protective treatment using MWCNT-CpG ODN that the molecule is able to generate a protective response at lower doses than free CpG ODN *in vivo*. Previous studies have indicated that free CpG ODN is able to provide a protective immune response against pathogenic bacteria such as *Salmonella* and *E.coli* (Gomis et al., 2003). In previous studies it was found that doses of 10 µg to 3 µg were able to significantly protect against a lethal bacterial challenge; however, below that dose proved to be ineffective. In this study we repeated the studies done in chickens with CpG ODN but added treatments of MWCNT-CpG ODN to see if there was improved protection at lower doses. It was observed that at doses of 10 and 1 µg both free and MWCNT linked CpG ODN were able to provide an effective protective response against a lethal challenge of bacteria (Figure 6.12). However, a difference between the two treatments was observed at a dose of 0.1 µg. At this concentration MWCNT-CpG ODN provided a protective response comparable to a treatment of 10 µg of MWCNT-CpG ODN or CpG while 0.1 µg of free CpG ODN did not provide protection and the rate of survival was similar to the negative control birds treated with PBS (Figure 6.13). MWCNT by itself also did not provide a protective immune response therefore the protection

provided by MWCNT-CpG ODN is due to the CpG ODN linked to the carbon nanotube. These observations indicate that MWCNT-CpG ODN is more effective than CpG ODN at lower doses at providing protection in chickens. This suggests that MWCNT-CpG ODN could lower the cost of protecting the birds against costly losses to bacterial infections.

The mechanism of the improved protection of MWCNT-CpG ODN compared to free CpG ODN was examined first by looking at cell recruitment to injection sites *in vivo*. It has been demonstrated in other studies that CpG ODN recruits immune cells to the site of injection (Mutwiri et al., 2004; Uwiera et al., 2001). The cells recruited to the site of injection in sheep were mainly lymphocytes and other mononuclear cells (Uwiera et al., 2001). Cell recruitment is important to stimulate an immune response as the immune cells converge on the CpG ODN and are stimulated to produce an immune response that prepares them for an encounter with a potential microbial pathogen. In this study with chickens, it was observed that the majority of the cells recruited to the site of injection were mononuclear cells, which includes lymphocytes and macrophages. Some early recruitment of neutrophils and heterophils was also observed but the majority of the cells were mononuclear cells especially with the passage of time. Table 6.2 reviews the severity of the cell recruitment to the site of injection. It was observed that free MWCNT at 10 µg did not recruit any cells or cause any inflammation. This indicates that *in vivo* this dose is likely not toxic towards the chicken or the chicken's tissue and cells. It observed that at higher doses of CpG ODN there was severe cell recruitment early which was sustained until day 7 (Figure 6.14A). This was not observed with the dose of 0.1 µg of CpG ODN (Figure 6.14B). The cell recruitment of MWCNT-CpG ODN began slower, but by day 2 had reached severe levels with doses of both 1 µg and 0.1 µg (Figure 6.14). After this time point, the severity decreases in chickens treated with MWCNT-CpG ODN. It was observed that the doses of both CpG ODN and MWCNT-CpG ODN that provided protection against a lethal challenge of bacteria all had severe cell recruitment at day 2. The treatments varied in severity on days 1, 3 and 7 but they had severe scores on day 2 in common. Also the treatment of 0.1 µg of CpG ODN only had moderate cell recruitment on day 2. This dose of CpG ODN proved to be ineffective at providing a protective immune response in chickens. This indicates that in order to have a strong protective immune response generated by CpG ODN in chickens there needs to be severe cell recruitment to the site of injection on or before day 2 post initial

treatment. The severity of the recruitment on day 1 and past day 2 seems not to matter for the ability to protect against lethal bacterial infections, at least in the case of MWCNT-CpG ODN. The lower dose of MWCNT-CpG ODN may be able to recruit cells more effectively than free CpG ODN, perhaps it is due to the stability provided *in vivo* by the MWCNT. It is still unclear why MWCNT-CpG ODN is more effective at lower doses than free CpG ODN but this study indicates that MWCNT-CpG ODN can attract immune cells at the proper level to generate a protective response at a lower dose then free CpG ODN.

7.6 Comparative innate immune gene expression between free and linked CpG ODN

To further investigate the mechanism of improved immune response to lower doses of MWCNT-CpG ODN compared to free CpG ODN the production of select innate immune cytokines produced by HD11 cells treated was examined. It has been demonstrated that key pro-inflammatory cytokines produced by stimulated innate immune cells are important to controlling certain types of infections (Blanks, 2007; Krieg, 2006). Here we found that the same cytokines are being produced by free and MWCNT-linked CpG ODN, but there is significantly more pro-inflammatory cytokines being stimulated with a lower dose of MWCNT-linked CpG ODN compared to free CpG ODN.

It has been observed in this study that the biological function of CpG ODN has not been altered by linking the molecules to MWCNT. An important part of the biological activity of CpG ODN is to stimulate pro-inflammatory cytokines such as interleukins. We observed that both free and MWCNT-linked CpG ODN were able to stimulate key inflammatory cytokines at about the same increased level at higher concentrations for IL-6, IL-1b and IL-12 using qRT-PCR (Figure 6.16A-C). However, it was observed that the production of these cytokines and IL-8 are dramatically increased with a lower dose of MWCNT-linked CpG ODN compared to the same dose of free CpG ODN (Figure 6.16). This may explain part of the increased protective immune responses observed. IL-6 is a key pro-inflammatory cytokine produced by macrophages and dendritic cells to help fight against infections (Heinrich et al., 2003). IL-12 is also typically produced by activated dendritic cells and macrophages. It plays an important role in the differentiation of naive T cells and can stimulate IFN-γ and TNF-α by natural killer cells

(Kalinski et al., 1997). IL-1b is an important cytokine in inflammatory responses such as bacterial infection and is also responsible for immune cell proliferation and differentiation as well as nitric oxide production in macrophages (Bencsath et al., 2003). IL-1b also stimulates the production of IL-8. Therefore the increase in IL-1b correlates with the increase in IL-8. IL-8 has the ability to specifically activate neutrophil granulocytes. IL-8 causes a transient increase in cytosolic calcium levels in neutrophils to aid in release of enzymes from granules which helps destroy invading pathogens (Krieger et al., 1992). IL-8 also acts as a chemotactic cytokine which attracts other immune cells to the site of infection (Smith et al., 1991). These cytokines act as a first defense against infections as well as recruit and activate adaptive immune cells to fight infections. It is unclear why MWCNT-linked CpG ODN is able to stimulate cytokine production more effectively in the HD11 cells at lower doses. It may be that MWCNT provides protection from degradation that allows more time for CpG ODN to stimulate these cytokines. The presence of more of these cytokines *in vivo* would stimulate a stronger, longer lasting response that could result in effective clearance of intracellular pathogens (Patel et al., 2008). From the gene expression study, there is a clear time course of innate immune activation. After 4 hours there is an improved up-regulation of early innate genes such as toll-like receptors, adaptor proteins and transcription factors. After 24 hours the downstream products of these early genes are also up-regulated at higher levels with lower doses of MWCNT-CpG ODN compared to the same dose of CpG ODN. Compared with the cell recruitment, it would seem that the lower dose of MWCNT-CpG ODN is able to recruit immune cells to the site of injection and after 24 hours there is significant up-regulation of key cytokines that prepares the host to fight potential infections. Further studies need to be done on the stability of the MWCNT-CpG ODN molecule to see if this response to lower doses is due to stability of CpG ODN or another reason.

7.7. Future directions

This project is a small step in the direction of using nanoparticles like MWCNTs as a drug delivery aid. Carbon nanotubes themselves can be used to deliver a wide variety of immune stimulating molecules besides CpG ODN. Nano delivery systems of different antigens and drugs could be possible to fight a wide variety of infectious diseases. Also carbon

nanotubes are not the only nanoparticles that can be used as a delivery vehicle for drugs. Other particles such as gold nanoparticles can also be conjugated to immune stimulating molecules like CpG ODN and host defense peptides and used to deliver and protect the molecules. Different nanoparticles can be used based on the need of treatment. They could be used to target specific tissue or cells which could be useful in treating disease like cancer by focusing on the tumor cells without damaging the healthy cells of the patient (Subramani et al., 2009). Nanoparticles can also be designed to deliver drugs and be released at a specific time using a photoregulated release of the cargo of the nanoparticle (Agasti et al., 2009). By carrying out similar studies on other nanoparticles, as have been conducted here, the most effective nanoparticles for a specific antigen can be used to improve the immune response to the molecule.

Further studies can be conducted on the use of MWCNT as a delivery vehicle of CpG ODN and other immune stimulating molecules. This project has established that CpG ODN when linked to MWCNT is able to provide an improved protective immune response at a lower dose then free CpG ODN in chickens. The *in vitro* studies indicated an improved cell proliferation and increased select innate immune expression. Further studies can be conducted *in vivo* to examine the innate immune gene expression post-treatment to see what has been observed *in vitro* is happening *in vivo* to find the mechanism of improved immune stimulation. Long term toxicity studies also need to be conducted to determine if consumption of MWCNT or MWCNT-CpG ODN chickens can result in the transfer of nanotubes to humans and result in the buildup of carbon nanotubes in the biological system. The use of MWCNT-CpG ODN in chickens would better serve the chicken industry by treating the birds *in ova* as mortality of chicks in the first week due to bacterial infections is a significant problem (Dar et al., 2009). *In ova* delivery of CpG ODN has been attempted with other delivery systems including lipid-based delivery without any success (Gursel et al., 2001). The use of MWCNT to deliver CpG ODN *in ova* may or may not improve mortality in newly hatched chicks. Further studies need to be conducted to see if this is a possibility. The use of nanoparticles to improve the delivery of immune stimulants is just beginning to be explored. This project proves that MWCNT can improve the effect of lower dose of CpG ODN. By lowering the effective dose of an immune stimulant or drug, the cost of using these highly expensive molecules will be lowered which, in

turn, could lead to increased availability of protective treatments for patients as well as animals in the livestock industry. With further studies, the potential of the approach of using nanoparticles in bio-medicine can be realized and will hopefully improve treatments against infectious disease.

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